PROCEEDINGS OF A SYMPOSIUM
VIENNA, 7–11 APRIL 1997
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TOWARDS LIVESTOCK DISEASE DIAGNOSIS AND CONTROL IN THE 21st CENTURY
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PROCEEDINGS OF AN INTERNATIONAL SYMPOSIUM ON DIAGNOSIS AND CONTROL OF LIVESTOCK DISEASES USING NUCLEAR AND RELATED TECHNIQUES JOINTLY ORGANIZED BY THE INTERNATIONAL ATOMIC ENERGY AGENCY AND THE FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS AND HELD IN VIENNA, 7–11 APRIL 1997

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What are the global problems in livestock development? How may scientific advances be harnessed to identify and solve such problems? What unique features of advances enable them to be applied for the benefit of agriculture?

To attempt to answer these questions, an examination of statistical data involving production criteria identifies the livestock areas on which experts should concentrate. The Food and Agriculture Organization of the United Nations Committee on Agriculture, in its fourteenth session in 1997 in Rome, outlined the trends in livestock development. A few of the committee's conclusions serve to show the areas for application of existing and emerging technologies:

— From 1975 to 1995, there was a trend away from livestock as a multifaceted farming system and towards a more intensive and specialized production system.

— Global meat production increased over the last two decades from 110 to 195 million tonnes, yet the contribution from developing countries has trebled from 33 to 101 million tonnes and now exceeds production from developed countries by 94 million tonnes.

— Viewed from a regional perspective, the changes in meat production are more dramatic. For example, overall Asian production rose by 293%, mainly as a result of increases in poultry and pork meat, which have risen by 397 and 283%, respectively.

— The growth in milk production in developed countries has increased by 105%.

— Globally, egg production has risen by 92%, with production from developing countries increasing by 287% and now exceeding that of developed countries.

While such results have been mainly due to increases in herd and flock sizes, there has also been a substantial increase in overall productivity. For example, beef production increased from 1975 to 1995 by 22%, while the global cattle herd only grew by 12%. Pork production increased by 100%, while the herd population grew by only 32%.

The indication is that production of livestock in developing countries is now the dominating factor for food sustainability. Aside from the disease elements, which are the more obvious subjects of this symposium, there are other adverse consequences that affect aspects of developing country success in production of livestock, such as land degradation, deforestation, collapse of mixed farming systems, pollution, global warming and loss of biodiversity. The concentrated approach to farming also elicits new disease problems.

The ultimate goal of livestock science is to increase food security. This symposium looks into the 21st century and contemplates the application of science to
production as a complex of socioeconomic problems. The subject matter of the symposium not only considers the impact of developments in serology and molecular biology (which might be expected in view of their direct relevance to methods for diagnosing diseases), but also addresses some of the broader issues, such as epidemiology, vaccines, information networks, geographical information systems and socioeconomic factors.

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OPENING SESSION

Chairperson

M.H. JEGGO
FAO/IAEA
This symposium is about livestock. Livestock are vital to sustainable agriculture and contribute significantly to the food security of many communities, particularly in developing countries. They are a source not only of food (meat and milk), but also of manure and draught power. Animal products are also a source of disposable income for many farmers in developing countries. Mixed farming systems that include livestock offer many advantages over crops alone. Indeed, many farmers in developing countries operate production systems where there is strong interaction and interdependence between livestock and crops. Trends of the last decade have shown that production of livestock in developing countries is now the dominating factor for food security and sustainable agricultural productivity. This trend is expected to continue into the next century.

However, livestock diseases remain a key constraint to livestock production in developing countries. Diseases like rinderpest, foot-and-mouth disease, brucellosis, trypanosomosis, tick borne diseases such as anaplasmosis, heartwater and East Coast fever, contagious bovine pleuropneumonia and Newcastle disease have caused great economic losses in various parts of the world. The control, and ultimate eradication where possible, of these and other diseases is important for the economies of many nations. It is for this reason that both the IAEA and the FAO have put great emphasis on the control and eradication of livestock diseases.

The success of any disease control or eradication programme relies heavily on the robustness and efficacy of the diagnosis, surveillance or seromonitoring method or methods being used. Nuclear based and related techniques such as enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) have played and continue to play a vital role in this regard.

The aim of this symposium is to review existing and emerging techniques used in disease diagnosis and control and to carefully put them in context for use in developing countries in the future. Putting the techniques in context for use in developing countries is vital, for techniques should not be used for their own sake or as an end to themselves. Rather, careful consideration should be given to their most relevant and cost effective use in solving practical problems. Often, existing highly relevant techniques can be overlooked in the understandable drive to go ‘high tech’. The
consideration of technologies has to be examined in terms of sustainable impact: what do they offer, in what time frame and at what cost? This process of 'problem oriented' research and application is central to the approach of the Joint FAO/IAEA Division in its programmes in developing Member States.

Having said that, I should also point out that we are in an exciting period of scientific and technological changes. The advent of modern biotechnology in the mid-1970s heralded many new possibilities for solving problems in all branches of biological science, including animal science. The elegance of the science involved and the simplicity of emerging techniques can now be fully exploited in developing countries, with a relatively low economic burden for specialized equipment and infrastructure. Thus, the opportunities offered by modern biotechnology, in many cases, can no longer be regarded as the sole domain of developed countries, but also of developing ones. Indeed, the technologies can be regarded as having extremely high impact possibilities in developing countries as compared to developed countries in that they offer solutions to problems hitherto insurmountable. Some of these uses and their future potential will be discussed in this symposium.

Let me now turn to a related subject, that of computing technology. Many of the methods used are totally dependent on the rapid collection, storage, retrieval, analysis and display of data; that is, they call for the use of modern computing technology. The huge increase in cheap computing power and sophisticated software should be viewed as an integral part of technological advancement in disease diagnosis and surveillance. Computers can be viewed as being essential in GIS (Geographical Information System) technology, in molecular epidemiology, gene sequencing, mathematical modelling, drug design and also in training. I know that this point will be illustrated in many areas discussed at this symposium.

We are looking into the 21st century at this symposium. We have to ask ourselves: what are the global problems in livestock development into the 21st century? We have to ask ourselves: how may advances be harnessed to solve such problems? Looking at the programme of the symposium, I see that we are to cover virtually all aspects related to disease control in the areas of epidemiology, serology and molecular biology, as well as GIS and socioeconomics. I am very much encouraged by this realization that an integrated approach to disease control is necessary. I feel certain that we can look forward to the new century with optimism, which, if realized, will lead to increased livestock production and enhancement of food security in developing countries.
M.H. Jeggo  
Animal Production and Health Section,  
International Atomic Energy Agency,  
Vienna

I would like to take a few minutes to place this particular symposium in context both in terms of the Joint FAO/IAEA Division’s subprogramme in Animal Production and Health and in the more general context of global animal health.

Before doing so, it is pertinent to first look at our current mandate and then at the areas for which we are currently providing support. Our mandate states “to improve livestock production in developing countries through the support of problem oriented research that identifies the constraints on production and develops cost effective and sustainable solutions using nuclear based technologies”. I would like to focus your attention on a number of key phrases in this mandate: that the research we support should be problem oriented; that the solutions should be cost effective and sustainable; and that there must be a nuclear or nuclear related component to the work. Bear in mind that we are a division of FAO and it is the nuclear related link that identifies our activities within the overall FAO programme.

At present, we have activities in three key areas: animal production, veterinary drug residues and animal health. In the near future we anticipate becoming involved in aquaculture. Overseeing all these activities is a rapidly increasing programme of external quality assurance (EQA). EQA now touches all aspects of the subprogramme, and activities range from an accreditation scheme for veterinary laboratories using FAO/IAEA enzyme linked immunosorbent assay (ELISA) kits to introducing ‘good laboratory practice (GLP)’ to our own laboratory activities.

It should be noted that the subprogramme operates through sectional activities here at the Vienna International Centre and through activities conducted at the Animal Production Unit of the FAO/IAEA Agricultural Laboratory at Seibersdorf, some 40 kilometres from here.

When we focus on the animal health component of the subprogramme, it is appropriate to look back some eleven years to the origins of this current programme. At that time in 1986, a group of consultants were given the task of deciding where support could most effectively be provided in the field of animal health, given the mandate of this subprogramme. To undertake this task, it was decided that the nine consultants should visit 15 African countries and learn from them what they perceived as problem areas needing addressing. At a meeting following the country missions, the consultants group concluded that in many developing countries an inability to diagnose and monitor the major diseases affecting livestock was an enormous constraint in providing effective veterinary services and operating disease control programmes. While the reasons behind this inability were many, in part this was due
to the plethora of diagnostic techniques that had to be maintained in national veterinary laboratories. The experts concluded that a nuclear based diagnostic technique, the ELISA, could overcome many of the problems associated with other assays and was applicable for nearly all the major diseases affecting livestock in developing countries. They recommended that the animal health component now embark on a programme of support to national veterinary laboratories, focusing on the introduction and use of the ELISA for the diagnosis and monitoring of the major epizootics.

This consultants group meeting was followed the next year by a symposium of the Animal Production and Health programme similar to that which we are now involved in during this week. While dealing with many aspects of the subprogramme at that time, including nutrition and reproduction, the re-direction of the animal health component of the subprogramme was endorsed, and a number of aspects of how this support should operate were clarified.

Over the past ten years, therefore, the subprogramme has concentrated on providing support for improving animal disease diagnosis through the introduction and use of ELISA based technologies. This has not been a static or passive process, and the focus has changed during this period. Initially, we were concerned with the adaptation of research assays for use in developing country situations, with standardization and validation and with the development of methodologies for transferring ELISA technologies. After some five years we had standardized and validated assays for rinderpest, brucellosis, foot-and-mouth disease and trypanosomosis. We had established strong ties with the Office international des épizooties (OIE), and with the designation of our own laboratory as the FAO/IAEA, OIE and World Health Organization (WHO) ELISA Collaborating Centre. Thus, for the past five years we have progressively focused our support programme on using ELISA based systems within the context of national and international disease control and eradication programmes such as the FAO Global Rinderpest Eradication Programme. As part of this, and starting some three years ago, we have been developing a system to quality assure the use of FAO/IAEA ELISA kits. B. Van der Eerden will present a paper on this later in the week, but we see this as becoming a pivotal activity within the animal health programme, particularly with regard to livestock trade and the needs of the World Trade Organization (WTO) and the OIE for some system of veterinary laboratory accreditation. We have strongly resisted the temptation to enlarge the programme to encompass more diseases than we have the resources to support and have, to a large extent, remained within the confines of ELISA based technologies, although we are currently embarking on support for the transfer of polymerase chain reaction (PCR) technology, but this is still in its infancy.

This symposium, therefore, is perfectly timed to provide us with an opportunity to look at what is new. It offers us a chance to reassess our focus and ensure that the health component of the animal production and health programme remains appropriate to the needs of diagnosticians in the developing world, taking into account both
the diseases that need to be tackled and the technologies that can be harnessed to diagnose and effectively monitor control and eradication programmes.

But within the larger context of global animal health and diagnostic science I believe this symposium is even more appropriately timed. The past three to four years have seen a dramatic change of emphasis in national and international approaches to animal disease control and eradication. This has been brought about in part by the completion of the General Agreement on Tariffs and Trade (GATT) and the potential opportunity this offers in terms of increased international trade in livestock and livestock products, but also through new regional and global perspectives in terms of disease control and eradication as well as those of food security as emphasized during the recent FAO global summit. We now have the global eradication of rinderpest as a realizable goal in the next three to five years. We have eradicated foot-and-mouth disease from Europe, Indonesia and Uruguay, and declared the intention of its eradication from the whole of the Americas by the year 2009. We have a blueprint for the eradication of brucellosis across Europe and the Arabian Peninsula. We have witnessed the eradication of trypanosomosis from Zanzibar, with plans being developed to achieve the same in parts of Ethiopia.

At the same time there is a biological scientific revolution going on. Biotechnology and genetic engineering are beginning to offer solutions that were never thought possible five years ago. Only three years ago, PCR was considered a highly sophisticated research tool fit for use in only the most advanced of laboratories, but it is right now being used routinely in Kenya to confirm outbreaks of rinderpest. Advancing in tandem with information technology and computerization, we seem poised to benefit from a new, enormously powerful arsenal of weapons for use in livestock disease control and eradication programmes.

After my presentation, J.R. Crowther will highlight what modern biotechnologies are available and how they can assist us, but I would like to conclude by asking whether we are sure that we are making the most of these opportunities. In the international control programmes I have mentioned, it is not the introduction of new technology that is providing the impetus, but other factors such as trade pressures. Are senior managers of such programmes aware of how scientific advances could be used to assure success, reduce costs and provide innovative alternative approaches?

In convening this symposium we intended to specifically address this question. We believe that this symposium and its resulting publication will provide the perfect forum to look at what is currently available, what recent success has been achieved and what is potentially available in the near future. While we appreciate that many of the key decision makers/administrators in international organizations, in donor groups and, most importantly, in developing countries are not present today and during this week, we intend to ensure that the symposium publication will be produced as soon as possible after this meeting and distributed widely to provide the necessary information on how the latest scientific advances can assist livestock disease control and eradication.
Several major points need to be examined when considering the impact of biotechnology on livestock disease control and diagnosis, particularly when considering the potential of modern technologies in developing countries.

*Biotechnology is market oriented.* This profoundly affects the level of research and the pace of introduction of technologies, which has consequences for the availability of techniques, reagents and expertise to animal scientists in general. This stems from the identification, by commercial concerns, that the vital profitable market involves human beings directly rather than animals. This leads to several qualifying statements.

*The veterinary market is highly fragmented.* This is unappealing to commercial exploitation by those who are looking for large scale applications in a single field.

*Biotechnology is big.* Linked to the first point, this limits the application of technologies because of considerations and worries about returns on investment in a number of relatively small projects.

*Research is a vital catalyst.* Without fundamental research there is no possibility of applied research. Historically, the main progenitors of such research in veterinary medicine have come from the national governmental arenas of developing countries. This is no longer the case, and there is a severe reduction in the quantity of research being done in developing countries on diseases relevant to these countries.

Several other factors that favour the exploitation of biotechnologies in developing countries should also be stated.

The elegance of the science has led to the development of reagents and methods that are relatively easy to exploit by developing country scientists. The fundamentals of the technologies have been well characterized. What remains is for such techniques to be used to solve ‘local’ problems in developing countries.

Training is an important element in the exploitation of techniques, and there is a good depth of understanding by a large body of scientists in developing countries able to utilize new developments. Such training will be done more and more within developing countries rather than in developed countries through use of local scientists.

The main impetus for research into livestock diseases will come from developing country scientists. The realization by governments of the importance of livestock diseases in socioeconomic terms is a necessary prerequisite for sustained support of their scientists.

Linked to the previous statement is the expectation that there will be increasing pressure on governments to ensure quality in their ‘products’. One element is to be
nationally recognized as being free of diseases before trade is possible with other countries. Such a pressure is building up now through the dealing of standards in international trade agreements. Eventually, international recognition and certification of disease status will be necessary, and this will be enough for governments to rethink the cost–benefit equations concerning livestock diseases. Such a pressure should increase the exploitation of trained staff in the research and development areas.

International organizations still should have a key role in sustaining technology transfer. More particularly, they will be the only bodies able to set up and sustain quality assurance for monitoring results. This should be viewed as an extremely important role and one which will drive the successes of technology transfer and subsequent developments.

The major mistake that can be made is for developing countries to ‘leap-frog’ existing technologies for the sake of a perceived ‘high tech’ ideal. It is the responsibility of all those concerned with evaluating problems that projects, campaigns, etc., are ‘problem oriented’. There are often far more realistic methods able to answer questions, if the questions are first fully defined. Thus, a true vindication of new methods can only be made when they answer questions hitherto unanswerable by existing technologies and when a thorough and realistic cost–benefit exercise has been made. Again, the international organizations have a key role in evaluating projects, and they need to drastically address matters of co-operation in the field of livestock disease control to eliminate duplication of effort and dreadful project design.

The breadth of the knowledge covered by the symposium illustrates the complex nature of disease diagnosis and control. There is little doubt of the value of some technologies. These include the enzyme linked immunosorbent assay (ELISA) which, although it has been around for some 20 years, can still be regarded as the fundamental diagnostic technique now and will be for the foreseeable future.

Molecular techniques are assuming increasing importance, since they tend to be unequivocal in defining disease agents at the sequence level and have exquisite potential, theoretically detecting a single genome copy. The challenge here is to understand what such molecular techniques are telling us about the diseases in the context of past knowledge. Here we need a good deal of applied research to provide hard data. Where this has been done and a large amount of data exists (for example, in molecular epidemiological studies of rinderpest and foot-and-mouth disease), there can be little doubt as to their value.

The symposium also covers topics more closely relating to control of diseases in terms of epidemiology and management. Thus, the developments in computing cannot be ignored. The high power of modern computers, the speed and availability of software and the relative cheapness of computers all add to their exploitation in developing countries. This can be linked to the great possibilities in information technologies through computers and telephone links. The world is shrinking and with it the problems.
There are no substitutes for having a keen, well sustained, motivated veterinary staff with a good infrastructure for disease surveillance. This is often the key problem in developing countries and the major constraint to combating livestock diseases.

There are other factors that compound the problems. These include civil strife, local and global environmental changes, as well as more biological problems of disease agents changing through selective mutation and lack of pest control. All are compounded by alterations in farming systems, for example, intensive and multifactorial farming practices.

No single factor can be held as being totally dominant in its influence on disease per se. All have to be considered, and thus we all have the responsibility of re-examining methods of disease control from the national to the international levels. This symposium is a brief attempt at examining some of the areas of relevance to such an overview philosophy.
SEROLOGICAL ASPECTS

(Session 1)

Chairpersons

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ELISA AND THE JOINT FAO/IAEA DIVISION
Accomplishments and opportunities

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Abstract

ELISA AND THE JOINT IAEA/FAO DIVISION: ACCOMPLISHMENTS AND OPPORTUNITIES.

The enzyme linked immunosorbent assay (ELISA) is an inexpensive yet sensitive and specific tool in the diagnosis of livestock diseases. The use of the ELISA can be associated with many efforts at reducing the enormous wastage of animals caused by infectious diseases. The use of this technology in developing countries was recommended in 1983 by a consultants meeting called by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna. Since then, enormous resources have been committed towards the development and standardization of ELISA kits and their application, mostly in Africa, Asia and Latin America. The IAEA introduced training programmes, initiated regional research efforts, sponsored technical co-operation projects, provided ELISA readers and other equipment and dispatched experts/trainers to assist laboratories in developing countries. International standards for ELISA data expression and evaluation, reagent reference standards and an external quality assurance programme have been developed with assistance from consultants so that laboratories can meet minimal ELISA performance standards. Despite these efforts, the proper validation of ELISAs for application in some geographical areas is still difficult. Unique specificity problems occur because of unknown substances in sera of test animals. The application of ELISA in the field is difficult, and usually laboratories are required for the analysis of samples. Even in the laboratory the technique is easily ‘abused’, resulting in invalid data. Therefore, the ELISAs of the future that will be most effective in control and eradication of livestock diseases in developing countries should be simpler, more specific, less prone to error and portable for use in remote areas. They will probably consist of membrane based ELISA technologies accompanied by handheld portable
touch screen computers for data management. Inexpensive means of identification of tested animals are becoming available — a necessity for disease status certification. A guarantee of disease free status of national herds will assuredly improve the economic conditions of developing countries in the context of the global trade opportunities. The challenge is to provide that guarantee through creative applications of improved ELISAs or their assay relatives.

1. THE BEGINNINGS OF ELISA

1.1. The assay

Enzyme immunoassays combine the specific recognition of antibodies for their target molecules with the catalytic power of enzymes into a single sensitive and relatively simple test. Antibodies, bacterial and viral antigens, nucleic acids and many diverse molecules are detectable by an indicator system in which bound enzymes convert colourless chromogenic substrates into brightly coloured products. By far the best known and most widely used version of this technology is the heterogeneous enzyme linked immunosorbent assay (ELISA). Since its description in 1971 [1], it has been the focus of over 45,000 papers in the scientific literature. Some of the reasons that make the ELISA such a sustainable technique are summarized in Table I.

1.2. Launching ELISA at the Joint FAO/IAEA Division

The exploitation of ELISAs as inexpensive, sensitive and specific tools in the diagnosis of livestock infectious diseases cannot be denied. If applied in developing countries to detect and reduce infectious diseases, the enormous wastage of animals caused by infectious diseases might be brought under control. Accordingly, the use of this technology in developing countries was recommended in 1983 by a consultants meeting called by the then Joint FAO/IAEA Division of Isotope and Radiation Applications of Atomic Energy for Food and Agriculture Development [2]. The consultants’ recommendations were to:

(1) Increase specificity of established immunoassays;
(2) Identify problems associated with the application of ‘modern techniques’ in laboratories in developing countries for detecting antibodies/antigens;
(3) Train professional and technical staff in modern techniques, including immunoassays;
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(4) Encourage development of new immunoassays for diagnosing important bacterial and viral diseases of ruminants in tropical countries;

(5) Support implementation of the standardization of immunoassays between laboratories;

(6) Provide training in introduction, development and application of immunoassays.
Since 1983, large resources have been committed by the Agency towards the development and standardization of ELISA kits and their application, mostly in Africa, Asia and Latin America. The Agency has introduced ELISA training programmes for professionals and technical staffs of Member States, initiated regional research efforts to adapt ELISAs for detection of specific diseases in a country or region, supplied many laboratories with ELISA readers and other equipment to acquire and evaluate ELISA data and dispatched experts/trainers to assist laboratories in developing countries.

During the first ten years of the programme, efforts were made to refine the transfer of technology to developing countries so that they could apply validated ELISAs to their specific needs. Many problems in technology transfer and application have been encountered and addressed. Resolution of these problems has made it possible for diagnostic aids such as ELISAs to have a greater impact on national economies.

2. ELISA REAGENTS, PROTOCOLS AND LABWARE

2.1. ELISA reaction schemes

Dozens of reaction schemes have been devised for ELISAs, and reference to a textbook dealing with ELISAs is necessary for a review of the methods. The most commonly used methods initially were indirect and sandwich ELISAs. More recently, the advantage of competition ELISAs has been recognized, particularly in conjunction with availability of monoclonal antibodies (MAbs). The indirect and competitive systems have been the core of the Joint FAO/IAEA Division programme.

2.2. Microtitre plate technology: Adsorption of antigens to surfaces of wells

Antigen adsorption to wells of polystyrene microtitre plates was variable in early ELISAs because of poor plate production techniques and a lack of understanding of the physical chemistry of antigens adsorbed to the plastic. The ‘edge effect’ precluded use of the outer wells of microplates in early ELISAs. Optimal antigen concentrations were initially determined empirically; now plates are designed to accommodate antigens of at least two types with high reproducibility between plate wells. Antigens that are predominately hydrophobic require one type of plate, whereas other antigens bind best to surfaces that accommodate both hydrophobic and hydrophilic macromolecules. Research has clarified the rationale for use of various types of commercial polystyrene plate [3–5].
2.3. Sample handling and pipetting

As with any other serological assay, ELISA is prone to error when serum samples are degraded, either by bacterial contamination or physical factors. Sample collection, transport and storage are significant problems in developing countries where refrigeration and courier services may not exist. This problem may be the principal factor in driving ELISAs towards simplified protocols that would be appropriate as pen-side technologies, thus obviating the need for transport of samples.

Automated pipettors and diluters have reduced pipetting errors in well equipped laboratories. Such equipment also increases the testing capacity of laboratories. Because of the amplification effect in ELISA, accurate pipetting is essential, because small pipetting errors can translate to large discrepancies in results.

2.4. Plate washing systems and blocking reagents in wash fluids

Automated plate washers are usually found in well developed laboratories. When properly maintained, such washers can reduce variability in ELISAs and increase the efficiency of the laboratory. Surprisingly, however, these washers are a great potential source of error for ELISAs. If the probes that aspirate fluids from the wells are not aligned properly, the fluid remaining at the interface of the bottom and side of a microtitre plate well will not be aspirated. The remaining reagent inevitably affects the accuracy of the readings for that well, usually elevating the colour development because of excess antibody not properly removed by washing. Automatic washers must be cleaned often. The buffers and blocking reagents used in wash buffers are good substrates for bacterial growth. As a result, the manifolds of plate washers are easily contaminated, particularly in warm laboratories. Use of a simple wash bottle, with vigorous slapping of the plate on towelling to remove any trace of wash solution, has proved to be an excellent way of reducing problems of repeatability (between wells) and reproducibility (between plates or between runs) associated with plate washing.

To prevent binding of non-specific antibodies to the microplate wells, Tween-20 detergent is usually an effective additive to the phosphate buffered saline (PBS) washing buffer. For some antigen/antibody systems in ELISA, however, the detergent alone is insufficient to block all non-specific activity attributed to adsorption of antibody from the sample to the plastic. Studies have shown that inexpensive milk powder, purchased at a local food store and added at a concentration of 2% to the Tween-20 PBS buffer, is an excellent blocker. It is a cost effective replacement for more expensive blocking reagents such as bovine serum albumen, ovalbumen or gelatin. Addition of blockers to the washing buffer enhances its potential as a growth medium for bacteria. Thus, storage of these buffers for extended periods to prevent wastage is a primary problem in developing countries. Small amounts of
buffer should be prepared, kept refrigerated and expended in a timely way to prevent this problem.

2.5. Enzyme substrate choices

Peroxidase has become the most widely used enzyme in ELISAs [5]. It also affords the greatest analytical sensitivity among the enzymes commonly used, but is subject to inactivation by contaminated water or even polystyrene if Tween-20 is not included in the PBS wash solution. The substrate for peroxidase, hydrogen peroxide, must be used at the correct concentration to avoid interassay variations and enzyme inactivation. Typical peroxidases transfer hydrogen from the chromogenic hydrogen donor to the substrate, resulting in a change in colour of the chromogen. Lack of attention to details in preparing and using enzymes and substrates is lethal to ELISAs.

2.6. ELISA readers, computers, software and data expression

The Joint FAO/IAEA Division has provided ELISA readers and transferred computer hardware and software to many laboratories in developing countries on three continents. This has allowed acquisition of quantitative data and the means to adjust raw optical density data to the activity of standard samples included in the assay through various algorithms. The data from different runs of the assay are then comparable, both within and between laboratories. Computerization has also provided a means of encouraging the use of standardized plate formats for the Joint FAO/IAEA Division's kits and of collecting data for internal quality assurance assessments. The EDI computer program has been modified to accommodate the unique needs of developing countries. This program will be an essential component in establishing assurance that laboratories are producing ELISA results that comply with both internal and external quality assurance criteria.

A Joint FAO/IAEA Division consultants meeting was convened in 1992 to review aspects of ELISA data expression, definition of reference standards, quality assurance and criteria for validation of ELISAs. The consensus of that meeting was published [6] and serves as a guideline for ELISA standardization and validation for detection of antibodies in infectious disease diagnosis [7].

3. USES AND ABUSES OF ELISA KITS

The definition of what comprises a kit rests on considerations of test validation, the perceived objective of the kit, the 'market' or end users who are to exploit the kit and factors involved in sustainability.
Thus, the equation for a kit is complex, involving technical performance, supply and profit motives and continuity. Kits, at best, also have to be accepted by international bodies to fulfil their ultimate role of standardization of a given approach and allow harmonization with other tests measuring the same or similar factors.

It would be useful here to examine generalized ideas about kits in all fields of human and veterinary applications. From where do kits originate? It could be assumed that kits always fill a need identified by careful assessment of existing problems and the current solutions, resulting in a direct route from need to development to end product. This generally is not true. Rarely is there such a 'clean' scenario. Rather, some meagre research developments are made to prove the feasibility of an approach, followed by a relatively moderate amount of validation and then exploitation. The success of a kit and its long term benefits usually depend on the skill of the kit developer, the reagents selected and the care given to assay validation. Kit development and application take into account the profit motive as well as technical aspects. The possibilities for profit are great in the human medical sphere and concentrated on relatively few diseases, whereas the veterinary market is fragmented, centred more on developing country application and hence lacks the appeal to the commercial sphere.

Since kits can be poorly designed and improperly applied as a diagnostic test, it is probably useful to describe the ultimate kit. Such a description may then be examined against kits that are being used, or aid in designing better kits. It is important to note that, for biological systems, there is no perfect kit that can be applied universally with the same performance under any given conditions. All kits have their limitations. Kits may require modification of reagents/conditions/protocols to account for the many physical, chemical and biological variables that must be allowed for when applied under local conditions.

A kit should contain everything needed to allow testing, including software packages for storage, processing, demonstration and reporting of data. The reagents should be absolutely stable under a wide range of temperature conditions. The manual describing the use of the kit should be 'foolproof'. The kit should be validated 'in the field' as well as in research laboratories. All containers for reagents should be leakproof. The kit should contain internal quality control samples. External quality assessment should be included in the kit 'package'. Data on the relationship of kit results to those from other assays should be included. Attention should be paid to ensuring that all equipment used in the kit is calibrated (spectrophotometers, pipettes). Training courses in the use of the kits should be organized. Information exchange should be established to allow rapid 'on-line' help and evaluation of results where there are perceived problems. The internationally sanctioned supply and control of standards used in the kits should be maintained.

All these factors have been examined for the original reagents and resources supplied with the kits that have been used in developing countries through co-operative
efforts with the Joint FAO/IAEA Division and research oriented institutions. Thus, it is fairly obvious that the simple word 'kit' encompasses a complicated scenario. Several of the factors involved in ELISA kits are described below to illuminate the common problems that are often overlooked when considering use of ELISA kits.

3.1. Reuse of expendable supplies

Even when transfer of the technology to developing countries has been successful, it has been difficult to sustain the use of ELISAs in those countries due to the cost of expendable supplies and basic chemical reagents. Alterations in protocols are sometimes introduced because of fiscal constraints. This has led to reuse of plasticware such as pipette tips and even attempts to wash and reuse microtitre plates. In some situations, the same reagent boat (the vessel for use with multtip pipettors) has been used for pipetting antigen, conjugate and substrate solutions. These approaches degrade ELISA data.

If the intent is to reuse pipette tips, usually they are all placed in a single beaker containing water and possibly some detergent. When a sufficient quantity of tips is accumulated, they are 'washed'. Submerging and washing tips in a detergent solution does not necessarily remove traces of residual reagent. A stream of wash solution must be forced through each pipette tip to ensure that residual reagent will not interfere with reactions associated with subsequent use of the tip. For example, residual conjugate solution in a tip that subsequently is used for pipetting substrate solution may cause development of coloured product, leading to a false positive reaction. If tips are to be washed and reused, a minimal precaution is to always reuse tips for only one reagent. This requires placing of tips into separate containers after use. There is a need for a standard procedure for washing pipette tips to ensure that variability is not introduced into an ELISA because of contaminating residual reagents in pipette tips.

Use of plastic reagent boats, which hold solutions being pipetted by multichannel pipettors, is another potential source of variability in assays. Proteins of conjugate solutions are not easily washed from plastic vessels. If substrate solution is then pipetted from the same boat, residual conjugate may cause colour development in the substrate solution. Reagent boats should be used for only one type of reagent (e.g. conjugate solution) and labelled for reuse with only that reagent. This will preclude the contamination problem.

3.2. ELISA kits and the problem of water quality

Reagents provided with the FAO/IAEA and commercial ELISA kits invariably require dilution. The most common problem with use of kits in developing countries is the source of water used in buffers to make reagent dilutions, particularly conjugate
solution. Water containing impurities can inhibit enzyme activity or impair the enzyme's substrate. Water used for successful cell cultures is of the quality required to ensure that ELISAs will not be affected by poor water quality.

3.3. False assumptions about use of ELISA kits

It is not true that slight changes in protocol will have no effect on test results; although the changes may be slight, they can be very detrimental to the stability of ELISA results. It is essential to follow the protocols exactly, without deviation, to achieve minimal variability in the assay. Similarly, it is not feasible to make 'adjustments' to reagents in a kit and expect reproducible results. In a properly validated assay, reagents are balanced against each other; manipulation of one reagent may, therefore, affect the performance of other reagents, leading to inaccurate results. If a decision is made to alter reagents in the kit, then the kit needs to be fully revalidated.

The tendency is that ELISA kits for infectious diseases of domestic animals are initially validated using European or North American serum samples from animals indigenous to those areas. If such kits are applied to animals in tropical or subtropical ecosystems, it is highly likely that test results will not classify infection status with the same proficiency as when the assay is applied to cattle from temperate regions. Therefore, it is essential that a kit's performance characteristics be established under local conditions.

The requirement to reassess the performance under local conditions leads to other problems. For example, it is often difficult to acquire samples from animals of known infection status in developing countries. These samples are needed to establish specific 'cut-off' values among test results that separate infected from uninfected animals at acceptable levels of diagnostic sensitivity and specificity for the assay.

4. IMPLICATIONS OF ASSAY VALIDATION

A validated assay consistently provides test results that identify animals as positive or negative for antibody (or other analytes) and, by inference, accurately predicts the infection status of animals with a predetermined degree of statistical certainty [8]. The essential feature to remember is that kits per se are being 'validated' continuously through use. Any set of new conditions constitutes a basis for re-examination of that kit's performance. The continual assessment of performance and the need to make adjustments are inescapable aspects of laboratory/kit usage towards diagnosis of infection status and rely heavily on the training and ultimate expertise of operators, on defined standards and control reagents and on harmonization of results obtained from different sources.
The continuous assessment of performance is at the root of the need to provide well established methods for examining the quality of results on a day-to-day basis within specific laboratories (internal quality control, IQC). This is also an integral part of any externally moderated exercise in assessing laboratories using kits (external quality assurance programmes (EQAPs)).

Inherent in the validation process are a large number of considerations [8]. This paper cannot describe the specific details of methods used to ‘maintain’ and ‘tune’ an ELISA. However, five points will direct attention to the principal considerations:

1. Assay validation — what is a well validated assay?
2. Requirements for validating an assay.
3. Assay suitability for local conditions.
4. Resetting a cut-off in an assay.
5. New estimates of diagnostic sensitivity and specificity.

Diagnostic sensitivity (D-Sn) and specificity (D-Sp) are estimated initially when the assay is developed. These estimates are established by selection of a cut-off in the assay that separates test positive from test negative animals. The reference animals used for this purpose are deemed either infected or uninfected by some gold standard criterion. When the test is applied to a targeted population in a developing country, it is possible that many false positive results occur because of biological differences in the target population (compared with the reference gold standard population originally used to set the cut-off). Likewise, false negative results may occur because the assay is not sufficiently robust to perform properly under laboratory conditions in tropical developing countries. This necessitates re-establishment of the cut-off for the developing country based upon testing of samples from animals in the indigenous population. This is often nearly impossible because of the inability to establish the infection status of indigenous reference animals that would serve as the ‘gold standard’ for re-establishing the cut-off in the assay. For these reasons, estimates of D-Sn and D-Sp for a kit, as altered for use in the developing country, need to be constantly upgraded to lend ever greater levels of credibility to the validity of test results. The establishment of D-Sn and D-Sp of a kit used in a developing country remains a variable that is contentious and very difficult to establish.

Considerable attention, therefore, must be given to these factors if the assay is ever to achieve international recognition. Indeed, movement towards use of MAbs in a competitive format would help to alleviate this problem because of their inherent high degree of analytical and diagnostic specificity. As such assays become more commonplace, their enhanced performance would help to alter dogmas that have become established based on other, less exacting methods.

One force that is driving the move towards greater reliability in test results is the fact that assay validation criteria must be acceptable to the international
community. This is becoming an important consideration in the worldwide trading of livestock and livestock products, because certification of movement of animals will depend on data obtained in accredited laboratories by accepted and externally monitored tests.

5. THE FUTURE OF ELISA

The future of ELISA as an overall method of colorimetric reading of tests is ensured even with existing systems involving direct and indirect methods, sandwich methods and the same methods used in a competitive or blocking format. The inherent analytical sensitivity range of the ELISA is ideal diagnostically for the detection and quantification of antibodies. For certain uses in detecting antigen, the analytical sensitivity of a relatively simple ELISA can be too low, and methods for amplification of the signal through avidin/biotin regimens can be further exploited. The exploitation of ELISA in conjunction with molecular methods, in particular polymerase chain reaction technologies, will also accelerate.

MAbs provide the greatest revolution in serology by supplying exquisite specificity if required, so that ELISAs involving their use will continue to be exploited provided there is a sufficiently active pure research base to produce, define and supply MAb s in the future. Other areas of development include simplifying ELISA systems without loss of assay performance through the move towards competitive assays, membrane based ELISAs supplanting conventional ELISAs, visual reading, feasibility for use as pen-side tests and the use of handheld computers for recording data.

6. CONCLUSIONS

The use of heterogeneous solid phase ELISAs has revolutionized diagnostic serological methods. Such methods have been exploited in all branches of biological sciences for the measurement of antibodies and antigens from a large variety of samples under a great number of local conditions.

The versatility of the ELISA means that individual laboratories can devise their own assays easily. This is an advantage where there are few other facilities, but also a disadvantage when standardization of test protocols and reagents has to be a priority. Standardization is becoming increasingly important in terms of ratifying results from laboratories to allow freedom of livestock movement in trade.

With adequate recognition of the factors that cause variation in the ELISA, it is possible to provide kits that perform specific tasks to provide data to be used in disease management. This is not easy, because the variability of the test itself (including procedural and operator error) has to be addressed as well as the variables of the
biological systems being studied. Thus, kits at best provide a well defined system, but they need to be regarded as only as good as the last set of results. All tests should have both IQC and external monitoring to provide harmonization of data obtained from all laboratories. This will also provide confidence to international bodies that are given the task of ensuring test result validity based on quality assurance criteria. The ELISA does provide the possibility for absolute standardization at a particular time. It also allows continuous adjustment to methods, because the results are based on absolute, quantifiable readings that are linked to standard sera of known activity. The future is ensured for the basic (now conventional) approaches as well as for ELISAs involving the limits of ingenuity of molecular biologists, immunochemists and diagnosticians. Thus, tests will exploit other amplification systems and will be simpler, cheaper, with unequivocal results available more quickly.

An ultimate objective would be to make ELISAs virtually foolproof, so that non-trained personnel virtually anywhere could conduct them. This goal may not be achievable; in an examination of over 100 ‘incidents’ where laboratories have reported an insurmountable problem with an ELISA kit, 93% of the problems could be associated with the operator (and solved almost immediately) and not the reagents. Indeed, ELISAs are only tools. If misused, they will mislead. If properly used, they will be a major component in identifying livestock disease/infection problems on a worldwide scale.

REFERENCES

THE USE OF ELISA IN THE CONTROL OF RINDERPEST

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Abstract

THE USE OF ELISA IN THE CONTROL OF RINDERPEST.

The enzyme linked immunosorbent assay (ELISA) has played an important role in the control of rinderpest and will continue to play a major role as the world moves towards the eradication of rinderpest. In the early phases, control was achieved through the use of mass vaccination. Because of the thermolabile nature of the attenuated vaccine, seromonitoring was essential to determine the immune status of the national herd. This was accomplished by using first the indirect and later the competitive ELISA. Now, as countries move towards eradication and have ceased vaccination, the competitive ELISA is being used for serosurveillance to detect residual foci of infection. The introduction of the immunocapture ELISA for the detection of rinderpest antigens has allowed rapid, sensitive, specific, differential diagnosis of both rinderpest and peste des petits ruminants viruses. In the future, if recombinant vaccines are adopted for use in the eradication campaigns, ELISA may then be used for differentiation of vaccinated from infected animals. ELISA has been successfully introduced into many laboratories throughout the world to form the IAEA ELISA Network. This has been achieved through the use of fully field validated, standardized, quality controlled diagnostic kits using standardized protocols and after intensive training programmes. The quality of the results is maintained and assured through the use of both internal and external quality assurance programmes. The use of a single kit throughout Africa, the Middle East and Asia has greatly assisted in standardization, interpretation and international acceptance of results.

1. INTRODUCTION

One of the oldest virus diseases known to humanity, rinderpest, has devastated cattle populations for centuries. Since the advent of the attenuated tissue culture vaccine, control measures have been based mainly on mass vaccination programmes.
Although administration of a single dose of vaccine induces lifelong immunity, the vaccine virus is both thermolabile and sensitive to sunlight. It is therefore rapidly inactivated under tropical field conditions. This requires the use of a combination of freezers, fridges, vacuum flasks and ice making machines, forming a 'cold chain' to maintain the viability of the vaccine in the field. A successful vaccination campaign depends on the integrity of the cold chain, the potency of the vaccine, the efficiency of the vaccination teams and the extent of vaccine cover achieved throughout a country. Seromonitoring is essential to determine the immune status of the animals following vaccination. Indirectly, this will also give an indication of vaccine potency and vaccination team performance. In the past, the virus neutralization test was used for seromonitoring. This technique requires the use of tissue culture and the provision of sterile serum samples. Both of these requirements are difficult to achieve in many developing countries. The test itself is labour intensive, requires five to seven days of incubation and is read microscopically. It would be virtually impossible to test the required number of sera (approximately 20 000 per year) by using this method.

The enzyme linked immunosorbent assay (ELISA) offers many advantages. The reagents are extremely stable and usually freeze dried to give long shelf life. Commercially produced enzyme conjugates for many animal species are widely available, offering the opportunity for bulk purchase of quality controlled reagents, thus ensuring continuity of supply. The introduction of enzyme substrates, buffers and hydrogen peroxide in tablet form has greatly eased diagnostic kit formulation and test standardization. ELISA readers can read a 96 well plate in 5 s, allow statistical analysis of the data and link directly to a computer database. The ease with which large numbers of samples may be tested has permitted large scale epidemiological surveys that would have been impossible using 'traditional' assays.

Because of all the advantages mentioned above, an indirect ELISA was developed for the detection of antibodies to rinderpest.

2. INDIRECT ELISA

A simple indirect ELISA for the detection of antibodies against rinderpest virus was developed in 1982 [1]. Rinderpest antigen was pelleted from infected tissue culture supernatant (secondary bovine kidney cultures) by ultracentrifugation. Blocking buffer was phosphate buffered saline (PBS) supplemented with 3% bovine serum albumin and 0.1% Tween-20. Sera were tested at a dilution of one to two and detected by using rabbit anti-bovine IgG horseradish peroxidase (HRPO) conjugate. The substrate/chromogen combination used was hydrogen peroxide and ortho-phenylenediamine. Comparative testing against the virus neutralization test gave a 95% correlation. At this stage, the test was used purely as a research tool.
In 1985 the Institute for Animal Health (IAH) was asked to assist in the emergency programme for the eradication of rinderpest in Tanzania. Since this involved testing large numbers of sera and the local laboratory lacked facilities for tissue culture, it was decided to use the indirect ELISA. Large quantities of antigen were required so that antigen preparation was modified to an infected cell sonicate. This allowed the production of large batches of antigen and reduced batch to batch variation. The Madin Derby bovine kidney cell line was used for antigen production; this was more convenient than primary and secondary cell cultures and gave a more reproducible product. Sera were tested at a dilution of one to four (compared to the previous one to two), as this resulted in a better binding ratio. Owing to the expense of bovine serum albumin, alternative blocking agents were examined, and dried milk powder was shown to be optimal. The test performed extremely well under local conditions, and 80,000 sera were tested over a period of four years.

Following validation under field conditions in Tanzania a diagnostic kit was developed in collaboration with the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna. The non-infectious components were supplied by the IAEA, while the antigen and control antisera were provided by the IAH. The test was adopted by the Pan African Rinderpest Campaign (PARC) and over a period of five years successfully introduced into 21 countries in Africa. Technology transfer was achieved by the use of multinational training courses held in Africa, followed by assistance in establishing the assay in each individual laboratory. There has been technical support whenever needed and continuous updating of the kit as improvements evolved. The major problem was the quality of the local distilled water. In some cases, this drastically affected the test and resulted in reduced binding ratios and hence a poor separation of negative and positive populations. This was partly solved by the supply of sterile distilled water, sufficient to reconstitute the freeze dried kit reagents, and also by providing modern distillation and deionizing apparatus.

The indirect ELISA proved suitable for evaluating the immune response to rinderpest vaccination in cattle. However, as the PARC programme progressed and most of the cattle population had been vaccinated and become antibody positive, serosurveillance of other animal species became increasingly important. There was an increasing need to test sera from sheep, goats and game animals, all of which could possibly act as reservoirs of infection. Difficulties had been experienced in the development of an indirect ELISA for sheep and goat sera, because of the high non-specific reactivity of such sera. This was further complicated by the presence in some countries of a closely related virus, peste des petits ruminants (PPR). Antibodies to PPR cross-react with rinderpest virus in the rinderpest ELISA. The lack of commercially available HRPO conjugates suitable for the various species of game animals also posed problems.

The solution to all of these problems lay in the development of a competitive ELISA using a rinderpest specific monoclonal antibody (MAb).
3. MONOCLONAL ANTIBODIES AGAINST RINDERPEST VIRUS

Before producing MAbs, it was important to consider the structure and function of the various rinderpest viral proteins and compare their specificity and cross-reactivity with other related viruses, particularly PPR virus. Amino acid sequencing data have shown the H protein to be the most variable viral protein, with little sequence homology between rinderpest and PPR virus isolates [2]. Radioimmune precipitation data showed little cross-reaction between the H proteins of the two viruses compared to the F and N proteins, which had high levels of cross-reactivity [3]. DNA probes using the H gene and the N gene were both shown to be rinderpest specific [4]. The H protein is involved in host cell attachment, and antibodies to H protein have a major role in virus neutralization [5]. From the above evidence it was decided that MAbs against the virus haemagglutinin (H protein) would be most suitable for use in a rinderpest specific assay. Antisera from cattle vaccinated with an experimental vaccinia rinderpest-F recombinant vaccine were found to be virus neutralization positive but ELISA negative (using the standard ELISA antigen). When purified antigen was used in the ELISA instead of crude sonicated infected cell extract, high levels of anti-F antibody were detected in these sera. This indicated that some epitopes were not expressed on the crude ELISA antigen, probably owing to masking by host cell proteins, and highlighted the need for purified antigen preparations for immunization and screening procedures during the production of MAbs.

MAbs were produced against purified rinderpest vaccine strain virus and screened by indirect ELISA for specificity against rinderpest and PPR viruses. Those MAbs that were rinderpest specific were characterized by immuno-precipitation and Western blotting. All the MAbs were also evaluated for use in a competitive ELISA. One rinderpest specific MAb against rinderpest haemagglutinin (designated C1) was found to be suitable for use in a competitive ELISA. Using a similar procedure, MAbs against PPR virus were also produced, and a PPR specific MAb against PPR haemagglutinin (designated C77) was found to be suitable for use in a competitive ELISA.

4. COMPETITIVE ELISA

4.1. Rinderpest

The rinderpest competitive ELISA [6] was found to detect antibodies to a range of mild, virulent and vaccine strains of rinderpest virus and, unlike the indirect ELISA and the virus neutralization test, there were no cross-reactions with antibodies to PPR. Also, compared to the indirect ELISA, there was a far greater separation of positive and negative populations when sera were evaluated by frequency distribution analysis. As virus neutralizing antibody levels have been shown to correlate with
protection, it was imperative that any new assay should correlate with the virus neutralization test. In comparative studies using field sera from the Yemen, there was a 98% agreement between the competitive ELISA and the virus neutralization test. The major advantage of the competitive ELISA is the ability to test sera from any species whilst using a single enzyme conjugate. This will allow epidemiological studies on the role of sheep and goats and wildlife in the epidemiology of the disease. In the past this has been difficult because of high levels of cytotoxicity in these species serum and also the lack of species specific enzyme conjugates.

4.2. PPR

The PPR competitive ELISA [6] was found to detect antibodies to all strains of PPR and, like the rinderpest competitive assay, gives a good separation of negative and positive populations; it enables sera from all animal species to be tested by using a single enzyme conjugate. Although the MAb does not react with rinderpest antigen, some antibodies to rinderpest virus do compete in the assay. However, a combination of the two competitive assays allows differential diagnosis of the two related diseases. This has been confirmed by the examination of sheep and goat sera following vaccination with rinderpest vaccine in the Gambia. The use of the two competitive assays could differentiate between animals which had responded to vaccination and those which had failed to do so because of the presence of antibodies to PPR virus before vaccination. Antibodies to the two viruses are cross-protective; therefore, prior infection with PPR virus prevented replication of the attenuated rinderpest vaccine.

Both of these MAb based assays offer major advantages in standardization, which is a significant consideration in diagnostic kit development, especially for use in international eradication campaigns. MAbs, which are homogeneous in nature and available in unlimited amounts, offer the opportunity for all laboratories to have identical diagnostic reagents.

5. RINDERPEST ANTIGEN DETECTION

Although presumptive diagnosis may be based upon clinical observations or seroconversion of non-vaccinated animals, definitive diagnosis has always been based upon isolation of the infectious agent in tissue culture followed by neutralization of infectivity by virus specific antiserum. This is a long, involved process, requiring tissue culture and sometimes taking many weeks.

Rinderpest antigen may be detected by agar gel immunodiffusion and counter immunoelectrophoresis, and these techniques are widely used throughout Africa, Asia and the Middle East. Unfortunately, the techniques are very insensitive and lack specificity.
More sophisticated techniques such as radioactively labelled DNA probes have been used for a number of years for differentiating rinderpest from PPR virus strains [4]. They were used to diagnose for the first time the presence of PPR virus in India and also to demonstrate the relationship between 'phocine distemper' (seal morbillivirus) and rinderpest, PPR and canine distemper viruses. More recently, the polymerase chain reaction (PCR) has provided the ability to rapidly amplify a single copy of RNA to detectable levels. These techniques, while invaluable to the well equipped Reference Laboratories, have not been widely transferred to developing countries because of the cost of reagents and the lack of local facilities (problems which are now being overcome through IAEA support).

5.1. Immunocapture ELISA

An immunocapture ELISA has been developed by CIRAD-EMVT [7] in collaboration with the Joint FAO/IAEA Division and is now reaching the final stages of field validation. The test is sensitive and specific and allows the differential diagnosis of these two closely related viruses. The test is based on three MAbs, all of differing specificities. A broadly reactive MAb is used for trapping or capturing both rinderpest and PPR antigens; then rinderpest specific and PPR specific MAbs are used as detecting antibodies. These detecting antibodies are biotinylated, and specific binding is detected by using a streptavidin–HRPO enzyme conjugate. Once introduced into all laboratories, this will greatly reduce the delay between clinical diagnosis and confirmatory diagnosis in the laboratory.

5.2. Pen-side diagnosis

Although not an enzyme labelled immunoassay, chromatographic strip assays are analogous to immunocapture ELISA and offer a real opportunity for sensitive, specific pen-side diagnosis. The 'Clearview' technology, patented by Unipath, has been adopted for the detection of rinderpest antigens in ocular secretions. The basis of the test is the coating of anti-rinderpest MAb onto blue coloured latex particles. The reaction takes place on a nitrocellulose solid phase in a device the size of a microscope slide. Ocular secretions are added through a small sample window, where they rehydrate the lyophilized antibody labelled particles. If rinderpest antigen is present, it binds to the MAb, and the antigen/MAb/particle complex migrates along the nitrocellulose strip (owing to a wick effect) until it meets an immobilized band of anti-rinderpest capture antibody. If antigen is present it will be captured, resulting in the buildup of blue latex particles which will be seen as a blue line. Excess MAb labelled particles will continue to migrate down the strip until they are captured by an immobilized band of anti-mouse antibody. This will result in a further blue line. This
control line is to demonstrate that the sample has moved down the length of the strip. Samples are considered positive if two lines, a test line and a control line, are visible.

The test is in the final stages of optimization and validation, but has already yielded positive results when used in the field in Tanzania and Pakistan.

6. FUTURE DEVELOPMENTS

Research is under way to produce rinderpest antigens in expression vector systems such as baculovirus, yeast and *E. coli*. The use of synthetic peptides is also being evaluated. These may be selected either in the ‘classical’ manner by producing overlapping polypeptides based on sequence data, or by the use of the mimotope/pepskan strategy [8]. Vector expressed proteins and synthetic polypeptides offer the opportunity for highly defined, non-infectious antigens for use in diagnostic assays.

The rapidly developing techniques of bioluminescence, chemiluminescence, fluoroimmunoassay and the use of biosensors may well have a role to play in disease diagnosis in the future. This depends on the development of appropriate, economically priced readers and the supply of commercially produced reagents. At present, the increased sensitivity offered by these techniques is neither necessary nor appropriate for antibody detection, especially in developing countries.

7. CONCLUSIONS

ELISA has played a major role in control and eradication programmes for rinderpest. The indirect and competitive assays have allowed rapid analysis of many thousands of sera for seromonitoring. The results from seromonitoring have proved an invaluable management tool for national co-ordinators and have highlighted problem areas in the field. The introduction of the immunocapture ELISA has provided many laboratories with diagnostic capability for the first time.

One of the main advantages of these MAb based assays has been the degree of international standardization which has been achieved. At present, all scientists, whether in Africa, the Middle East or Asia, are using the same test and in most cases the same batch of reagents. This degree of uniformity helps immensely in border harmonization discussions since the data presented by each country are universally acceptable.

Although not an ELISA, the chromatographic strip assay offers the first real opportunity for a sensitive, specific pen-side test. Further pen-side tests are being developed for the detection of foot-and-mouth disease, PPR and bluetongue virus antigens. This will enable rapid differential diagnosis in the field.
Diagnostic methods are now being developed using an interdisciplinary approach involving molecular biology, immunology and epidemiology. Molecular biological studies have led to a better understanding of protein structure and function. Immunological research has resulted in an appreciation of the role and relative importance of individual proteins in the immune response to infection and possible mechanisms of protection. The requirement for highly sensitive and specific assays necessary for epidemiological studies has led to an amalgamation of these three sciences, resulting in today's increasingly sophisticated assay systems.

REFERENCES


BRUCELLOSIS: DEVELOPMENT AND SUCCESS USING ELISAs FOR DIAGNOSIS

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Abstract
BRUCELLOSIS: DEVELOPMENT AND SUCCESS USING ELISas FOR DIAGNOSIS.

Brucellosis is a highly infectious disease of a substantial number of animal species. Because of its pathogenicity to humans, the cost and the time involved, classical diagnosis by isolation of the causative microorganism is not commonly used for diagnosis of individual animals. Presumptive diagnosis has been made for about 100 years by using serological tests. Initially, a slow agglutination test was developed and later modified to increase the sensitivity and to decrease the time. Complement fixation and gel diffusion tests were also developed and, in some cases, widely used. The development of primary binding assays, in particular, enzyme immunoassays, has provided tools for diagnosis that are more accurate than conventional serological tests. The enzyme immunoassays, the indirect and competitive formats, have different uses but are both potent additions to the diagnosis of brucellosis in that they may be used for rapid and accurate assessment of antibody in milk and serum in the absence of interference by antibodies resulting from vaccination. Another type of primary binding assay, a fluorescence polarization assay, was also developed for the diagnosis of brucellosis. This assay, which is a homogeneous format, has the added advantages of being simple to perform, very rapid, potentially inexpensive and capable of being performed outside the laboratory.

1. INTRODUCTION

Brucellosis is an infectious disease caused by members of the genus Brucella. Some of the species are relatively host specific, for example, B. neotoma, which has only been isolated from the wood desert rat. Other species have multiple hosts, for example, B. abortus, which can infect humans, ruminants, camelids and others. Because of the public health implications, B. abortus infection of cattle has received the greatest attention in terms of diagnosis, prevention, eradication and research; however, other species, such as B. suis and B. melitensis, are of equal, or perhaps greater, importance as human pathogens. Since B. abortus has been most intensely studied, most of the following discussion deals with this species in its natural host, i.e. cattle.
Direct contact with infected materials by farmers, veterinarians and slaughterhouse workers and exposure to infected milk or dairy products are the main routes of spreading the disease from animals to humans. Humans are considered a dead end host, but infection is usually lifelong, with symptoms appearing at erratic intervals.

Because of the serious public health aspects of brucellosis, the economic implications and the barriers which the disease causes to international trade, many countries have instituted control and/or eradication programmes. Two major types of programme have been used. One involves vaccination of all calves under the age of eight months with \textit{B. abortus} strain 19, an attenuated live vaccine. The second type of programme involves identification and segregation (or removal) of infected animals. In most cases, control and eradication programmes consist of a combination of vaccination and identification of infected animals. One of the unfortunate side effects of vaccination with \textit{B. abortus} strain 19 is that it may interfere with diagnosis of field strain infected animals. Numerous efforts have been made to circumvent this problem. In the case of vaccinated calves, initial diagnostic testing does not begin for some ten months post-vaccination to allow vaccinal interference to decline. In some cases, vaccination of adult animals with a reduced dose of vaccine may also interfere, and in a small percentage of cattle actual infection with strain 19 occurs. The latter animals pose the same public health problems as those infected with field strains, and the bacterium is frequently shed in the milk. Another problem is that immunity due to vaccination is not lifelong. Some of the above difficulties may be overcome in the future by the use of alternate vaccines, for example, \textit{B. abortus} strain RB51, which lacks the antigens that normally result in diagnostic interference but still affords immunity.

2. DIAGNOSIS

The most accurate diagnosis of brucellosis involves isolation and identification of the causative microorganism. This is generally accomplished by removing and dispersing tissues and then attempting to get the organism to grow on laboratory media. Suspect colonies are then subjected to a variety of biochemical and serological tests for identification [1]. The procedures usually take about one week to be completed and require considerable expertise and stringent safety precautions. Other methods of diagnosis are presumptive and measure exposure by means of either activated cells of the cellular immune system [2] or humoral antibody in serum or milk [3, 4]. The former methods were found unsuitable for large scale diagnosis because enumeration relied on radioisotopes, and the time required was long (usually about one week). The latter, serological tests have been, and are, widely used.

Serological diagnosis of brucellosis was first described by Wright and Smith in 1897 [5]. Their test consisted of mixing \textit{B. melitensis} cells with patient serum and
observing for clumping of the bacteria (agglutination). Because of the relatively low specificity of this test and the 48 h incubation period, many modifications were made to reduce false positive reactions and testing time. These included simply heating the serum [6], treatment of the serum with chemicals, for example, 2-mercaptoethanol or rivanol, to destroy or remove IgM [7–9], the use of chelating agents [10] or using acidified antigen [11]. The latter procedure has been widely used as the Rose Bengal test (RBT), the buffered plate agglutination (BPAT) and the card test. They offer the additional advantage of being very rapid, with a completion time of less than ten minutes.

In spite of the modifications, the agglutination tests gave an unacceptable number of false positive reactions. This led to the development of the complement fixation test (CFT) [12] and the immunodiffusion test [13]. Using a rapid agglutination test as a screening test and a second, more specific test for confirmation provided a reasonably accurate diagnostic procedure [8]. However, because of the technical problems with the confirmatory tests, primary binding assays for the diagnosis of brucellosis were developed.

3. ENZYME IMMUNOASSAYS FOR DIAGNOSIS

Primary binding assays do not require antibody to perform any secondary functions, such as agglutination, activation of complement or precipitation. Rather, these assays only rely on the antibody to perform its main function, which is interaction with its antigen. This interaction is then measured by a second reaction involving a detection system. The most commonly used primary binding assays are enzyme immunoassays in which the detection system relies on the interaction of an enzyme, usually attached to an anti-species reagent, with its substrate and a reporter molecule. There are two commonly used types of enzyme immunoassay: the indirect and the competitive formats. The adaptation of these assays to the diagnosis of brucellosis will be discussed below.

3.1. Indirect enzyme immunoassay

The indirect enzyme immunoassay (IELISA) was first described by Engvall and Perlmann [14], and its adaptation to detection of anti-Brucella antibody was initially performed by Carlsson et al. [15]. This was followed by a large number of reports over the next ten years (see the reviews in Refs [16, 17]). Nearly all these reports used crude lipopolysaccharide antigen, prepared as described in Ref. [18] or soups of bacterial antigens, immobilized on a polystyrene matrix. After removal of unbound materials by washing, diluted test serum was added. After a suitable incubation period, the unbound materials were again removed by washing and the
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<sup>a</sup> Polystyrene tubes were 12 mm x 75 mm Falcon Plastics 2054. Plates were NUNC 69620. The plates were stable for at least one year when frozen at -20°C. Antigen coated and dried plates were stable for at least one year when stored at 22°C.

<sup>b</sup> PBST refers to 0.01M phosphate buffer, pH7.2, containing 0.15M NaCl and 0.05% Tween-20. PBST/EDTA is the same buffer, pH6.3 with 15mM EDTA and 15mM EGTA.

<sup>c</sup> RaBlg (H&L) is rabbit anti-bovine Ig (H and L chain) and MaBlgG<sub>1</sub> is monoclonal antibody to bovine IgG<sub>1</sub>. 
$\text{H}_2\text{O}_2$ is hydrogen peroxide (substrate), 5AS is 5-aminosalicylic acid and ABTS is 2,2'-azino-di-(3-ethyl-benzthiazoline sulfonate-6). Manual readings were performed in a spectrophotometer at the appropriate wavelength. Spectrophotometer readings used an automated 96 well plate reader (Titertek). OD assessment was kinetic (several readings over a time period), target (OD reading of a strong positive serum taken at 4 minutes was extrapolated to 1.0) or timed (a single reading at 10 min).

d Data handling was manual, using the Agriculture Canada VACS computer via a modem or using a spectrophotometer connected, programmed microcomputer.

e The assay format refers to the number of days per week the assay could be performed. With the shorter incubation periods, assays could be run several times per day.

f Sensitivity and specificity estimates were not available for the 1977 assay version. For the 1980 assay, the cut-off values were arbitrary ELAT units. For the 1984 and 1997 assays, a percentage of a strong positive serum (%P) was used as the cut-off value between positive and negative results. NV refers to non-vaccinated cattle and Vac refers to animals vaccinated with *B. abortus* strain 19.

detection system was added. The detection system usually consisted of an anti-bovine globulin reagent, raised in rabbits or goats and specific for all the antibody isotypes and the immunoglobulin light chain. This antiglobulin reagent was conjugated with an enzyme, usually horseradish peroxidase (HRPO). The detection reagent was added to the reaction, and if antibody was present in the serum the detection reagent was bound, the substrate was catalysed and the reporter chemical changed colour. Because of the reagents used in the early IELISAs, the sensitivity and specificity of the assays were not a marked improvement over the CFT. In addition, most of the assay procedures required five to seven hours to be completed. This was an advantage over the CFT, which usually required overnight incubation.

Improvements started to appear when the O-polysaccharide of *B. abortus* lipopolysaccharide was chemically characterized [19, 20] and when monoclonal antibodies to animal immunoglobulins became available. These advents, combined with the addition of divalent chelating agents to reduce non-specific serum protein interactions, eventually led to the development of an assay that could be performed in just over one hour, with sensitivity and specificity values of 100% and 99.4%, respectively, in a non-vaccinated bovine population [4]. A comparison of the assay formats developed at the Animal Diseases Research Institute (ADRI), Agriculture Canada, are presented in Table I. From the table, it is clear that the specificity of the IELISA, especially among non-vaccinated cattle, was always very high; however, the sensitivity was improved considerably with each successive generation of tests. Other marked improvements include the decrease in assay time from seven hours to just over one hour and the storage conditions for the antigen coated plates, allowing testing at any time. It has also been shown that antigen coated plates may be reused up to five times [21].
The IELISA was extended to the detection of anti-\textit{Brucella} antibody in milk. As for the serum antibody assay, the format has changed and, with it, improvements were made. The current in-use assay has the same basic format as the serum antibody assay except that the milk samples are diluted 1:1 with PBST/EDTA, allowing testing of pooled milk samples without loss in sensitivity [4]. The sensitivity and the specificity of the IELISA for milk were shown to be 97.5\% and 99.96\%, respectively, in individual milk samples.

In summary, progress in technology has allowed development of highly sensitive and specific IELISA procedures for detection of serum and milk antibody to \textit{B. abortus}. The current utility of the IELISA is mainly for milk testing, where serial use of this technology has been shown to eliminate or decrease the incidence to below 1\% in infected dairy herds [22]. This is a considerable advance in that a non-invasive, non-disruptive and inexpensive sampling procedure may be used to screen pooled milk samples for antibody. Positive reactions may then be confirmed on an individual animal’s milk and/or serum. This technology has considerable future in dairy areas where the incidence of brucellosis is relatively high.

3.2. Competitive enzyme immunoassay

Competitive enzyme immunoassays (CELISAs) were developed by Nielsen et al. [23], MacMillan et al. [24] and Nielsen et al. [25]. This assay format is one in which a pretitrated amount of antibody of known specificity (it may be polyclonal or monoclonal, although monoclonal antibodies have several advantages) competes for a limited number of antigen epitopes with antibody in test serum. Binding of the competing antibody is then measured directly or by using an anti-species reagent, conjugated with an enzyme. In contrast to the IELISA, development of colour in the CELISA is indicative of a negative test.

The CELISA test format has several advantages over the IELISA in that it is technically simple and rapid to perform, and sensitivity may be adjusted by altering the concentration of the competing antibody. The CELISA also has the ability to differentiate antibody produced in response to \textit{B. abortus} strain 19 vaccination and antibody arising from infection with pathogenic strains. This differentiation probably results from differences in specificity and affinity of vaccinal antibody from antibody due to field infection. This differentiation also allows elimination of antibody responses due to exposure to cross-reacting microorganisms such as \textit{Yersinia enterocolitica} serotype O:9. In addition, the CELISA may in theory be applied to the detection of antibody to smooth lipopolysaccharide of any species of \textit{Brucella} in any species of host. To date, the CELISA has been validated for detection of bovine antibody to \textit{B. abortus}, bison antibody to \textit{B. abortus} (and possible \textit{B. suis} type 4), porcine antibody to \textit{B. suis} and caprine antibody to \textit{B. melitensis}. 
Improvements in specificity were accomplished by adding chelating agents to reduce non-specific serum protein interaction and by using a conjugated anti-mouse reagent pre-absorbed with bovine serum.

In summary, the CELISA in its current format may be performed in just over one hour. This assay has considerable potential as a combination screening and confirmatory test, because of its sensitivity and specificity, 100% and 99.9%, respectively.

3.3. Data handling

A number of commercial software programs are available for use with spectrophotometers capable of optical density (OD) readings of 96 well plates. However, since none of the programs were ideal for the brucellosis serology, in-house software has been developed. This software was designed to include a number of features (Table II). It was intended for diagnostic use and allows assessment of up to eight controls and 88 test samples per plate, in addition to the calculation of the test status (positive or negative) based on controls included in each plate. It allows continuous monitoring of control values for quality control purposes and indicates whether the

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TABLE II. FEATURES OF ADRI SOFTWARE FOR BRUCELLOSIS SEROLOGY

May be used with either IELISA or CELISA assay formats
Indicates acceptability of data based on controls
Identifies plate, date, time, technician and test kit
Calculates the threshold value in %Inhibition or %Positivity and OD units
Identifies four controls: BAC++, BAC+, BAC− and BACc
Identifies the status of the controls (inside or outside confidence limits)
Calculates the mean of the duplicate control values for each
Prints each duplicate value (%P or %I) and OD values
Identifies upper (UCL) and lower (LCL) confidence limits
Sequentially lists sample numbers
Allows sample description
Provides status of each sample (positive or negative) based on the controls on the plate
Prints OD values and %P or %I for each sample
Allows for 88 test samples per plate
Allows for editing of control values
Provides continuous quality control assessment of control samples in the previous 50 plates
Has capability to output quality control data
Has screen and/or disk and/or printer output
Has plate recall
Has capability to change spectrophotometer parameters (for some spectrophotometers)
values for each plate are within acceptable limits established continuously on the basis of the last 50 plates. The software assesses spectrophotometer output based on a point-in-time reading, usually at ten minutes of substrate/chromogen incubation. This allows for batch processing of plates, a useful function in a diagnostic laboratory.

In conclusion, enzyme immunoassays have been developed to the point where they are capable of replacing current conventional serological tests and providing greater accuracy and confidence in the results in a shorter time and, in some cases, at less cost. Both IELISA and CELISA have been streamlined for diagnostic use, and electronic assessment of laboratory results and data analysis further enhance the utility of these assays for the diagnosis of brucellosis. A remaining problem for ELISAs and serological tests in general is the availability of standardized reagents and protocols to allow international standardization of the assays. With ELISAs this may be overcome by making available standardized procedures and reagents — antigen, monoclonal antibody and control sera — through international offices such as the Food and Agriculture Organization of the United Nations (FAO), the IAEA or the World Health Organization (WHO).

4. OTHER SEROLOGICAL TESTS FOR BRUCELLOSIS

A fluorescence polarization assay has been adapted to brucellosis serology [26]. Fluorescence polarization assays were developed nearly 50 years ago (reviewed in Ref. [27]), but found use mainly for the detection of drugs.

The assay format is homogeneous, requiring no washing procedures. Serum is diluted, and a background fluorescence polarization reading is taken. This reading is subtracted from the reading obtained after the fluorochrome labelled antigen has been added to the test to yield a net result of polarization due to antigen (and antibody interaction) after a second assessment. The antigen is a small molecular weight polymer of the O-polysaccharide of \( B. \) \( abortus \) lipopolysaccharide. The antigen is covalently conjugated with fluorescein isothiocyanate. Aside from the serum diluent buffer, this is the only reagent required for the test.

The basis of the assay is that a small fluorochrome labelled molecule rotates rapidly when in solution. The speed of rotation depends on a variety of factors, but is inversely related to the size of the molecules. The speed of rotation may be measured by exposing the antigen to polarized light and then measuring the depolarization. When the antigen is added to a sample containing antibody, the resulting antigen–antibody complex will reduce the rate of rotation of the antigen because of the size increase of the complex. The reduction in rotation also reduces the amount of depolarization of the incident light, and this reduction may be measured and gives an indication of the amount of antibody present in the sample.
The assay takes less than five minutes to perform, consisting of an initial reading of the diluted serum, addition of labelled antigen, a two minute incubation stage to allow antigen–antibody complex formation and then a second reading to assess complex formation. The results for specificity and sensitivity for bovine antibody to B. abortus are 99.9% and 99.02%, respectively. The specificity using sera from B. bortus strain 19 vaccinated cattle was 99.2%.

The assay is very simple to perform, and with the advent of portable polarization assessment equipment, it will be possible to use the test outside the laboratory. The cost should be low; the only reagents required are buffer and labelled antigen. The assay is performed in a disposable borosilicate glass tube.

In summary, the fluorescence polarization assay is an alternative to the ELISAs. The results obtained to date indicate an assay performance similar to the CELISA. The advantages of the assay are manifold, including rapidity, estimated low cost, simplicity and use outside the laboratory environment.

5. CONCLUSIONS

Enzyme immunoassays and other primary binding assays for the detection of antibody in bovine brucellosis have not yet reached their full potential as diagnostic tests. There are a number of reasons for this, including current lack of standardized protocols and reagents (the only available standardized test is the Office international des épizooties (OIE) approved version of the IELISA from the Joint FAO/IAEA Division). Because of the improved performance of these tests, however, it is inevitable that eventually they will be accepted and become available for diagnosis. This will enhance the ability of diagnostic laboratories to make accurate decisions regarding the infection status of animals, therefore allow the elimination of such animals from herds and eventually lead to control and eradication of the disease.

REFERENCES


APPLICATION OF ENZYME LINKED IMMUNOSORBENT ASSAYS TO THE DIAGNOSIS OF THE PATHOGENIC ANIMAL TRYPANOSOMOSES

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Abstract

APPLICATION OF ENZYME LINKED IMMUNOSORBENT ASSAYS TO THE DIAGNOSIS OF THE PATHOGENIC ANIMAL TRYPANOSOMOSES.

The pathogenic animal trypanosomoses comprise a number of diseases caused by different species of vector borne trypanosomes. In Africa, trypanosomes transmitted by tsetse flies (Glossina spp.) constitute a major constraint on socioeconomic development; over ten million km² of sub-Saharan Africa are infested by the fly and 400 million livestock are at risk from infection with Trypanosoma congolense, T. vivax and T. brucei. Diseases caused by trypanosomes also occur outside the tsetse belt. Two trypanosome species are transmitted mechanically by haematophagous biting flies, mainly tabanids, and thereby have achieved a wider geographical distribution; T. evansi occurs in North Africa, Asia and South America, while a non-tsetse-transmitted form of T. vivax is found in South America. Animal trypanosomoses are difficult to diagnose: there are no pathognomonic signs, and detection of parasites is problematic. Therefore, considerable effort has been expended to develop serological assays with improved diagnostic sensitivity for use in obtaining information on the incidence and prevalence of infection and in the management of disease in the individual animal. Many different tests have been used over the past 70 years, but it is only recently that attention has been paid to test standardization, principally because of the introduction of enzyme linked immunosorbent assays (ELISAs) for the detection of trypanosomal antibodies and antigens. Although a variety of serological diagnostic techniques have been available for many years, there is still a lack of reliable data on trypanosomosis prevalence that makes economic justification and planning of control difficult to support. Clearly, enzyme immunoassays could provide this basic information. Additionally, as the emphasis moves from eradication to control of the tsetse transmitted trypanosomoses, there will be a greater need for surveillance and diagnosis of infection in individual animals. Both antigen and antibody assays can fulfill these roles, but differences in the biology of tsetse transmitted and mechanically transmitted trypanosomoses, as well as the different approaches to their control, are likely to be a major consideration in determining which is most appropriate.
1. **ANIMAL TRYPANOSOMOSES**

Trypanosomes are haemoproteozoan parasites that occur among representatives of every taxonomic class in the Vertebrata, from fish to mammals. Most trypanosome species appear to have little or no pathogenic effect on their hosts but in mammals they are implicated as causal organisms in a number of serious diseases of humans and domesticated livestock. In livestock, trypanosomes cause an acute to chronic disease characterized by irregular fever, progressive anaemia, loss of condition, infertility, abortion and, in the absence of treatment, death. There is considerable variation in their effect depending on the species and strain of trypanosome, the species of livestock infected and precipitating factors such as intercurrent infection, malnutrition, climatic factors and work.

1.1. **Tsetse transmitted animal trypanosomoses**

Three species of trypanosomes, *Trypanosoma congolense*, *T. vivax* and *T. brucei*, are transmitted by the bites of tsetse flies (*Glossina* spp.) and cause the diseases in sub-Saharan Africa often referred to as 'nagana' in cattle, sheep, goats and horses. Their biology is complex; the trypanosomes undergo a cycle of development in the vector before infective metacyclic forms are produced. Many different species of tsetse fly can transmit trypanosomes, and wild animals can harbour the organisms without ill effects, thereby acting as permanent reservoirs of infection. The tsetse transmitted trypanosomoses have had the greatest impact of any animal disease in preventing cattle raising in extensive areas of rangeland [1]. Some ten million km$^2$ are infested by tsetse flies, and raising cattle in these areas is difficult or impossible. The economic impact of trypanosomosis is difficult to assess, and the much quoted figure of US $4 billion (10$^9$) [2] is based on flawed data.

1.2. **Non-tsetse-transmitted animal trypanosomoses**

The requirement for cyclical development in tsetse flies has been instrumental in limiting *T. congolense*, *T. vivax* and *T. brucei* to Africa. However, two trypanosome species have lost the ability to develop in the insect vector and adapted to mechanical transmission by haematophagous biting flies such as *Tabanus*, *Haematopota*, *Chrysops* and *Stomoxys*. Transmission of infection by these trypanosomes is by mechanical means, on the mouthparts of flies contaminated with trypanosomes during feeding. One of them, *T. evansi*, which is derived from *T. brucei* [3], has become the most widely distributed pathogenic trypanosome, occurring in Africa north of the tsetse belt, in Asia and in South America. The other, a mechanically transmitted form of *T. vivax*, is restricted to South America. These trypanosomes affect camels, cattle, buffalo, horses, pigs, sheep and goats, but their economic importance is more difficult
to determine because there is not the obvious impediment to raising livestock, namely, the exclusion of animals from areas of potential grazing, as observed with the tsetse transmitted trypanosomes. In addition, there are very few data on the actual distribution of the trypanosomes and their prevalence, largely owing to the lack of sensitive diagnostic tests. However, at the end of the last century and at the beginning of the present century, trypanosomosis caused by *T. evansi* (known as ‘surra’) was a major problem in buffalo, camels and horses, causing severe epidemics in China, Indonesia, India and the Philippines. The epidemics were characterized by high prevalence of infection and mortality from 30–100%. Surra currently affects over 100,000 buffalo every year in southern China, and epidemics of disease in draught buffalo and dairy cattle have been reported recently in Indonesia, Viet Nam, Cambodia, the Philippines and Thailand [4].

2. DIAGNOSIS OF TRYPANOSOMOSES

Although there may be marked clinical signs of infection with pathogenic trypanosomes, they are not sufficiently distinct to provide a sound basis upon which to make a presumptive differential diagnosis of infection. Trypanosome infections are characterized by fluctuating parasitaemias in which parasites appear in the peripheral blood. Hence, the demonstration of trypanosomes in the blood is the most widely adopted diagnostic technique used routinely in the field [5].

Although various modifications of blood examination have produced tests which are capable of detecting as few as ten trypanosomes [5], it is often difficult or impossible to demonstrate parasites in the blood [6, 7]. Parasitological tests are not sufficiently diagnostically sensitive to identify all infected animals and cannot be relied upon for diagnosis and treatment of the individual. Nevertheless, where prevalence is high and where treatment is to be administered on a herd basis by the application of chemotherapeutic drugs, even a test with a relatively low diagnostic sensitivity will suffice. However, where control campaigns against tsetse transmitted trypanosomes are effective and the disease prevalence declines, individual rather than block treatment might also become necessary and post-control surveillance will require more sensitive diagnostic tests. In addition, in situations where mechanically transmitted trypanosomes occur, in camels or in cattle and buffalo infected with *T. evansi*, treatment is rarely applied on a herd basis and individual animals are selected for treatment [3]. Furthermore, in southeast Asia, the lack of accurate information on the distribution and prevalence of infection with *T. evansi* can be attributed to the continued reliance, for providing this information, on parasitological tests of low diagnostic sensitivity. This lack of accurate data precludes definition of the extent of the problem, makes planning for control difficult and makes assessing the economic losses due to trypanosomosis impossible [3]. For all these reasons, it is
therefore necessary to use tests which are more sensitive diagnostically than the currently employed parasitological techniques [6, 8].

Serological assays have been considered to answer this requirement and have been used extensively for detecting trypanosomes in infected animals [6, 8]. An excellent example of the successful control of trypanosomosis — the eradication of *T. equiperdum* from Canada in the 1920s — relied on the complement fixation test (CFT) [9]. However, this is possibly the only example of successful use of serodiagnosis applied to control and eradication of trypanosomosis. This might be due to the fact that in spite of the many applications of serological tests, there has been, until recently, very little attempt to evaluate them critically in terms of their sensitivity and specificity. The advent of enzyme linked immunosorbent assays (ELISAs) [6, 8] has revived interest in the potential for serology as an aid in the control of trypanosomosis. Unfortunately, most of the reported uses of ELISA in trypanosomosis have failed to address the basic questions of diagnostic sensitivity and specificity which are needed for interpretation of the findings from the tests; the term 'diagnosis' is used frequently but without any clear understanding of the validity of the data upon which the decision is based. This deficiency in evaluation is probably the single most important issue requiring urgent consideration for the future application of ELISAs in the control of trypanosomosis.

2.1. ELISAs for the detection of trypanosomal antibodies (Ab-ELISAs)

It is axiomatic in the diagnosis of *T. congolense*, *T. vivax* and *T. brucei* that the ultimate aim of any test is to discriminate between infections with the different trypanosome species, since it is possible that an individual animal can be infected with one, two or all three species. In fact, with Ab-ELISAs little attention has been given to the use of species specific diagnostic assays and most work has been done with ill defined antigens, incapable of such discrimination. Ab-ELISAs generally have a common methodology, irrespective of the laboratory developing the assay; soluble antigens are prepared from large numbers of trypanosomes harvested from the blood of mice by separation on anion exchange cellulose [10]. After harvesting, the parasites are disrupted by ultrasonication and the soluble proteins collected by removal of cell debris by centrifugation. Such protein extracts can be stored frozen, with or without enzyme inhibitors, for considerable periods and form the basic antigen for use in assays. Assays are invariably carried out in microtitre plates, although occasional modifications are used.

2.1.1. Ab-ELISAs for detection of tsetse transmitted trypanosomoses

In initial applications of ELISA, antigens prepared from *T. congolense*, *T. vivax* and *T. brucei* were compared [10] in order to determine if any species specificity
existed or if there was any advantage in using a particular trypanosome species. None of the tests was found to cross-react with the non-pathogenic *T. theileri* and other haemoprotozoa including *Theileria parva*, *Th. annulata* and *Babesia divergens* [11]. However, it was clear that the quality of antigen preparation, and possibly the species of trypanosome used for antigen, did influence the results with pathogenic trypanosomes. Antigens did not seem to show any pronounced species specific reactivity, but those prepared from *T. brucei* and *T. congolense* showed marked differences in diagnostic sensitivity and specificity. It was equally clear that the antibody response in individual animals varied considerably and that secondary, or even tertiary, infection in an already infected animal had no detectable effect on the antibody response measured by ELISA. Post-treatment, antibodies persisted for up to three months. Further tests did not confirm these differences in diagnostic sensitivity between antigens. ELISA was found to show a similar degree of sensitivity to the indirect fluorescent antibody test (IFAT) [12]. In a survey carried out in Zambia [13], Ab-ELISA and IFAT gave a high level of agreement, and estimated serological prevalence rates were three times higher than prevalence rates estimated by parasitological tests. Ab-ELISA was also found to be a useful tool in surveys in southern Tanzania [14].

2.1.2. Ab-ELISAs for detection of non-tsetse-transmitted trypanosomoses

Possible differences in antigen preparations were also investigated in studies with *T. evansi* in rabbits [15]. Antigens were prepared from three different isolates and clones of *T. evansi* and rabbits infected with those same trypanosome stocks. No significant differences between the antigen preparations were found, but the responses of the rabbits varied with the particular infecting organism. Antibodies could be detected by 15 days after infection and until approximately three months after treatment. Ab-ELISA was compared with different serological tests in a study of *T. evansi* in camels, goats and sheep in the Sudan [16]. Experimentally infected camels produced antibodies within 10–15 days of infection; antibodies persisted throughout infection and disappeared by two to three months after treatment. The sample estimate of sensitivity of the assay applied to naturally infected camels was 87%, compared with the IFAT value of 97%. Samples tested in this work came from a biased population, namely, animals brought to veterinary clinics by the owner on suspicion of infection. Many parasite negative animals gave positive results. Where it was possible to carry out a screening test on a healthy herd, 5% of animals tested as positive, demonstrating the potential value of the assay in identifying infected animals before they showed clinical signs of infection. No information on the diagnostic specificity of the tests was obtained. Over 50% of sheep and goats sampled from various locations in the eastern Sudan were antibody positive [17], although none of them was parasite positive. Nearly 70% of camels sampled at similar locations were
antibody positive, and over 10% were found to be infected. These findings suggest that small ruminants may play a role in the transmission of *T. evansi*, possibly acting as symptomless reservoirs of infection. Although there is presumptive evidence of infection, it has not been subsequently confirmed. As an alternative to specific anti-camel IgG antibody conjugates, protein-A conjugates have been used successfully, the assays showing significant correlation with standard antibody detection ELISAs [18].

Ab-ELISAs have also been used as screening tests in other geographical regions. In Indonesia, surveys have confirmed the widespread distribution of *T. evansi* in buffalo [19], and in an epidemic of *T. evansi* in Madura Island, Central Java, over 45% of buffalo and 25% of cattle were found serologically positive by ELISA [20]. Ab-ELISA was found to be a useful diagnostic tool in deterring the spread of *T. vivax* in the eastern Caribbean [21]; much concern was expressed over the potential threat imposed by this parasite, and it was considered that screening tests using ELISA should be carried out periodically to monitor the situation. Neither of these examples of the use of ELISA in screening for trypanosomosis provided information on the diagnostic sensitivity and specificity of the assays. Although the CFT is still used routinely for screening of horses for dourine, Ab-ELISAs are considered to be more sensitive than CFTs [22].

2.1.3. **Standardization and critical evaluation of Ab-ELISAs**

It has been recognized that Ab-ELISAs are deficient in a number of areas in relation to standardization. The most obvious source of variation is in the preparation of antigen due to the differences in trypanosome species, the large numbers of strains and the lack of adequately controlled techniques for producing antigens. One possible way to reduce variation is to utilize procyclic forms of trypanosomes — the organisms present in the tsetse fly which lack the surface coat antigen present on the bloodstream forms of the parasites. One study [23] confirmed that procyclic forms of *T. brucei*, tested against serum from cattle infected with *T. congolense*, *T. vivax* or *T. brucei*, gave a highly positive correlation with the results obtained using bloodstream forms. The sonication and centrifugation of the proteins is still an imprecise way of preparation and the complex antigenic make-up of the resulting soluble antigens cannot easily be replicated in producing different batches of material, or when attempting to compare antigens prepared from different trypanosome species. Fractionation of crude antigens of *T. brucei* and *T. congolense* by gel chromatography [24] has enabled production of antigens with species specific characteristics which could be used in differentiating infections. These antigens have not been tested, however, and their usefulness awaits further research. It has been suggested that the reproducibility of tests could be improved by a more rigorous standardization of assay conditions in terms of temperature, volume of reactants, number of replicates, and introduction of blocking proteins to reduce background signal as well as by
monitoring the progress of the reaction and final reading on the development of a control sample rather than a function of time [25]. With these conditions in place, it was found that antigen preparations influenced the results, and reactions between homologous components \((Tb/Tb, Tc/Tc)\) gave better results than heterologous reactions.

2.2. ELISAs for the detection of trypanosomal antigens (Ag-ELISAs)

Ab-ELISAs have not been comprehensively evaluated to determine their role in providing information on the distribution and prevalence of trypanosomes, in evaluating the efficiency of control measures or in surveillance in areas where trypanosomosis control has been effected. Nevertheless, it has become accepted that the persistence of antibodies after treatment is a major impediment to the use of these tests for such purposes and that, since it is not possible to distinguish between active and past infections, the tests do not provide a reliable means of determining treatment. Considerable effort, therefore, has gone into developing assays that do provide evidence of current infection, and for this purpose assays that detect circulating trypanosomal antigen were considered ideal [6]. In this context, it is worth recalling the conclusions of a rigorous study undertaken in the Sudan of the ability of antibody and antigen detection tests to discriminate between infected and non-infected camels [26]. The detection of circulating antibodies by Ab-ELISA was considered likely to be the best test for identifying infected camels with either patent or non-patent infections, although there was some indication that antigen detection might also be useful [27]. Antigen detection ELISAs (Ag-ELISAs) are all based on the double antibody sandwich technique. Capture antibody is adsorbed to the microtitre plate; after reacting with the test sera, the same antibody, conjugated with horseradish peroxidase, is added as an indicator.

2.2.1. Ag-ELISAs for detection of tsetse transmitted trypanosomoses

The first experimental studies on Ag-ELISAs, using polyclonal antibodies to \(T. evansi\) and \(T. congolense\), showed that in rabbits trypanosomal antigens could be detected 4–14 days after infection [27]. Since the antibodies were raised against crude antigen preparations there were considerable cross-reactions with other trypanosome species, and, in fact, the assays could be used to detect antigens in animals infected with \(T. vivax\) and \(T. brucei\). Following treatment, antigens were no longer detected by 14 days after drug administration. At the same time, trypanosomal antibodies remain high [27]. In order to enable species specific diagnosis, a number of monoclonal antibodies (MAbs) were produced that specifically recognized antigens present only in \(T. brucei\), \(T. congolense\), \(T. simiae\) and \(T. vivax\) [28–31]. In the case of \(T. brucei\) MAbs, there were cross-reactions with species in the same genus, i.e.
T. evansi, T. gambiense and T. rhodesiense [28]. The MAbs were used to develop Ag-ELISAs for detection of antigens in cattle infected with tsetse transmitted trypanosomes [32]. Antigens of T. congolense, T. vivax or T. brucei were detected 8–14 days after infection and, even when parasites were not detected in the blood, antigens were still present. Conversely, trypanosomes could often be detected before antigens appeared in the serum in the acute stage of infection, but this was considered to be due to the absence of antigen at this time, before trypanosomes had been destroyed by the host’s immune response. Administration of trypanocidal drugs removed parasites from the blood, and antigen was cleared from the serum within 14 days. However, in the case of T. congolense, antigens reappeared some time later, although there was no evidence of a relapse in parasitaemia [32].

Further experimental studies examined long term infections of T. congolense, T. vivax and T. brucei in cattle and goats and compared parasitological techniques and Ag-ELISA in detecting infection over a period of three years [33, 34]. Antigens could be detected constantly over the period of investigation in over 90% of samples from goats and 80% of samples from cattle infected with T. vivax or T. congolense. Over the same period, only 10% of blood samples from goats and 19% of samples from cattle had patent infections [33]. Similar findings were obtained with T. brucei: 80% of samples were antigen positive, whereas trypanosomes were detected in only 16% of blood samples [34]. Hence, antigens could be detected more frequently than the organisms themselves. In field application of the tests [35], 131 cattle were identified with trypanosome infections, mostly T. vivax and T. congolense; no cases with T. brucei and only three mixed infections were found (Table I). In contrast, by Ag-ELISA most of the infections were mixed, including all three trypanosomes. T. brucei

<table>
<thead>
<tr>
<th>Trypanosomes</th>
<th>MHCT</th>
<th>Ag-ELISA (individual categories)</th>
<th>Ag-ELISA (all categories)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. congolense</td>
<td>51</td>
<td>8</td>
<td>90 (71%)</td>
</tr>
<tr>
<td>T. vivax</td>
<td>77</td>
<td>18</td>
<td>107 (85%)</td>
</tr>
<tr>
<td>T. brucei</td>
<td>0</td>
<td>2</td>
<td>86 (68%)</td>
</tr>
<tr>
<td>Tc/Tv</td>
<td>3</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Tc/Tb</td>
<td>0</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Tw/Tb</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Tv/Tc/Tb</td>
<td>0</td>
<td>73</td>
<td></td>
</tr>
</tbody>
</table>
was found in over two thirds of the animals. *T. brucei* is extremely difficult to detect microscopically [5], so it is quite possible that infections with this species went undetected. However, the high level of mixed infections has not been substantiated by more sustained parasitological examination, and so the findings cannot be confirmed. Fifty per cent of samples collected from animals that did not show infection were also positive with trypanosomal antigen.

2.2.2. **Ag-ELISAs for detection of non-tsetse-transmitted trypanosomoses**

The *T. brucei* group specific MAb [28] has also been used in assays for the detection of antigens in animals infected with *T. evansi*. In experimental infections in rabbits, antigens were detected five to ten days after infection and were present throughout the infection. The test was 100% sensitive with sera from infected buffalo [36]; in camels a value of 92% was obtained [36] but these values were based on small sample sizes. Neither of these studies gives values for diagnostic specificity. A modification of the assay using polystyrene tubes was said to yield results similar to those of conventional ELISA [37]. In a comparative evaluation of the MAb based [28] and the polyclonal based [27] assays, in camels in Kenya the tests were found to have a sensitivity of 83% and 63%, respectively, but with the two tests used in parallel, the sensitivity increased to 92% [38]. A comparative study of the diagnostic value of Ab- and Ag-ELISA in camels in Kenya demonstrated that both assays were highly sensitive [39], although the authors concluded that Ab-ELISAs were unlikely to differentiate between infected and uninfected animals. The MAb was also used in the detection of antigens in horses in Argentina infected with *T. evansi* [40]. Antigens appeared in the serum 7–21 days after infection and increased steadily thereafter. In outbreaks of 'mal de caderas', 58 (74%) of 78 infected horses were antigen positive; and of 47 horses that were involved in the outbreak but did not have parasites, 35 (75%) were antigen positive [40].

Although it was considered that the MAbs produced for the assays recognized antigens which were ubiquitous throughout the various trypanosome subgenera, there is some evidence that, in South America, strains of *T. vivax* may not be recognized by that particular MAb [41]. The assay was found to have a low sensitivity to Guyanese strains of *T. vivax*. Ag-ELISA tests have also been used in the diagnosis of *T. equiperdum* [42].

2.2.3. **Application of Ag-ELISAs in estimating levels of trypanotolerance in *N'dama* cattle**

Although the Ag-ELISAs have not been fully evaluated in terms of diagnostic sensitivity and specificity, and it is difficult to determine with confidence the likelihood of an individual animal harbouring infection, they have been used to identify
cattle that are deemed to have a high level of trypanotolerance [43, 44]. The assays were considered to (a) accurately identify trypanosome species including mixed infections, (b) identify animals with non-patent infections as having a superior ability to control infection and (c) more accurately measure the duration of infection and the ability of the animal to control anaemia [44]. It is possible that too much emphasis has been placed on the ability of the tests to identify infected animals correctly, and it is probably premature to conclude that the antigenaemic animals that showed no daily reduction in weight gain were infected animals with a greater ability to withstand the pathogenic effects of trypanosomosis.

2.2.4. Standardization and critical evaluation of ELISAs

A major initiative in the standardization of Ag-ELISAs as well as a positive move to enable technology transfer has been made under two co-ordinated research programmes managed by the IAEA and funded mainly by the Netherlands Government [45, 46]. Under these programmes, fourteen different laboratories in Africa are carrying out validation of the Ag-ELISAs and also using the tests in various investigations involving vector control and chemotherapy or chemoprophylaxis of the tsetse transmitted trypanosomoses. The programmes gave particular consideration to the use of stringent quality control of the assays, an essential prerequisite for universal application of the assays, and the guidelines proposed by the IAEA [47] were followed in carrying out test standardization. The results of these studies [46] have established that the assays used in this programme have a high diagnostic specificity, exceeding 95% with *T. congolense*, *T. vivax* and *T. brucei* assay systems. There appeared to be a variation in the ability of ELISA to detect infection according to the stage of infection. ELISA was more effective in detecting chronic infections whereas parasitological techniques were more likely to detect infection in the acute stage of the disease. The sensitivity for *T. brucei* was high, but in the case of *T. congolense*, parasitological techniques were more effective [46]. ELISA also revealed that a larger number of mixed infections occurred in infected animals than was likely to be revealed by parasitological techniques. It was therefore concluded that parasitological and ELISA techniques were complementary and by combining the tests the diagnostic sensitivity was increased [46].

Studies have also been carried out in Indonesia, involving the validation of two Ag-ELISAs, one prepared by the CTVM (2G6) and another, also used in the IAEA programme for *T. brucei* detection, Tbr7. These two assays were compared with Ab-ELISAs detecting IgG or IgM antibodies for diagnosis of *T. evansi* in buffalo. Quality control parameters similar to those recommended under the IAEA co-ordinated research programmes were followed in standardizing the assays. The studies involved investigations in experimentally infected buffalo with both a primary and a secondary
TABLE II. ESTIMATES OF DIAGNOSTIC SPECIFICITY (%), WITH 95% CONFIDENCE INTERVALS (CI), FOR THE 2G6 Ag-ELISA AND Tbr7 Ag-ELISA AND FOR THE IgM-ELISA AND IgG-ELISA, USING SERA FROM AUSTRALIAN BUFFALO (n = 263) AND CATTLE (n = 80)

<table>
<thead>
<tr>
<th>ELISA test</th>
<th>Species</th>
<th>Specificity (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>2G6 Ag-ELISA</td>
<td>Buffalo</td>
<td>75</td>
<td>(70, 80)</td>
</tr>
<tr>
<td>Tbr7 Ag-ELISA</td>
<td>Buffalo</td>
<td>78</td>
<td>(73, 83)</td>
</tr>
<tr>
<td>2G6 Ag-ELISA</td>
<td>Cattle</td>
<td>68</td>
<td>(56, 78)</td>
</tr>
<tr>
<td>Tbr7 Ag-ELISA</td>
<td>Cattle</td>
<td>100</td>
<td>(96, 100)</td>
</tr>
<tr>
<td>IgG Ab-ELISA</td>
<td>Buffalo</td>
<td>92</td>
<td>(86, 96)</td>
</tr>
<tr>
<td>IgM Ab-ELISA</td>
<td>Buffalo</td>
<td>55</td>
<td>(46, 64)</td>
</tr>
</tbody>
</table>

infection, followed by field studies in central Java. For estimates of diagnostic specificity, serum samples from cattle and buffalo from Australia were used.

The diagnostic specificity estimates obtained from buffalo and cattle sera and calculated with associated 95% confidence intervals for both Ag-ELISAs and for Ab-ELISAs are shown in Table II. The specificity of 2G6 Ag-ELISA was similar to the specificity of Tbr7 Ag-ELISA using buffalo sera but was significantly lower using cattle sera (p < 0.001). IgG-ELISA specificity was significantly higher than IgM-ELISA specificity (p < 0.001) (Table II).

In experimental infections, there was considerable variation in the patterns of antigenaemia seen in individual buffalo. Antigenaemia was first detected 7–42 days after the primary and secondary infections by both 2G6 Ag-ELISA and Tbr7 Ag-ELISA. Some buffalo had similar 2G6 specific and Tbr7 specific profiles of antigenaemia, whereas in other buffalo the two antigens appeared at different stages of infection, with differences in the pattern of development of antigenaemia. Both Ag-ELISAs detected peaks of antigenaemia in many buffalo from 42 days post-infection and declining antigen levels thereafter. Estimates of sensitivity of 2G6 Ag-ELISA and Tbr7 Ag-ELISA calculated for different stages of the secondary *T. evansi* Bakit 362 showed that the sensitivity of Tbr7 Ag-ELISA was 83% at 15 days post-infection and was significantly higher than that of 2G6 Ag-ELISA at 15–36 days post-infection (p < 0.05). For both Ag-ELISAs, the highest sensitivity estimates were obtained 57–64 days post-infection. The observed differences in sensitivity reflect the underlying fluctuations in serum trypanosomal antigens that occur in buffalo. After
TABLE III. ESTIMATES OF DIAGNOSTIC SENSITIVITY (%), WITH ASSOCIATED 95% CONFIDENCE INTERVALS (CI), FOR THE 2G6 AND Tbr7 Ag-ELISAS AND IgG AND IgM Ab-ELISAs, USING SERA FROM INDONESIAN BUFFALO SHOWN TO BE NATURALLY INFECTED WITH *Trypanosoma evansi* BY MICROHAEMATOCRIT TECHNIQUE (n = 100), BY MOUSE INOCULATION (n = 39) AND BY EITHER MICROHAEMATOCRIT TECHNIQUE OR MOUSE INOCULATION (n = 139)

<table>
<thead>
<tr>
<th>Infection category</th>
<th>ELISA test</th>
<th>Sensitivity (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHCT positive</td>
<td>2G6 Ag-ELISA</td>
<td>67</td>
<td>(7, 76)</td>
</tr>
<tr>
<td></td>
<td>Tbr7 Ag-ELISA</td>
<td>80</td>
<td>(71, 87)</td>
</tr>
<tr>
<td></td>
<td>IgG Ab-ELISA</td>
<td>86</td>
<td>(78, 92)</td>
</tr>
<tr>
<td></td>
<td>IgM Ab-ELISA</td>
<td>90</td>
<td>(82, 95)</td>
</tr>
<tr>
<td>MI positive</td>
<td>2G6 Ag-ELISA</td>
<td>79</td>
<td>(64, 91)</td>
</tr>
<tr>
<td></td>
<td>Tbr7 Ag-ELISA</td>
<td>82</td>
<td>(67, 93)</td>
</tr>
<tr>
<td></td>
<td>IgG Ab-ELISA</td>
<td>87</td>
<td>(73, 96)</td>
</tr>
<tr>
<td></td>
<td>IgM Ab-ELISA</td>
<td>92</td>
<td>(79, 98)</td>
</tr>
<tr>
<td>MHCT/MI positive</td>
<td>2G6 Ag-ELISA</td>
<td>71</td>
<td>(63, 79)</td>
</tr>
<tr>
<td></td>
<td>Tbr7 Ag-ELISA</td>
<td>81</td>
<td>(5, 88)</td>
</tr>
<tr>
<td></td>
<td>IgG Ab-ELISA</td>
<td>89</td>
<td>(84, 94)</td>
</tr>
<tr>
<td></td>
<td>IgM Ab-ELISA</td>
<td>88</td>
<td>(84, 94)</td>
</tr>
</tbody>
</table>

Treatment with trypanocidal drug, antigen clearance was variable. In some individuals antigen disappeared, but in others antigenaemia persisted after treatment for up to 74 days after the primary infection and up to eight months after the secondary infection.

There was a wide range of serum antibody responses in individual buffalo. Buffalo were positive by antibody detection tests by 7–42 days post-infection, but, in contrast with the Ag-ELISAs, usually remained positive, particularly by the IgG-ELISA, throughout the remainder of the monitoring period. In some buffalo, antibody responses were low and not all antigenaemic buffalo had antibody responses. In the
primary infection, the sensitivity of the IgM-ELISA was higher than that of IgG-ELISA on most sampling days post-infection. The IgM-ELISA had a sensitivity of 50% on day seven post-infection, whereas the sensitivity of IgG-ELISA increased more slowly but was higher in the later stages of infection. With the IgM-ELISA and IgG-ELISA, the highest sensitivity estimates were obtained 50–64 days post-infection. In the primary infection, the ranking of the different diagnostic tests in descending order of frequency of positive results was MI > IgG-ELISA > IgM-ELISA > 2G6 Ag-ELISA > Tbr7 Ag-ELISA > MHCT for buffalo shown to be parasitaemic. In this group of buffalo, 25% (19, 30)\(^1\) of all weekly samples were positive by 2G6 Ag-ELISA compared with 15% (11, 20) by Tbr7 Ag-ELISA. By antibody detection tests, 34% (28, 41) of all weekly samples of parasitaemic buffalo were positive by IgM-ELISA and IgG-ELISA. In the secondary infection, 54% (47, 62) of all weekly samples from parasitaemic buffalo were positive by 2G6 Ag-ELISA compared with 76% (69, 82) by Tbr7 Ag-ELISA, 54% (47, 62) by IgM-ELISA and 82% (76, 87) by IgG-ELISA. The ranking of the tests in descending order of frequency of positive tests for secondary infection was IgG-ELISA > Tbr7 Ag-ELISA > MI > 2G6 Ag-ELISA > IgM-ELISA > MHCT.

Estimates of diagnostic sensitivity were based on infections in 139 buffalo naturally infected with *T. evansi* by MHCT (n = 39) and by MI (n = 100). The diagnostic sensitivity of the 2G6 Ag-ELISA and Tbr7 Ag-ELISA were estimated based on results of MHCT, or MI, or a combination of these tests, as shown in Table III. Using buffaloes positive by MHCT alone, the Tbr7 Ag-ELISA had a significantly higher sensitivity than the 2G6 Ag-ELISA (p < 0.05). The diagnostic sensitivities of the IgM-ELISA and IgG-ELISA were also estimated on a similar basis.

Positive and negative predictive values for the different tests are shown in Table IV. The ranges of positive predictive values, calculated for theoretical true prevalence values of 0.10 to 0.90, were 0.24 to 0.96 (2G6 Ag-ELISA) and 0.29 to 0.97 (Tbr7 Ag-ELISA). Positive and negative predictive values of the Tbr7 Ag-ELISA were higher than those of the 2G6 Ag-ELISA. With a true prevalence of 0.50 and a 20% cut-off value, the positive predictive values of the 2G6 Ag-ELISA and Tbr7 Ag-ELISA were 0.74 and 0.79, respectively. The results for the antibody assays showed that, as true prevalence values increased, the positive predictive values of the IgM-ELISA and IgG-ELISA increased and the negative predictive values decreased. At a theoretical test prevalence of 0.50, the positive predictive values of the IgM-ELISA and IgG-ELISA were 0.88 and 0.92, respectively.

\(^1\) All confidence intervals are 95%.
TABLE IV. POSITIVE PREDICTIVE VALUES (PPV) AND NEGATIVE PREDICTIVE VALUES (NPV) CALCULATED FOR VARIOUS THEORETICAL TRUE PREVALENCE VALUES (P) FOR 2G6 AND Tbr7 Ag-ELISAs AND FOR IgG AND IgM Ab-ELISAs

<table>
<thead>
<tr>
<th>P</th>
<th>2G6 Ag-ELISA</th>
<th>Tbr7 Ag-ELISA</th>
<th>IgG Ab-ELISA</th>
<th>IgM Ab-ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PPV</td>
<td>NPV</td>
<td>PPV</td>
<td>NPV</td>
</tr>
<tr>
<td>0.10</td>
<td>0.24</td>
<td>0.96</td>
<td>0.29</td>
<td>0.97</td>
</tr>
<tr>
<td>0.30</td>
<td>0.55</td>
<td>0.86</td>
<td>0.61</td>
<td>0.91</td>
</tr>
<tr>
<td>0.50</td>
<td>0.74</td>
<td>0.72</td>
<td>0.79</td>
<td>0.80</td>
</tr>
<tr>
<td>0.70</td>
<td>0.87</td>
<td>0.53</td>
<td>0.90</td>
<td>0.63</td>
</tr>
<tr>
<td>0.90</td>
<td>0.96</td>
<td>0.22</td>
<td>0.97</td>
<td>0.31</td>
</tr>
</tbody>
</table>
3. CONCLUDING REMARKS

There is a dogma that Ag-ELISAs have more potential than Ab-ELISAs for discriminating between the infected and the non-infected states. However, too little work has been done in critically comparing these tests for this to be accepted unconditionally. For instance, the studies briefly reported here indicate that persistence of trypanosomal antigens in animals treated with trypanocidal drugs is likely to be as significant a factor in interpretation as is the persistence of trypanosomal antibodies in Ab-ELISAs. Also, the sensitivity and specificity of Ab-ELISAs may be higher than those of the currently available Ag-ELISAs. More work is required in evaluating ELISAs before they can be properly utilized in serodiagnosis; the foregoing account has illustrated how infrequently attention has been paid to establishing diagnostic sensitivity and specificity, and the Ab-ELISAs require defined antigens to reduce variability in their preparation. There are also major conceptual barriers to be overcome, notably, using the results of serodiagnosis at the levels of both the individual and the population, to determine a treatment strategy.

Enzyme immunoassays are likely to be used for several different reasons: to estimate the distribution of disease, to estimate prevalence, to monitor the effects of implementation of a control strategy, or to carry out surveillance in areas in which control measures have been applied. Further development of the assays will determine whether one or the other (or both) of the assays will be most appropriate. Enzyme immunoassays do have an important role to play in the overall understanding of the epidemiology of animal trypanosomoses. Currently, there are plans to use Ab-ELISAs in the Regional Tsetse and Trypanosomosis Control Programme to monitor cattle in areas that have been cleared of tsetse fly. In southeast Asia, although epidemics of trypanosomosis caused by *T. evansi* occur, overt clinical disease is often not seen in areas where the disease is endemic. Serological surveys have shown high prevalence values, but control of trypanosomosis is based on treatment of infected animals identified by simple parasitological tests or by clinical signs of disease in response to outbreaks. This method of control fails to address the underlying problem of carrier animals, because treatment of animals with overt evidence of infection or disease will not diminish the number of cases as effectively as the treatment of all infected animals. The method is beneficial to the individual, but of little relevance to the larger number of animals with non-patent infections whose presence contributes more widely to the overall morbidity and provides a constant source of infection. Unlike the tsetse transmitted trypanosomoses, wildlife reservoirs of infection are unimportant. Given this circumstance and the non-cyclical mode of transmission, a control strategy based solely on the use of chemotherapy should prove effective if all infected animals can be identified. Enzyme immunoassays should provide the means by which this can be accomplished, and it is in this area that the assays are likely to make their most significant impact.
REFERENCES


[15] LUCKINS, A.G., et al., Comparison of the diagnostic value of serum immunoglobulin levels, an enzyme-linked immunosorbent assay and a fluorescent antibody test in


DEVELOPMENT AND USE OF ELISA IN THE CONTROL OF FOOT-AND-MOUTH DISEASE

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United Kingdom

Abstract

DEVELOPMENT AND USE OF ELISA IN THE CONTROL OF FOOT-AND-MOUTH DISEASE.

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating disease that requires laboratory investigation for definitive diagnosis. Highly specific and sensitive assays are needed to obtain a rapid result and so optimize the efficiency of FMD diagnosis and formulation of appropriate control measures. The development of the enzyme linked immunosorbent assay (ELISA) has produced marked improvements in the diagnosis of FMD. An indirect sandwich ELISA has replaced the complement fixation test for FMDV virus antigen detection and a liquid phase blocking ELISA (LPBE) has replaced the virus neutralization test for the detection of serum antibodies. The LPBE is also employed to test the suitability of available vaccine strains for use in current outbreaks. The assays mainly use polyclonal antisera, but monoclonal antibodies have brought greater precision to virus strain characterization studies and have the potential to replace polyclonal sera in other assays. The provision of ELISA kits enables national FMD laboratories in developing countries to carry out their own assays in the first instance and allows effective and more immediate decisions to be made on control. At present, there are no reliable mass serum screening laboratory tests (i.e. ELISA) that can distinguish vaccinated from infected and carrier from non-carrier animals. These groups of animals have to be considered potential carriers of FMD virus and a source of new disease and thus need to be excluded from internal and international trade. ELISAs employing reagents to bioengineered non-structural proteins of FMD virus hold considerable promise for the discrimination of naive, convalescent and vaccinated animals, and work is in progress for their refinement.

1. INTRODUCTION

Foot-and-mouth disease (FMD) is regarded as economically the most important viral disease of livestock worldwide. Severe impositions on a country’s internal and international trade in livestock and meat products may result from a single outbreak. All cloven hoofed animals are susceptible to infection. In endemic areas, major consequences of FMD are through incapacitation of draught animals for ploughing,
harvesting and transport. FMD is a major constraint on efforts to upgrade the quality of livestock through introduction of improved breeds of animals, which are often more susceptible to disease than indigenous stock.

Although control and eradication methods may vary from a stamping-out policy in FMD free areas to vaccination in endemic areas, an essential feature is accurate information on the serotype of the virus causing outbreaks. There are seven antigenically distinct serotypes of FMD virus: O, A, C, SAT1, SAT2, SAT3 and ASIA 1. It is the most highly contagious animal virus known and has the potential for massive and explosive spread upon introduction to a highly susceptible animal population in a previously FMD free area. The ever increasing global movement of people, livestock, their germ plasm and products provides increasing opportunity for transmission of FMD virus. Early recognition in the field coupled with rapid laboratory diagnosis of the viral agent is of prime importance in limiting the spread of any infection. In endemic regions, vaccination remains the cornerstone of any control policy, and information on the causative serotype is essential for vaccine selection. In either situation, the faster this information is obtained, the more rapidly can appropriate zoosanitary measures be implemented to control and eradicate the disease. Consequently, laboratory assays need to be highly sensitive and specific; this paper describes the development of FMD enzyme linked immunosorbent assays (ELISAs).

2. FMD DIAGNOSIS

FMD is characterized in cattle by fever, anorexia, depression and drop in milk yield, followed by the development of vesicles on the tongue, udders and feet. The vesicles rapidly increase in size and rupture, which leads to excessive salivation and lameness. However, there are a number of other conditions which produce clinical signs similar to these of FMD, and differential diagnosis in cattle is required from the diseases of vesicular stomatitis (VS), bovine viral diarrhoea, infectious bovine rhinotraceitis, mucosal disease, rinderpest, bovine papular stomatitis and calf diphtheria. The most important diseases in the differential diagnosis of FMD in pigs are swine vesicular disease and vesicular exanthema of swine, and in sheep, foot rot and bluetongue.

2.1. Development of ELISA for FMD virus antigen detection

Definitive diagnosis of FMD is based on the demonstration of FMD virus or antigen in tissue samples. The definitive diagnosis involves identifying viral antigen in vesicular specimens submitted from animals in the early stages of disease, without recourse to tissue culture growth, amplification of virus or the delay that this entails. The diagnostic sample of choice is epithelium from a vesicular lesion. When tissue samples cannot be obtained, diagnosis may be based on the demonstration of specific
antibodies in serum samples, but is complicated by uncertain history of vaccination or the possibility of previous infection.

The complement fixation test (CFT) had long been the test of choice for FMD virus antigen detection, but it has many disadvantages: pro- and anti-complementary factors commonly invalidate the assay, test results are read subjectively and can vary between test operators, the CFT is complex, and reagents have a short shelf-life and must be stringently maintained. Most importantly, however, the CFT is relatively insensitive.

The intention to utilize ELISA in routine FMD virus diagnosis had been evident ever since the effectiveness of ELISA procedures in FMD virus research was first demonstrated [1, 2]. Hamblin et al. [3] explored the possibilities of an indirect sandwich ELISA using bovine immunoglobulin (Ig) as a trapping antibody and guinea pig Ig as a detecting antibody. The assay was more sensitive and specific than the CFT on freshly harvested vesicular epithelium, but failed to markedly improve upon the CFT in the direct typing of samples of poorer quality. Other ELISA procedures failed to combine sensitivity and type specificity simultaneously [4, 5] until this was achieved by Roeder and Le Blanc Smith [6] with an ELISA using high titred, type specific rabbit instead of bovine antisera as the trapping antibodies. This ELISA, with minor modifications, was run in parallel with the conventional CFT in the World Reference Laboratory (WRL) for FMD in routine diagnosis on submitted field samples for a period of eight months [7]. Its superiority for diagnosis was demonstrated, and the assay adopted for use. Other groups around the world similarly demonstrated this superiority [8-10], and the procedure has been almost universally adopted for FMD primary diagnosis.

Briefly, the ELISA procedure is as follows. Rabbit antisera specific for the different serotypes of FMD virus are passively adsorbed to polystyrene microwells. With the addition of the test sample, antigen (if present) is trapped by the immobilized antibodies. Specific guinea pig anti-FMD virus detecting antibodies are then added, which react with the trapped antigen. The bound guinea pig antibodies are detected by means of the rabbit anti-guinea pig Ig conjugated to horseradish peroxidase. With the addition of substrate/chromogen solution, a colour product develops that may be measured and interpreted with respect to the antigen content of the test sample. As this is a solid phase assay, suitable washing procedures to remove unbound reagents are needed at each step.

An important aspect in the evaluation of the assay was the necessity to block the antiserum reagents against anti-bovine Ig activity. Purified antigens used to raise antiserum contain trace bovine Ig. This is derived from the normal bovine serum supplement to the Eagle's medium used for growth of cells employed for virus propagation. Resulting antisera thus contain both anti-FMD virus and anti-bovine antibody components and will bind to any bovine Ig attached to the solid phase or trapped by rabbit antibody and lead to an eventual false positive result. However, the
adverse activity of the anti-bovine Ig is eliminated by mixing equal volumes of the guinea pig antiserum (and conjugate) and non-immune bovine serum. Blocking the rabbit antiserum in this manner tends to increase the background colour unacceptably and is hence avoided; in any event, it is not necessary to do so if later antiserum reactants in the ELISA are blocked.

Homogenates of positive FMD virus epithelial samples contain high concentrations of extraneous protein that will compete for available sites on microwells in an indirect assay. The particular advantage of the indirect sandwich ELISA is that the presence of FMD virus type specific rabbit antibody on the plate will select the FMD virus antigen from the epithelial suspensions and ensures that its amount is semiconcentrated to increase the chances of its detection. Other advantages of the ELISA include the following:

1. Increased sensitivity — ELISA has produced marked improvements over the CFT in primary diagnosis. In the WRL, 70–80% of confirmed virus positive submissions are typed by ELISA on suspensions of vesicular samples received from disease outbreaks. This compares with values of between 5% and 20% previously encountered using the CFT. The main reason why this figure is not 100% is a reflection of the quality of the submitted specimen. Samples are received from countries worldwide, and a high proportion of received material is of poor quality, containing insufficient amounts of antigen for detection by ELISA. This can be regarded as a result of taking samples late in the course of disease when amounts of antigen are waning. Ideally, ELISA should be used in combination with cell culture to amplify low amounts of antigen to a concentration detectable by ELISA. In the absence of cell cultures, more than one sample should be submitted from the herd to increase the chance of detection.

2. ELISA is able to serotype degraded preparations of FMD virus efficiently, which is useful for carrying out diagnosis on samples subjected to the adverse pH conditions of an incorrect transport buffer or to elevated temperatures between collection and laboratory receipt.

3. Complement is not involved in the ELISA, so pro- and anti-complementary activities are of no importance.

4. ELISA is more economical in the use of type specific antisera; antiserum dilutions in the thousands are common.

5. ELISA plates can be read spectrophotometrically, i.e. objectively, giving greater accuracy to results. The use of a computer linked to the spectrophotometer aids in the interpretation of results, their storage and retrieval.

6. ELISA is rapid, highly reproducible and simple to perform; the reagents have a long shelf-life and can be readily standardized. Considerable time can be saved in the assay by employing immunoplates sensitized with rabbit antisera, which can be stored ready for use at either 4°C or −20°C.
Crucial to the success of ELISA is the quality of the antiserum reagents used in the assay. Essential requirements of a diagnostic serum are that it should be high in titre, exhibit no or very low heterotypic reaction and have a broad homotypic reaction. A standardized procedure for producing inactivated and purified 146S antigens of FMD virus as immunogens for the production of reference antisera has been adopted [11] and provides more reproducible methods for antiserum production [12]. Field virus isolates can be of widely divergent subtypes of markedly different antigenicity. Careful selection of strains for preparation of reference diagnostic antisera is necessary to ensure that selected reagents have a broad spectrum of reaction for virus typing; otherwise, there can be risk of failure to detect all strains. Experience and investigation have shown that antisera against certain strains of FMD virus fulfil this criterion better than others [13, 14]. The FMD virus serotype that presents most problems is type A. Strains within this serotype can differ to such an extent that it is difficult to ensure adequate diagnosis by use of a single antiserum strain. A more comprehensive coverage of type A virus strains is achieved by use of a combination antiserum produced by the simultaneous animal inoculation of several antigenically different type A virus strains.

2.2. Development of ELISA for FMD serology

As previously stated, serology is not the method of choice for the primary diagnosis of FMD, but serological investigations are important for: (1) epidemiological surveys for estimating the incidence/prevalence of disease, (2) monitoring the efficacy of vaccination programmes and for an indirect assessment of vaccine strain suitability, and (3) the screening of sera to demonstrate the absence of antibodies before international movement of animals or their germ plasm (semen, embryos).

As in the field of antigen detection, Crowther and Abu Elzein [15, 16] were the first to show that ELISA could be used for FMD virus serology. Antibody titres could be determined from calibration curves derived from an indirect ELISA. These and other assays were sensitive and specific but the correlations between virus neutralization (VN: the ‘gold standard’) and ELISA titres varied. Hamblin et al. [17, 18] developed a liquid phase blocking ELISA (LPBE) that mimics the VN test whereby ELISA titres can be correlated with VN titres. An ELISA serum titre of 1:40 corresponds to a VN titre of 1:16 and was considered positive. The LPBE has since been successfully run in many other FMD laboratories and is generally accepted as a replacement for the VN test.

The test is based on specific blocking of the FMD virus antigen in liquid phase by antibodies in the test serum sample. Rabbit antisera specific for the different serotypes of FMD virus are passively adsorbed to polystyrene microwells. After the test serum has been allowed to react with the specific FMD virus antigen, the test serum/antigen mixture is then transferred to an ELISA plate coated with FMD virus
trapping antibodies. The presence of antibodies to FMD virus in the serum sample will result in the formation of immune complexes and consequently reduce the amount of free antigen trapped by the immobilized rabbit antisera. In turn, fewer guinea pig anti-FMD virus detecting antibodies will react in the next incubation step. After the addition of enzyme labelled (HRP) anti-guinea pig Ig conjugate and substrate/chromogen solution, a reduction in colour development will be observed when compared to controls containing free antigen only.

Benefits in assay have been achieved from more reproducible results and their quicker issue, the lower percentage of false positive results, and not least the elimination of tissue culture cells during assay and the disadvantages this entails through invalidation of test procedures resulting from poor cell growth, variation in virus sensitivity, contamination by bacteria and fungi and susceptibility to the cytotoxic effects of some test sera. An additional advantage in disease security of this ELISA is that inactivated antigen does not alter the sensitivity or specificity of the LPBE and can be used instead of live virus [19]. Laboratories handling certain live serotypes of FMD virus could increase the spectrum of their tests by using inactivated antigens of other serotypes. Countries could themselves evaluate the likely immunity of their animals against exotic strains of FMD virus that might enter from outside. This could be of particular appeal to those countries whose land borders such areas of endemic FMD and where cross-border movement of animals is difficult to monitor. It would thus comply with the recommendations of the Food and Agriculture Organization of the United Nations (FAO) that national laboratories should keep in stock and have the capability of diagnosing all seven serotypes of FMD virus. Other laboratories lacking the high security facilities required for the manipulation of live virus might also be sanctioned to carry out FMD serological assays.

3. VIRUS STRAIN CHARACTERIZATION

Once the virus type has been confirmed, further investigations on the antigenic characterization of the virus strain are carried out in order to identify an appropriate vaccine strain and for epidemiological evaluation of a likely origin of the outbreak strain. The former aspect is approached by use of an ELISA using antisera against current available vaccine virus, and the latter aspect by a combination of nucleotide sequencing and monoclonal antibody (MAb) profiling analysis by ELISA.

3.1. Vaccine selection

The LPBE has been incorporated into a two stage system for comparing the antigenic relatedness of contemporary field strains to reference vaccine strains [20]. In the first stage of the procedure, a contemporary field strain is grown in BHK-21
cells and titrated against guinea pig reference vaccine strain antisera using the indirect sandwich ELISA. The strength of reaction indicates the respective vaccine strains with which the field isolate has the greatest affinity. In the second stage, the most closely related vaccine strains are examined further as potential vaccines for the control of the outbreak using the LPBE. Bovine antisera collected 21 days after vaccination with these selected vaccine strains are titrated against the field isolate as well as against the homologous vaccine strain viruses. The $r_1$ values are determined as follows:

$$r_1 = \frac{\text{titre of bovine antiserum against isolate}}{\text{titre of bovine antiserum against vaccine virus}}$$

$r_1$ values close to 1 suggest that the isolate is not significantly different antigenically from the vaccine strain, but values <0.2 suggest that there is a highly significant serological variation from the reference vaccine strain.

In this manner, advice can be given as to the suitability of an existing vaccine against the virus strains prevalent in the area, and control measures by vaccination can be implemented immediately. If the strain is markedly different, the need to select a contemporary field strain for adaptation as a new vaccine strain virus can be considered.

It is recognized that this is an in vitro system, but the particular advantage of this method is that advice about the most suitable vaccine based on antigenic relatedness can be given rapidly. Over several years during which the in vitro system has been used, field experience has corroborated the advice given.

The success of this work partly depends upon vaccine virus strains that are currently in use by manufacturers being made available for comparative studies together with 21 day post-vaccinal sera. These tests for vaccine suitability can now be carried out with inactivated antigen, thus allaying the fear of vaccine manufacturers when supplying live seed vaccine virus strains and further improving the service that FMD reference laboratories can provide.

The test gives no information about vaccine potency, but the LPBE has been applied for evaluating the likely protective immunity in vaccinated cattle in Argentina as a replacement or partial replacement for the cattle challenge test [21]. Results suggest that a post-vaccination serum titre of $\log_{10} 2.1$ could be taken as the minimum titre compatible with protection. Vaccines producing serum titres of $<\log_{10} 1.5$ should be rejected automatically, and those inducing titres $>\log_{10} 2.1$ approved directly without the need for cattle challenge. It would only be necessary to employ cattle challenge experiments for those vaccines inducing serum titres of between $\log_{10} 1.5$ and $\log_{10} 2.1$. This approach would be beneficial on ethical, time and economic grounds and would reduce the risks associated with challenging the animals with live virus.
3.2. Monoclonal antibodies

Viruses are complex structures composed of different proteins that in turn consist of multiple antigenic determinates (epitopes). Polyclonal antisera consist of heterogeneous populations of antibodies which recognize many different epitopes. Consequently, the study of small antigenic differences between virus isolates is difficult. However, FMD virus strain characterization has been advanced by the availability of MAbs produced by cells derived from single cell clones and capable of reaction with a single epitope. They are therefore ideal reagents for rapid identification and differentiation of virus isolates in epidemiological studies and in vaccine evaluation [22, 23].

Rabbit polyclonal sera are used in an indirect sandwich ELISA to trap a pretitrated, single dilution of each virus sample and reacted with a single, predetermined dilution of each MAb in the panel and compared for reaction with guinea pig polyclonal serum. Following the addition of anti-species conjugate, the assay is developed by substrate/chromogen solution. Percentage reactivity values for each virus can be derived against each of the MAbs and measure whether viruses share epitopes. Comparisons can therefore be made between new and previously isolated viruses to indicate a likely origin of an outbreak and for the establishment of a possible epidemiological link. In addition, field and vaccine strains can be compared for the selection of an appropriate vaccine for control measures, or for decisions as to the need for creating a new vaccine if the current vaccine strain virus is considered inappropriate.

4. DEVELOPMENT OF ELISA KITS FOR FMD DIAGNOSIS

As the benefits of ELISA became more widely appreciated, the WRL received an ever increasing number of requests from FMD laboratories around the world for suitable diagnostic reagents. Sending diagnostic samples to the WRL or regional laboratories is costly, and there is an inevitable time delay before results are obtained. The availability of a sensitive ‘off-the-shelf’ test would be beneficial in allowing countries to run tests in the first instance independently of reference laboratories. The ELISA is readily adaptable to technology transfer to other countries, and the possibility of supplying ELISA kits for diagnosis therefore had considerable appeal.

FMD virus type specific antisera can be supplied in a freeze dried and standardized condition, which greatly simplifies procedures [24]. There would be considerable advantage if it were also feasible to supply FMD virus antigens in a freeze dried condition as controls for antigen detection and for use in the LPBE. Investigations have consequently been carried out to explore the effect of freeze drying and subsequent storage on FMD virus antigenicity and the reactivity of freeze dried FMD virus antigens in the ELISA [25].
It has generally been found that some additive solution must be made to virus suspensions to facilitate freeze drying and to preserve their biological activity during the freeze drying process. A diverse range of additives were thus investigated for their suitability, as historically other investigators have found the freeze drying of FMD virus to be especially problematical. Antigen potency, as judged by titration in ELISA, was unaffected by freeze drying and throughout subsequent storage at −20°C and 4°C with or without additives having been made to virus suspensions before freeze drying. However, freeze drying antigens without additive were predisposed to rapid loss of activity at the elevated storage temperature of 37°C. This was in contrast to the prolonged maintenance of antigen strength resulting from the use of many of the additive solutions that were employed. Together with other studies performed on the maintenance of infectivity of freeze dried antigens, the cryoprotectant of choice was 10% sucrose and 5% lactalbumin hydrolysate. The study demonstrated that freeze drying of antigens did not compromise their type specificity in ELISA or drastically alter the reactivity for serological tests.

The fact that certain solutions prolonged the survival of antigens in the freeze dried state offers the possibility of shipment and short term storage of antigens without the requirement of refrigeration and would protect against damage due to heat that might occur with delays in transit in tropical countries. Once in the laboratory, reagents in a freeze dried condition can be stored indefinitely.

ELISA kits for antigen detection and serology have since been formulated using freeze dried reagents of rabbit and guinea pig antisera, conjugate, inactivated antigens and positive and negative bovine control sera, and combined with commercially available powder, tablets or capsules of phosphate buffered saline, coating buffer, chromogen and substrate solely requiring reconstitution with deionized/distilled water. Such kits have now been supplied to national FMD laboratories in many countries with standardized protocols, often through liaison with the IAEA in Vienna, and have been used successfully in disease surveillance, control and eradication programmes.

Particular areas of interest where FMD kits using freeze dried inactivated antigens would be of value include epidemiological surveys for virus or antibody, monitoring of vaccination coverage and indirect assessment of vaccine strain suitability. These are important considerations in the formulation of a policy for a country (or for a region within a country) in which vaccination is to be the basis of disease control.

An example of their use is an FAO/IAEA funded research programme in Latin America to evaluate the usefulness of WRL ELISA kits for the diagnosis of FMD and VS as compared with conventional methods carried out in collaboration with the Pan American Foot-and-Mouth Disease Center (PAFMDC) in Brazil. Successful validation of the antigen detection ELISA resulted from its use in some nine countries, and the assay is now used routinely. The subsequent goal is to use PAFMDC kits
for serological surveys of the incidence/prevalence of these diseases and to monitor
the effectiveness of national control programmes aimed at their control, e.g. vaccina-
tion schemes against FMD. The third aim is to generate the data necessary to enable
international acceptance of test reagents and protocols in order that they may subse-
quently be used in other regions of the world. Additional benefits arise from the
strengthening of collaborative links between FMD laboratories and regional reference
centres and the WRL to improve the control of FMD.

A similar programme is now operating in the diagnosis and control of FMD in
South-East Asia where it is also recognized that the best way to tackle the problem is
a regional one. There was a considerable variation in the existing diagnostic capabil-
ities of national FMD laboratories (ten countries), and the minimum requirement has
been to establish the ELISA for antigen detection in the first instance. Laboratory
activities have now been strengthened and extended by the use of antibody kits for
checking vaccines and for epidemiological surveys. Both assays are being utilized to
determine the epidemiology of the disease in the region as an aid in determining the
optimal strategies for disease control.

5. FUTURE DEVELOPMENTS IN ELISA FOR FMD DIAGNOSIS

At present, polyclonal antisera are used in the antigen detection ELISA, but
investigations are being made to identify suitable MAbs to each serotype for replace-
ment. Such reagents will have advantages in improvement of test standardization and
specificity and perhaps sensitivity, and provide a potentially infinite source of reagent.
However, many MAbs have a particularly high strain specificity that can be a draw-
back in non-recognition of other strains within the serotype, but this can be overcome
by mixing several MAbs into appropriate panels to achieve the desired degree of
reaction. Another advantage from such panels is that serotyping, strain
characterization and vaccine selection might be performed simultaneously and overall
results issued yet more rapidly.

Infected animals can become carriers of FMD virus for many years and can be
a potential source of new outbreaks. A main priority of any FMD free country is to
avoid introduction of the virus; therefore, serological tests should be highly sensitive
and specific to reduce this risk. The LPBE is type specific, but this can be a drawback
for import/export testing because a serum sample needs to be tested against antigen
of every serotype of likely concern.

An objective of many FMD research groups is to develop a serological test suit-
able for mass serum screening that can discriminate between vaccinated and infected
and between carrier and non-carrier animals. The LPBE measures antibody to the
structural proteins of FMD virus and, as these are produced following both infection
and vaccination, does not discriminate vaccinated from infected animals.
Non-structural proteins (NSPs) of FMD virus are produced following infection, while vaccination induces antibody principally to the structural proteins. The NSPs were commonly referred to as the virus infection associated antigen (VIAA) and are conserved between serotypes. This has considerable appeal as, theoretically, infection with any serotype of FMD virus could be detected by one assay using VIAA. Considerable efforts made in attempts to develop a VIAA ELISA have foundered through lack of consistent, reproducible results.

Biotechnology for antigen production has considerable appeal in terms of the potentially unlimited supply of standardized material that can be generated and, through removal of the need to manipulate live virus, in terms of disease security. The major antigenic component of VIAA is the viral RNA polymerase, i.e. NSP, 3D. Bioengineered forms of 3D have been examined as potential candidates for use in diagnostic tests. However, increasing evidence suggests that 3D is not the best antigen to use to differentiate vaccines from infected animals because vaccination can induce antibody to 3D. This has led to the examination of other NSPs for suitability. As an example of such studies, Mackay [26] has developed an indirect, profiling ELISA using MAbS and \textit{E. coli} expressed antigens to NSP Lb, 2C, 3A, 3D and 3ABC. The assay successfully detects antibody to 2C, 3A, 3D and 3ABC in sera from cattle infected with any of the seven FMD virus serotypes and, in the majority, Lb. Conversely, only 3D is detected in sera from vaccinated cattle. The potential therefore exists for NSP assays to discriminate between normal, infected and vaccinated animals on the basis of the reaction of sera with structural proteins and NSPs. Further development is in progress.

6. DISCUSSION

The ELISA has improved the efficiency of FMD diagnosis in all the major areas of interest: antigen detection, serology, strain characterization and vaccine evaluation. Failure to correctly diagnose FMD because of insensitive assays can have serious repercussions as illustrated by events in Greece in 1994. FMD in sheep on the island of Lesbos was misdiagnosed in the field because of mild clinical signs, which was compounded by local laboratory failure to type antigen in submitted samples. It was only after sheep were moved onto the Greek mainland that samples were sent to the WRL and FMD was confirmed, by which time disease had spread and hence exacerbated control.

An important requirement for the control of disease is the ability to investigate rapidly the likely sources of outbreaks. The application of ELISAs and monoclonals has greatly reduced the time taken to characterize field isolates, and advice on the most appropriate vaccine for emergency use and the probable origin of outbreaks can now be more speedily provided.
The transfer of ELISA techniques to provincial laboratories in the form of kits can contribute greatly to improving the universally accepted role of diagnosis for effective disease control. Kits are useful in countries in which vaccine is used to contain disease, especially under extensive systems of husbandry where there is a need to monitor the efficacy of vaccine administration and the resulting immunity; the kits greatly facilitate large scale epidemiological surveys.

It has been essential to consider both vaccinated and convalescent animals as potential carriers of virus and source of new disease, but it is hoped that the development of NSP ELISAs will ultimately lead to a reliable serological assay suited to mass serum screening that will distinguish between antibody to FMD vaccination and FMD infection and between carrier and non-carrier animals.

REFERENCES


BIOSENSORS

New developments and opportunities in the diagnosis of livestock diseases

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Abstract

BIOSENSORS: NEW DEVELOPMENTS AND OPPORTUNITIES IN THE DIAGNOSIS OF LIVESTOCK DISEASES.

Phenomenal growth in the field of biosensors has been observed in recent years, with applications in a wide range of disciplines, including food, environment and medical analysis. The need for high sensitivity, speed and accuracy of analytical measurements has stimulated considerable interest in developing sensors as diagnostics tools. Substances with recognition powers are available naturally in the form of antibodies, enzymes, cell receptors, nucleic acids and lectins. These can be used as the sensing element in biosensors, and therefore a wide range of analytes can be detected and measured using these devices. A wide range of transducers are also feasible. Biosensors are suitable devices to fulfil the rapid monitoring needs of the diagnostic market. They have the great advantage over many other analytical methods in that they can be incorporated into simple to use instruments. Immunochemical sensors are powerful analytical devices which enable the identification of a wide range of target molecules. These devices combine the selectivity of antibodies with the sensitivity and rapid measurement of a biosensor. The rapid development of immunosensor technology during the last few years opens new perspectives for the development of relatively inexpensive applications to monitor livestock and their products. The paper deals with recent developments in biosensor technology and assesses the current and potential use of biosensors in the diagnosis and control of livestock diseases.

1. INTRODUCTION

Biosensors are analytical devices, constructed by combining a biological sensing element (e.g. enzymes, antibodies, microorganisms, DNA, receptors) with a chemical or physical transducer to obtain a measurable signal (Fig. 1). Four major types of transducer are used: electrochemical (electrodes), optical (optrodes), mass (piezoelectric or surface acoustic wave devices) and calorimetical (thermistor or heat sensitive sensors). Biosensors are becoming important in a wide range of analyses
because they have the advantages of high sensitivity and rapid measurement and can be used in situ, rather than requiring samples to be sent for laboratory testing. Biosensors have been developed for both specific analytes and broad spectrum monitoring. Miniaturization, reduced cost and the improved processing power of modern microelectronics have further increased the analytical capabilities of such devices and given them access to a wider range of applications [1]. In addition, the market for diagnostics continues to grow; for example, over 10 million penicillin assays are performed on the milk of 12 million cattle at dairy farms in the United States of America each year [2]. Fish and meat freshness instruments, based on the determination of nucleotide related compounds to indicate whether the product is fit for human consumption, have been introduced into the market. A staggering diversity of research on biosensors is reported in the literature and offers a seemingly infinite number of biosensor possibilities, but most of this research has been concentrated on small niche applications.

2. THE BIOLOGICAL COMPONENTS

A biosensor exploits the selective binding capabilities inherent in biomolecules. This recognition of properties of biological components can be applied to produce either an affinity or a catalytic sensor. Molecules such as nucleic acids, protein lipids and their derivatives, enzymes, etc., can all be used as the sensing element in

FIG. 1. Schematic diagram of a biosensor.
biosensors. In catalytic sensors, it is the change in the concentration of a component resulting from the catalysed reaction that is detected. In the case of an affinity sensor, it is the binding event itself that is monitored.

Enzymes are proteins that act as specific catalysts for a large number of biochemical reactions, and they are used extensively in biosensors as the catalytic component. By far the most important for biosensors have been the oxidoreductases, catalysing oxidation or reduction events using either oxygen or cofactors such as nicotinamide adenine dinucleotide (NAD). Whole cells of living organisms, such as bacteria, yeast, fungi, plant and animal cells or even tissue slices, have also been used as the recognition component by interrogating their general metabolic status. This usually involves detecting oxygen or substrate consumption, producing carbon dioxide or metabolites, detecting bacterial luminescence, or direct electrochemical sampling of the electron transport chain [3].

Affinity sensors use mainly antibody–antigen binding reactions, but other biological components such as cell receptors, single stranded DNA (to bind and detect its complementary sequence) and lectins (plant proteins used to bind carbohydrates) have also been used. Combinations of the above biological components have been utilized to provide new or improved analytical capabilities.

While biological reagents offer sensor opportunities that are often not currently feasible by other means, the poor stability of these materials is frequently the stumbling block preventing commercialization. Methods for improving the stability of these compounds, such as the use of soluble, positively charged polymers, for example as diethylaminoethyl (DEAE) dextran and lacitol, may overcome some of the storage problems often associated with biosensors.

3. TRANSDUCERS

The four major classes of transducers used in biosensors are based on electrochemistry, optics, thermometrics and piezoelectricity. Electrochemical devices usually monitor the current at a fixed voltage (amperometry) or the voltage at zero current (potentiometry), or measure conductivity or impedance changes. Impedance is the total electrical resistance to the flow of an alternating current being passed through a given medium. Typically, during measurement impedance decreases while conductivity and capacitance increase.

Optical transducers use a number of principles, such as the effect of the biological event on light absorption, fluorescence, refractive index or other optical parameters. Thermometric devices operate by measuring enthalpy changes during the biological reaction. Sensors based on piezoelectric principles use the change in the resonant frequency of wave propagation through a piezoelectric material. These principles can be used to measure mass, viscosity or density changes at the sensor
surface. Further information on transducers used in biosensor devices can be acquired from Ref. [4].

4. BIOCATALYTIC SENSORS

Electrochemical and, in particular, amperometric transduction approaches have dominated research activity in biocatalytic sensors for many years. An enzyme amperometric biosensor is an electrochemical device incorporating an enzyme which converts a substrate into a chemical that can be detected at a fixed potential by means of the flow of current. The simplest biosensor approach is to measure amperometrically the oxygen depletion or hydrogen peroxide production associated with the reactions involving oxidase enzymes. The combination of simplicity, ease of manufacture, high sensitivity and the availability and low cost of instrumentation is highly attractive. There are several biosensor products on the market based on amperometric transduction, including the MediSense blood glucose analyser and the Yellow Springs biosensor systems. Coupling the redox centre of enzymes to electrode surfaces via redox mediators has been an area of great interest to many researchers. Biosensors based on this format use the chemical mediators to shuttle the electrons resulting from the catalytic oxidation or reduction of the substrate of interest to the electrode. The aim is to increase the sensor stability and improve current densities. Much of the work is centred on the use of redox mediator compounds (such as ferrocene, tetrathiafulvalene and tetracyano quino dimethane) and their derivatives [5]. Careful selection of the mediator can also eliminate interference from other substances in the sample. Alternatively, interferences can be alleviated by the membranes used in sensor construction [6], or by introducing catalytic surfaces selective for the oxidation of hydrogen peroxide, such as rhodinized carbon [7] or hexacyanoferrate modified carbon [8].

Various approaches to produce implantable sensors have been performed during the last 20 years. The development of implantable sensors (in vivo biosensors) for monitoring glucose in diabetic patients has received much attention, especially for use as a hypoglycaemia alarm or as a closed loop insulin delivery system. Most of the in vivo glucose biosensors have a lifetime of between 24 and 48 h, and problems of biocompatibility and interference still remain to be overcome. Implantable biocatalytic devices for blood gases such as oxygen and carbon dioxide are also being developed. The major weaknesses of most of the in vivo biosensors are insufficient long term stability, lack of biocompatibility and the problem of recalibration in vivo.

The measurement of lactate concentrations is important in exercise and critical care medicine to show the level of metabolic stress on the body. The application of blood lactate measurements to racehorse training and fitness assessment have been reported in the literature [9, 10]. Reduced glutathione (GSH), N-(N-L-\(\gamma\)-glutamyl-L-
cysteinyl) glycine, is the major intracellular thiol found in mammals and is important for the detoxification of some drugs. Defects in the levels of glutathione and its metabolizing enzymes are used as a marker in the investigation of certain cancers in cattle [11, 12]. Biosensors have been developed to detect this compound in bovine blood [12, 13].

Enzyme inhibition sensors are the most commonly reported enzyme based biosensors for the detection of toxic compounds. These sensors are based on the selective inhibition of specific enzymes by classes of compounds or by the more general inhibition of enzyme activity [14]. Organic phase enzyme electrodes (OPEEs) have been used for the analysis of such compounds as cholesterol, alcohols, organic peroxides and phenols. This technique has the advantages of ease of sample preparation from materials such as fats and oils and increased enzyme stability [15].

Whole cells have been used as biocatalytic elements for several applications. Biosensors based on this approach generally exhibit multireceptor behaviour but can offer increased stability when compared with enzyme based sensors. Whole cell biosensors have been used for environmental monitoring, exploiting the broad sensitivity of such devices to a wide range of toxins. A range of such biosensors have been developed, in particular for water monitoring. The method comprises the use of mediators to detect the metabolic activity of the cells, which decreases sharply in the presence of toxins [3].

Potentiometric approaches have also been widely investigated for the construction of biosensors, primarily using ion selective electrodes (ISEs). However, potentiometric enzyme based devices are particularly prone to interference from pH changes and also drift due to critical dependence on the reference measurement.

Optical biocatalytic sensors have been developed based on the use of fluorescence dye and its interaction with immobilized enzyme. Optical sensors are usually based on optical fibres or planar waveguide films. On the basis of a pH optode, different fibre optic biosensors have been constructed for glucose, urea, penicillin and creatinine [16].

5. AFFINITY SENSORS

The use of antibodies in various immunosensor configurations has had a wide range of applications, although the development of an immunosensor is more complex than enzyme sensors. For an immunosensor or immunoprobe, either the antibody (Ab) or the antigen (Ag) constitutes the biospecific component. Immunosensors can be divided into classes depending on the transducer technology employed: piezoelectric, electrochemical, optical and thermometric. In the field of affinity sensors, optical devices have a clear advantage over electrochemical methods because of their ability to monitor binding reactions directly. The major drawback to
the application of optics to chemical sensor applications remains the high cost of many optical components, but these costs are constantly falling.

Optical techniques such as surface plasmon resonance (SPR) and evanescent wave (EW) have shown promise in providing direct measurement of Ag–Ab interactions occurring at the surface–solution interface, the surface usually consisting of a glass prism on a gold or silver metal layer. SPR is a phenomenon which occurs when a beam of light is directed onto a glass–metal interface, which results in changes in the resonance angle [17]. The resonance angle is sensitive to changes in the refractive index and dielectric constant at the interface up to a distance of ~1000 nm from the actual metal surface. Immobilization of an Ab on the surface causes a measurable shift in the resonance angle; on binding of Ag to the immobilized Ab, a further change will occur. The binding induced shift in resonance angle (expressed as resonance units, RU) is approximately linearly proportional to the concentration of bound Ag (or Ab, if Ag is pre-immobilized) for typical biological systems.

The SPR based biosensor system (BIAcore™) developed by Pharmacia (Uppsala, Sweden) represents a significant breakthrough in immunosensor technology. However, a major problem associated with this approach is the high degree of non-specific binding to the metal surface that occurs when the immunosensor is presented with real samples, particularly whole serum containing many different proteins [18].

Amersham International is another company that has been active in research in SPR based immunosensors for infectious diseases and has developed SPR based immunotechnology that employs antibodies labelled with latex particles (beads). The beads amplify the change in refractive index at the sensor surface–solution interface which occurs when the antibodies bind to the immobilized antigen layer and thus cause a larger change in the resonance angle [19].

EW based systems have been incorporated into immunosensor designs in both direct (no signal generating tracer used) and indirect (fluorochrome label) formats [20]. EWs are set up when light travelling through a waveguide or optical fibre, in direct contact with a solution, undergoes total internal reflection at the waveguide. The evanescent light waves propagate into the solution and decay exponentially with distance from the surface–solution interface. Chemical or biological reactions occurring at or very close to the interface perturb the EW, giving rise to a change in signal which, in the case of an Ab–Ag interaction, can be related to the amount of binding. The measured parameter may be absorbance, fluorescence or light scattering. Serono Diagnostics (Woking, UK) is developing a fluorescence based EW immunosensor that incorporates a novel capillary fill design. The system consists of two glass plates separated by a narrow capillary gap of ~100 μm. The lower plate acts as an optical waveguide and contains on its surface an immobilized layer of antibodies. The system benefits from the capillary fill system, which draws a fixed volume of sample into the space between the plates, regardless of bulk sample volume
Affinity Sensors (Cambridge, UK) has created a new generation of EW sensors, resulting in the development of the IAsys technology. This advanced optical biosensor system (IAsys and IAsys Auto+) permits analysis of the biomolecular interaction in real time. Reusable cuvettes offer a choice of derivatized sensor surfaces and chemistries. The ligand is simply and efficiently immobilized onto the sensor surface, and binding of analyte can be detected immediately.

Ab based electrochemical biosensors generally rely on the use of electroactive labels, usually employing enzyme labelling and amplification techniques. Detection by electrochemical sensors can be inexpensive and may achieve very low detection limits. Different types of electrochemical immunosensors have been developed and include potentiometric, capacitative, conductimetric and amperometric devices [21]. An electrochemical immunosensor is marketed by Molecular Devices Corporation (USA) using an enzyme labelled and amplified system based on light activated potentiometric sensor (LAPS) transduction.

Piezoelectric transduction approaches and, in particular, surface acoustic wave (SAW) devices have gained attention recently. Piezoelectric materials (usually quartz crystals) may be brought into resonance by the application of an external alternating electric field. The frequency of the resulting oscillation is determined by the mass of the crystal. The principal attraction of piezoelectric immunosensors is their ability to monitor directly the binding of Ab–Ag reactions encountered in affinity sensing [21].

Many immunosensors have been developed, and with minor adaptation these can replace immunoassays and enzyme linked immunosorbent assay (ELISA) kits used to screen livestock diseases. At present, immunodiagnostic tests are used for on-farm monitoring of livestock reproduction (milk progesterone) and quality control of foodstuffs originating from livestock production (authenticity and adulteration testing) [22]. An immunosensor for progesterone measurement in milk of dairy cattle was reported by van der Lende et al. [23]. For the successful application of any on-farm immunodiagnostic tests in practice, the sample containing the analyte should be easily obtainable, and the test should be simple to perform and interpret. The disappointing world market for on-farm milk progesterone tests is probably due to lack of ease in performance and interpretation. These tests can be developed to a new generation of rapid immunosensors for real time and on-site applications.

The use of antibiotics and chemotherapeutics in animal husbandry has led to the occurrence of veterinary drug residues in all types of food of animal origin. Because of the specification of toxicologically based maximum residue levels for a large number of substances, existing control strategies need even faster and more sensitive methods to meet new and more rigorous regulations [24]. An SPR based immunoassay has been developed to detect sulfamethazine (SMZ) in milk [24]. Immunoassay is also used for analytes such as veterinary drugs, pesticides and micotoxins in agrifood matrices [25]. Immunosensors may not have commercial
devices for diagnosis of livestock diseases in the immediate future, but their long term development potential is promising.

6. DNA BIOSENSORS

Nucleic acids, the building blocks of DNA and RNA, are universally present in all living cells and may be used as a general indicator of microbial biomass [26]. The polymerase chain reaction (PCR) is a widely used method for amplifying trace amounts of DNA for analysis. To date, it has been mainly used for qualitative analysis, but the need for quantitative information has resulted in further development of PCR protocols [27]. Quantitative PCR methods are shown to be extremely sensitive, and fully automated detection systems which are rapid, simple to use and inexpensive are being developed.

DNA biosensors are currently used in two areas of application — the detection of infectious diseases and the detection of genetic abnormalities. Detection of infectious diseases has been generally approached using immuno-based sensors because this technology is relatively well established and the performance is good. However, DNA based probes offer advantages in certain assays for infective agents because, for example, they can target the infectious virus itself. PCR is now widely used to amplify the signal in DNA probes, but it takes several hours to obtain the results. There is intense current interest in microsystems for DNA analysis. An electrochemical DNA sensor based on a gold electrode modified with DNA probes and an electroactive hybridization indicator has been reported in the literature [28]. Other types of detection system have also been applied in DNA probes, such as the use of an enzyme label, an SPR based biosensor [29], an EW biosensor [30] and an acoustic based sensor [31].

7. AUTOMATED SENSOR FABRICATION TECHNOLOGIES

One of the major obstacles hindering the broader commercialization of in vivo and in vitro sensors is the lack of a suitable means of automated production. The importance of automated manufacturing technologies has been clearly demonstrated in the commercial production of devices such as the ExacTech blood glucose biosensor (MediSense, Inc., Cambridge, MA, USA), where large numbers of inexpensive, reproducible electrochemical devices are required. Techniques such as screen printing, which is widely used for the fabrication of printed circuit boards in the electronic industry, and ink jet printing, which is used in dot matrix printers, have been adapted for biosensor fabrication. The ability to print materials at high precision and speed is very desirable for the mass production of analytical devices such as
biosensors [32, 33]. Airbrush and Cavro printing have recently been developed, under microprocessor control, as a means of depositing solutions for the manufacture of analytical devices. These deposition techniques are rapid and also flexible with regard to the ink characteristics, which make them promising techniques for printing materials on biosensor devices. Thin film deposition has also been used. This involves the application of material through a mask, under vacuum, via evaporation due to heating, or by placing the substrate to be coated between two electrodes (sputtering). The advantage of this process lies in its ability to create patterns less than 1 \( \mu \text{m} \) thick, making it suitable for the construction of microsensors [1].

Thin film sensors are generally produced by a variety of vapour deposition techniques, electrochemical methods and more recently by the use of Langmuir–Blodgett technology. Thin metallic films, usually deposited by vapour deposition, have been used extensively for the production of sensors utilizing SPR. These sensors have been primarily used for affinity sensing.

An area of intense research activity has been the use of silicon microfabrication for both electrochemical and, more recently, optical sensors. Silicon fabrication technology has been proffered as a means of mass production of biosensors. The capability of on-chip electronic signal amplification and data processing are very attractive. The low fabrication costs and high device reproducibility would also be advantageous.

Miniaturization of the sensor to needle size is a common engineering approach to obtain a sensor suitable for subcutaneous implantation. Therefore, efforts have been directed towards improving the electrode design and selecting the membrane material necessary for proper operation of such a sensor [34]. The development of new transducers such as piezoelectric crystals and the application of semiconductor technology will help in miniaturizing biosensors and will facilitate the fabrication of disposable sensors.

Advances in membrane technology are very important in biosensor development. An inner membrane, usually with a smaller pore size, prevents smaller interferential molecules from reaching the electrode. An outer membrane retains the biological molecules and prevents the working electrode ink from being washed away, thus stabilizing the sensor. It also acts as a defusion barrier and excludes interferential macromolecules.

8. THE MARKET PLACE AND FUTURE DEVELOPMENTS

There has been much research into biocatalytic and, more recently, into affinity sensors, which has resulted in a number of products worldwide. A wide range of biosensor devices has been demonstrated in the laboratory, but the relatively high
development costs have restricted commercialization to large markets. Medical applications have dominated the drive to produce biosensors, especially for home care or personal use owing to the huge market potential. There are a number of commercially available biosensors (Table I [1]), the most successful being the ExacTech device marketed by MediSense, Inc. This device uses a disposable single-use biosensor strip to measure glucose concentration in a drop of blood in 20 s.

Biosensors in general are at different stages of technological and commercial development. Biocatalytic sensors are advanced in their development; however, their widespread use has yet to be achieved, with commercial success mainly limited to medical applications, but with many future opportunities in food analysis, industrial process control and environmental monitoring. The size of the market for in vivo diagnostics is about US $10 billion (10^9) per year worldwide, and blood glucose monitoring is the prime target. Other medical applications for biosensors include the measurement of lactate, alcohol, paracetamol, cholesterol, cancer markers and infectious diseases.

Affinity sensors are mainly at a development stage, and most of the market to date has been for research applications, but with future opportunities in the biomedical sector. Academic and industrial interest in affinity sensors has escalated recently, and their market is predicted to increase greatly over the next few years. The products available worldwide are the IAsys system (Affinity Sensors, UK), the BIACORE instrument (Pharmacia, Sweden) and the Threshold Affinity sensors (Molecular Devices Ltd, USA). The high cost of these instruments (US $80 000–120 000) opens up the market for cheaper and more portable instruments. Application areas for affinity sensors can cover a broad number of analytes in medical diagnostics, food and agricultural applications, environmental analysis and military uses. There has been a rapid growth in the non-sensor diagnostics industry covering the areas listed above, such as immunoassay and ELISA kits and dipstick tests, which could be replaced by more rapid and robust sensors.

To date there is much interest in environmental monitoring and food applications. The move towards more stringent monitoring and increased public awareness of health issues are the major forces behind developments in these areas. However, the need for biosensors in these fields has been small, and only a handful of devices have made it to the market. Instrumentation for these applications needs to be robust, reliable over a wide range of operating conditions and simple to use. Opportunities exist for on-line [35] and off-line monitoring during manufacture and in shelf-life monitoring during distribution and storage. The use of sensors to replace the existing methods offers great potential due to the almost universal nature of the measurement. However, the demands placed on sensors in the various fields of application are very different, and these need to be addressed before usable devices will emerge.

The implementation of new analysis technologies such as biosensors and immunosensors in the food industry is becoming very attractive. Target analytes for
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TABLE I. (cont.)

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food analysis include sugars (glucose, sucrose and lactose), alcohols, penicillin and acetic acid. Opportunities are also apparent for the detection of salmonella, listeria and E. coli 0157 in animal products. There is intense interest in monitoring water supplies, soil and the atmosphere using rapid analysis methods. Biosensors for pesticides, herbicides and other toxic compounds are being developed for environmental applications [36]. However, they are mostly for research use, and very few are commercially available.

Extensive research is being carried out to offset the limitations of biosensors, such as their relative instability, and improve their biocompatibility. Techniques such as SPR and integrated optical waveguides have been developed to permit real time monitoring of biomolecule-surface interactions. These techniques are a major advance in understanding the mechanism of biocompatibility. A critical aspect, therefore, in improving sensor performance is understanding which chemical
properties should be engineered into a biomaterial so that its biocompatibility can be optimized.

Molecular imprinting is an emerging technology for the creation of selective recognition sites in synthetic polymers. This technique entails the polymerization of functional monomers in the presence of an important molecule (template analyte). The obtained polymers can exhibit a surprisingly high degree of stereo- and regio-specific selectivity, making the commercial use of such tailor-made affinity materials in several areas, including as antibody mimics, a realistic possibility [37, 38]. Affinity ligands for the detection of a broad range of analytes are being developed on the basis of combinatorial chemistry. These ligands could be used as the recognition element in future developed sensors.

The future trends in biosensor development will be towards further miniaturization to allow the production of multisensor arrays and integration into complex systems. Array based sensors can be expected to lead to capability for mass screening of samples.

9. CONCLUSIONS

There are numerous areas of medical, veterinary diagnosis, environmental, food and agriculture research where there is a long felt need for a reliable, real time, simple to use, inexpensive analytical system that can be tailored to a particular application. In general, classical methods can suffer from various disadvantages, including long analysis time, high instrumentation cost, lack of sensitivity and lack of amenability to the on-line monitoring of industrial processes. The successful development and application of a commercial instrument is dependent on the instrument's ease of use, sensitivity and analysis time, as well as cost. Many of the products on the market differ significantly in instrument size, sensitivity, application and purchasing cost. Biosensor systems which are relatively small, portable instruments, have an on-site application and are relatively inexpensive are desirable.

Biosensors can be designed for disposable 'one shot' applications, but it is also possible to produce multiple use devices. Developing marketable biosensor technology is difficult in today's rapidly changing environment. Many biosensor products are being developed in research laboratories, but very few find their way through the product development process to release and become a success in the market place. Despite the significant improvements that have been made in recent years, there are still challenges. The main challenges to be addressed for biosensors for future use are in the areas of surface science, to optimize biocompatibility, and the engineering of biological components for selectivity, sensitivity and stability. Machine manufacturing techniques are essential for the widespread introduction of reliable biosensors into new areas. Another challenge to introducing real sensors into
real systems lies in addressing ‘niche’ markets and changing requirements in complex systems. The sensor array offers opportunities here by being potentially tunable. The near-infrared (NIR) transcutaneous monitoring of glucose is currently simply based on pattern recognition. The use of a multianalyte measurement (multisensor arrays) with compatible software is also an area that might be important in the future.

Enzyme based and antibody based biosensors have been reported for detection of a range of analytical compounds; however, these sensors must compete with the fairly well established chemical and immunoassay techniques. To be successful, these biosensors must offer improvements on the available analysis methods. The impact of biosensor and immunosensor technology on the diagnosis of livestock diseases has been significant, but its impact on on-farm testing has not yet been fully met. The development of such methods, which will aid in the diagnosis and improve the health and welfare of the treated animals and the quality of their products, is highly desirable. It is clear that the field of livestock disease monitoring presents a number of niche opportunities for biosensors.

REFERENCES


PEN-SIDE TESTING

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Wilton, Connecticut,
United States of America

Abstract

PEN-SIDE TESTING.

Pen-side diagnostic testing is often required to provide real time information about the health status of an animal or herd. The rapid results are generally needed in the face of a disease outbreak where the diagnostician/clinician is presented with dead or dying animals. Another use of pen-side tests is as part of a preventive medicine/herd health programme, where the information can be used for determining pathogen incidence and prevalence in the herd, identifying potential carrier animals or evaluating subtle changes in overall herd health as a result of management changes. Still another area is as a part of health certification before sale or stocking, or during processing following slaughter. Advanced testing methods, which in the past were only appropriate for use in a laboratory setting, have now been formatted for use in the field. Enzyme immunoassays (EIAs) and immunochromatography have greatly enhanced diagnostic pen-side capabilities. These test systems allow for the rapid, sensitive and specific detection of target antigen or antibody and require little or no scientific equipment. Because of the wide format flexibility these immunochemical technologies allow, they have come to dominate the diagnostic industry, both in the laboratory and in the field. Using liquid phase or precipitating chromogens, the tests can be visually interpreted and do not need instrumentation. Immunochromatography, a simplified assay system, has found widespread application as a field or pen-side test. The entire assay only takes only one or two steps, and the results appear within 5-10 minutes. In the future, DNA based diagnostics will become more adaptable for use in the field. The DNA hybridization systems are at present cumbersome and require elevated temperatures. Companies are currently working to develop test formats that provide results in less than an hour, have a limited number of steps and can be performed at lower temperatures.

1. INTRODUCTION

Almost every food animal industry directive states a need for rapid, easy to use, sensitive and specific diagnostic tests for economically important diseases. To meet these needs, an ideal testing method is proposed that should be able to use whole blood or complex tissue as a sample and should not require further processing. It should require no volume measurement to provide quantitative data. Also, it should be able to be performed in one to three steps (preferably one), yield results in under
10 min (preferably 2 min) and lend itself to automation (running many samples with minimum human involvement). In addition, the test method should be 'sensitive and specific' [1]. However, it is more difficult to obtain consensus on these two parameters in terms of how sensitive is sensitive enough, or how specific this ideal test method needs to be in order to meet the needs of the industry.

In this paper, the concept of the ideal test method is used as a backdrop for discussing present, and perhaps future, pen-side diagnostic technologies. The reason is quite simple: most diagnosticians/practitioners expect a great deal from the technology that they are using, without understanding the limitations of the technology or what the results they obtain actually mean. There are several important considerations that should be taken into account in the evaluation of a pen-side test and in deciding how the test may or may not fit this concept of an ideal diagnostic. One consideration is why the pen-side test is being performed. One routine use of a pen-side test is in the case of an acute outbreak of disease with dead or dying animals. Here, the need is to obtain information for immediate action, such as initiating therapy, deciding whether to cull and slaughter, or do nothing and implement a policy of benign neglect. In this instance an antigen capture or identification system is the most appropriate technology. This is a situation where there is usually a great deal of antigen present (depending on the agent), so having the most sensitive assay system available may not be as important as the time it takes to obtain the necessary result. Therefore, an agglutination or immunochromatography assay may meet the diagnostian's needs.

Another reason to perform testing in the field is as part of a preventive medicine/herd health programme. In this case the information can be used for determining pathogen incidence and prevalence in the herd, identifying potential carrier animals or evaluating subtle changes in overall herd health as a result of management changes. In this situation the rapid feature of the assay system may not be as important as the test's sensitivity; therefore, an enzyme linked immunosorbent assay (ELISA) or DNA hybridization assay system may be more appropriate. Still another situation that might call for pen-side testing is as a part of health certification before sale or stocking, or during processing following slaughter. In this case an assay system that is both rapid and sensitive is needed, and as such an enhanced immunoassay or a 'next generation' polymerase chain reaction (PCR) might be the detection method of choice.

2. NON-IMMUNOLOGICAL/MOLECULAR METHODS

It is not a high technology approach to pen-side testing, but any such discussion should begin with the most basic of techniques, namely, physical examination. Although there are few pathogens that cause pathognomonic lesions, there is a good deal of diagnostic information that can be gleaned from grossly examining the animal
or herd. For example, one can get a sense of how many animals are visibly affected and to what degree, and also an indication of what organ systems are involved, e.g. gastrointestinal (diarrhoea), respiratory (coughing and nasal discharge) or integument/skin (ulcerations or haemorrhage). Often a tentative diagnosis is aided by assessing the time of year, geographical location and ambient temperature. Certain diseases appear in association with a rise or fall in temperature or the appearance of intermediate hosts or vectors.

The next level of diagnostic examination may include a field microscope that uses a mirror and the sun as the light source. This simple but cost effective approach can be used with wet mount, squash preparations, impression smears and faeces as sample sources. This technique can be used in combination with certain simple staining procedures for the detection of motile bacteria, many parasites and/or their eggs and, in some cases, viral infections. Although it may not be possible to exactly speciate the identified pathogen, that might not be absolutely necessary in order to implement cost effective treatment or control measures.

3. IMMUNOLOGICAL TESTS

Agglutination assays utilizing latex beads or other particles, such as tanned erythrocytes or colloidal particles, can be rapid, relatively inexpensive and simple to use. This system can be used to detect antibodies or antigens. For detecting antibodies, the particles are coated with the antigen of interest and mixed with a blood or serum sample on either a cardboard or plastic card, or a microscope slide. If the animal has pathogen specific antibodies present they will bind to the antigens, resulting in cross-linking or agglutination of the particles. Conversely, for testing for antigen, the particles are coated with antigen specific antibodies. If the sample contains target antigen, agglutination will occur when the sample is mixed with the antibody coated particles. This agglutination reaction usually occurs in 2 to 5 min and can be visually interpreted, thereby making it a viable pen-side test method for both antibody and antigen detection. A shortcoming of particle agglutination, however, is its lack of sensitivity. In terms of detecting whole viruses or bacteria, the limits of the particle agglutination system are generally useful at about $10^7$ organisms/mL. This technology can be very useful in an acute outbreak situation when there is abundant antigen or antibody present (depending on the agent), but is less useful in detecting carrier animals or subacute infections.

The evolution of enzyme immunoassays (EIAs) has been integral in advancing pen-side testing. These various assay systems allow for a wide array of format flexibility. Some of the first EIA formats applicable for use in the field were performed in test tubes. The test tube format can be used as indirect or competitive systems to detect antibodies or antigens, or a capture ELISA to detect antigen. In the indirect or
competitive formats, antigen is precoated on the surface of the tubes. The sample (preferably whole blood) is added, and during the incubation period the antibodies in the sample bind to the antigen. After washing to remove unbound antibodies, the detection phase begins. In the case of the indirect system, an anti-immunoglobulin antibody conjugated to an enzyme is added. After another short incubation period, the enzyme substrate is added and a colour change occurs that is proportional to the amount of antigen specific antibody in the sample. A variation on this indirect method is to have the antibodies in the test serum compete with enzyme conjugated monoclonal antibodies directed against the targeted antigen when the two are added simultaneously to the antigen coated tubes. If there are antibodies in the test serum, they will competitively bind to the antigen coated on the tube, thus displacing the conjugated monoclonal antibodies. After washing away unbound antibodies and adding the chromogenic substrate for the enzyme, the degree of colour change is inversely proportional to the amount of antibody present in the test serum. In other words, less colour change results when testing animals that have pathogen specific antibodies in their blood, because these antibodies successfully out-compete the conjugated monoclonal antibodies, which are then removed during washing. Therefore, when the substrate is added there is no conjugated enzyme present to induce the colour change. It should be mentioned that this competitive format can also be used to test for antigen in a sample. In this case, antigen specific antibodies are bound to the surface of the tube, and the test sample and an enzyme conjugated target antigen are added. The antigens compete with one another in binding to the antibodies, and unbound antigens are removed during the wash step before the chromogen is added. The resulting degree of colour change is again inversely proportional to the amount of target antigen in the sample. This antigen competition format is in widespread commercial use for detecting antibiotic residues in milk.

The antigen capture ELISA tends to be the more common assay system used in pen-side testing because the diagnostician usually wants immediate information on what is occurring in a particular animal or herd at the time of testing. A serological test to detect antibodies collects more historical information. In other words, the animal has been exposed to the agent some time in the past, and there has been sufficient time for the animal to develop a detectable antibody response (seroconversion). The capture ELISA determines whether the pathogen or another target antigen is present at the time of testing. In this format, the tube is coated with the first of two antibodies, normally referred to as the 'capture' antibody. The sample is added and the antigen, if present, is captured by the antibody. After the removal of unbound material by washing, the 'reporter' or 'detector' antibody is added. This second antibody is most often conjugated to an enzyme or biotin, here taken to be an enzyme conjugate. After a short incubation step, followed by washing to remove unbound conjugated secondary antibody, the chromogenic substrate is added. With
the capture ELISA system, the degree of colour change is proportional to the amount of antigen that was 'captured' from the sample.

The test tube format has several advantages and disadvantages as a pen-side testing method. First, the disadvantages. When running numerous samples, simultaneously manipulating many tubes at one time can be awkward in the field. Second, the solutions are usually added to the tubes with dropper bottles, which can slow processes considerably if many drops have to be added to a large number of tubes. Lastly, the packaging for such test formats tends to be bulky and requires a considerable amount of storage space. As to advantages, the tube test format can be very sensitive and provide semiquantitative results when visually compared to a set of standard controls. For example, a pen-side tube test is available for detection of a soluble surface protein of a gram positive bacterium of fish called *Renibacterium salmoninarum* (KwiK-Dtect, DiagXotics, Inc., Wilton, CT, USA). The test provides semiquantitative results down to about 10 ng/mL of pathogen specific antigen. This 10 ng/mL is not the limit of sensitivity of the test, but rather the limit of the ability of most human eyes to distinguish more subtle colour changes. In the development of the product, it was found that everyone could distinguish the colour difference in 10 ng/mL increments, but only about one half of those beta testing the product could tell the difference between 3 ng/mL increments. Other advantages of tube formats are that they are relatively rapid (10 min to 1 h) and inexpensive to manufacture and use.

At present, there is a rapid evolution occurring in the field of handheld instrumentation. Of particular interest to the pen-side testing sector are the advances being made in manufacturing fluorometers and luminometers that are miniaturized and can be worn in the field on one's belt. These field instruments, when fully developed, will allow for the development of next generation assays that are even faster and more sensitive than those currently available. Fluorometric immunoassays (FIAs) can be either heterogeneous or homogeneous. The heterogeneous assay requires separation of the labelled and unlabelled specimens. Most commercial FIAs use a solid phase antigen or antibody system. In the solid phase antigen assay, an antigen bound to the surface of a solid support (tube or microtitre plate) competes with free antigen and labelled antibody. After equilibrium has been achieved, unbound fluorescently labelled antibody is removed by washing and the amount of bound labelled antibody is measured. Most of the fluorophore or fluorescent compounds used to label the antibodies or antigens are organic ring structures and will only fluoresce after the absorption of light. The emission wavelengths of light are measured as the compound goes from the excited to its ground electronic state. FIAs are very similar to standard EIAs, the major difference being that instead of labelling the antibodies or antigens with enzymes and having a colorimetric readout system using a chromophore, the FIA is a fluorometric assay using a fluorophore. Chemiluminescent immunoassays (CLEIAAs) on the other hand can utilize many of the same enzymes commonly used in EIAs to generate their signal. Antibodies or antigens conjugated to peroxidase, alkaline
peroxidase or glucose oxidase can be used to oxidize a substrate such as luminol. This oxidation reaction must occur directly in front of the light detector to capture the immediate light signal.

The major disadvantage of FIAs has been the problem of serum samples tending to autofluoresce owing to bilirubin and other aromatic proteins. Advances have been made in instrumentation and immunochemical reagents such that certain analytes may be detected at a concentration of $10^{-15}$ M [2]. However, a more common range of sensitivity attributed to FIA is $10^{-9}$ to $10^{-12}$ M [3]. CLEIAs take the level of sensitivity even lower and can reach the femtomolar or even attomolar levels. Again, the drawback to using these technologies in the field has been the size and cost of the instrumentation required to obtain a result. Several companies are close to having relatively inexpensive (about US $400–800) handheld instruments that will then allow the use of FIA and CLEIA diagnostics pen-side.

Standard EIA, FIA and CLEIA detection systems utilize a liquid phase chromogenic, fluorogenic or chemiluminescent substrate. Alternatively, a precipitating substrate can be, and is, used in a number of different rapid EIA formats. The first such format is that of the immunodot. Unlike the tube and microtitre plate ELISAs that use a solid plastic support, the immunodot utilizes a membrane as its support. The types of tests (i.e. direct, indirect, competitive and capture ELISAs) and the steps required to perform the assay are similar to the methods described above, with the exception of the last step where a precipitating chromogenic substrate is used in lieu of a liquid phase chromogen. One of the most commonly used substrates is NBT/BCIP, which results in a dark purple to black precipitate that can be visually interpreted. Another difference between liquid phase ELISAs and the immunodot system is that rather than adding solutions to the wells or tube and then dumping them out at the end of the incubation period, in the immunodot the liquids are drawn through the membrane. This has the added benefit of increasing the surface area on which the reactions can take place, thus making them quite sensitive (ng/mL). The immunodot system also lends itself to many configurations, such as membrane-bottomed microtitre plates, sheets of nitrocellulose or glass fibre, or dots on a ‘dip-stick’.

An interesting variation on the immunodot system is currently being exploited by a company in Boulder, Colorado, USA, called BioStar, Inc. The system uses a change in refractance as a means of interpreting the results; rather than plastic or a membrane as the assay platform, a silicon wafer is used. The antibodies or antigens (depending on what is being assayed for) are bound to the surface of the wafer. The assay uses the same reagents and protocol as has already been described for the various other ELISAs. In the final step, the alkaline phosphatase conjugate acts on the precipitating substrate NBT/BCIP. The precipitate builds up on the surface of the silicon wafer to change the angle of refractance. This change in refractance can be visibly interpreted and also quantified with the use of an inexpensive handheld instrument.
A simplified immunochromatography assay system has found widespread application as a field or pen-side test. As an example of the test system, antigen specific antibodies are bound to the surface of coloured latex beads or immunogold colloidal particles, much the same way the surface of a microtitre plate is bound with capture antibodies. The antibody coated beads are then incorporated into a porous pad and dried. The pad is glued to a long membrane, to the top of which is adhered another pad that serves as a wick. In the middle of the membrane is striped a thin line of a second antibody specific to the antigen in question. At the top of the membrane just below the wick pad is usually striped another thin line of antibodies; however, this time the antibody is directed against the species of immunoglobulin used to coat the particles (e.g. rabbit anti-mouse if a mouse monoclonal antibody is used to coat the particles). When the sample is added it rehydrates the first pad, and if the target antigen is present in the sample it is bound by the antibodies coated on the particles. As the fluid in the sample is drawn up the membrane to the wick pad as a result of capillary action, the particle–antibody–antigen complex becomes bound to the antibodies in the first stripe. If there is no antigen in the sample, the particle–antibody complex continues to migrate past the first stripe. Excess material continues to migrate up the membrane in either case and then is bound by the anti-species antibodies in the top stripe. This top stripe serves as an internal control to show that the entire test system worked. As the coloured particles are captured by the membrane bound antibodies, they appear as a coloured line on top of where the second antibodies are striped. The whole assay is one step, and the results appear within 5–10 min, making it an almost ideal pen-side test.

4. MOLECULAR DIAGNOSTICS

The use of nucleic acid probes as diagnostic tools is in the process of moving from the laboratory into the field. At present there are several commercially available DNA based dot blot hybridization assays available in the human, veterinary and aquaculture health care professions. These assays often utilize a DNA–DNA hybridization system followed by an EIA detection system. The sample is generally boiled to separate the strands of DNA (if the target DNA is double stranded) before being dotted onto a nylon or nitrocellulose membrane. The membrane is baked to adhere the nucleic acid securely to its surface before it is exposed to a stringent buffer solution to create the proper microenvironment to promote target DNA hybridization to a labelled DNA probe. This DNA probe, which is a mirror image of the target DNA, has previously been modified by coupling a reporter group such as digoxigenin (DIG), biotin or a fluorochrome (in the following example, DIG is used as the label). The DIG labelled DNA probe (DIG probe) is boiled to separate the double strands before it is added to the membrane containing the samples. The DIG probe will
hybridize with the target DNA in the sample, if it is present. Following hybridization, the unbound DIG probe is removed by washing before a buffer system is used to change the microenvironment of the membrane from one that is conducive for nucleic acid chemistry to one that promotes immunochemical reactions. After the transition, the membrane is incubated with anti-DIG antibodies conjugated to alkaline phosphatase. The membrane is then washed to remove unbound antibody before the precipitating substrate NBT/BCIP is added. Those samples that have target DNA present will be visible as dots on the membrane.

The DNA dot blot hybridization detection system can at present loosely be classified as a pen-side test in that it can be performed outside the laboratory. However, it generally takes between 5 and 8 h to obtain results and requires the use of equipment to boil water (to separate the strands of DNA) and a means of heating the membrane to a temperature that efficiently promotes the hybridization of the target and probe DNA (42 to 65°C). One company (Siagene, Inc., Bothel, WA, USA), has managed, with the aid of proprietary technology, to reduce the hybridization time to 20–30 min and the time it takes to perform the entire assay to less than 1 h. This new advance makes it more feasible to consider this diagnostic technology a pen-side test method.

REFERENCES


MONOCLONAL ANTIBODIES

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Abstract

MONOCLONAL ANTIBODIES.

Monoclonal antibodies (MAbs) are antibodies having a single specificity for a given antigenic site (epitope). The development of hybridoma technology and the relative ease by which MAbs can be prepared has revolutionized many aspects of serological applications in diagnosis and differentiation of disease producing agents. The property of monospecificity offers advantages in diagnostic applications over polyclonal sera in that tests can be defined exactly with regard to the antigen detected and the affinity of reaction between the given antigenic site and the monoclonal reagent. In addition, MAbs offer better possibilities for test standardization, because the same reagent can be used in different laboratories. Such an MAb can be supplied by a central laboratory or 'grown' from hybridoma cells, ensuring that the resultant product is identical from laboratory to laboratory and that the part of the test involving the MAb reaction is the same. The methodologies for inoculation regimes, mice, cloning methods, selection of fusion partners, etc., have been validated extensively in developed country laboratories. The decision to establish a MAb production facility must be examined on a strict cost–benefit basis, since it is still expensive to produce a product. There are many MAbs available that should be sought to allow exploitation in developing tests. If a production facility is envisaged, it should produce reagents for national needs, i.e. there should be a clear problem oriented approach whereby exact needs are defined. In the field of veterinary applications, MAbs are the central reagent in many immunoassays based on the enzyme linked immunosorbent assay (ELISA). The development of specific tests for diagnosing diseases is dominated by MAbs and has been fuelled by a strong research base, mainly in developed countries allied to developing countries through the study of related diseases. Thus, there are very many assays dependent on MAbs, some of which form the basis of kits available commercially. There must, however, be a realistic pricing structure and some identification of the needs of developing countries to balance the cost of the kits, which are generally far too expensive. There is extensive literature concerning the preparation, characterization and application of MAbs. Areas of their use as therapeutics, the prophylactic prevention of diseases,
or the development of anti-idiotypic vaccines are not examined. There is also no mention of engineered MAbs, in which recombinant technologies are used to produce bispecific antibodies, chimeric antibodies and antibody fragments. These areas are being exploited in the human fields where, in terms of process biotechnology, kilograms of MAbs are routinely produced. It is reasonable to consider such exploitation in the veterinary sphere, whereby animals are protected by designer antibodies against defined epitopes. The main danger in the use of MAbs is that the research base necessary to understand the biology of host/pathogen relationships and the epidemiology of disease is being constantly eroded.

1. INTRODUCTION

Monoclonal antibodies (MAbs) are by definition antibodies with a single specificity for a given antigenic site (epitope). The development of hybridoma technology and the relative ease by which MAbs can be prepared has revolutionized many areas dealing with serological applications in diagnosis and differentiation of disease producing agents.

The property of monospecificity offers advantages in diagnostic applications over polyclonal sera in that tests can be defined exactly in terms of the antigen detected and in terms of the affinity of reaction between the given antigenic site and the MAb reagent. In addition, MAbs offer better possibilities for standardization of tests since the same reagent can be used in different laboratories. Such an MAb can be supplied as a reagent by a central laboratory or ‘grown’ from hybridoma cells; in both cases there is assurance that the resultant product is identical from laboratory to laboratory and that the part of the test involving the MAb reaction is the same.

This is not true for polyclonal antibody (PAb) based tests where there are large differences in the quantity of antibodies produced in serum against given specific sites on a disease agent and also a high variation in the quality (ability to bind) of antibodies against the individual antigenic sites. The result is that PAbs show a high variability from animal to animal (including variation in different species used to elicit antibodies). This means that the polyclonal serum is subject to variation even when the same antigen is used to produce antibodies in individual laboratories. PAbs are also affected detrimentally by physical factors (e.g. temperature and freeze thawing) since the concentrations of distinct antibody populations contained in the serum may be affected in different ways. This results in variation in the effective avidity of the serum in time. The avidity of polyclonal serum is also affected by dilution, since avidity depends on exactly what population of antibodies remains. Effectively, PAbs can be profoundly affected in terms of their specificity on dilution. MAbs are also subject to physical damage, but here it is an ‘all or none’ phenomenon. If a particular set of conditions affects one antibody, then all that population is affected in the same way. Thus, the titre (activity related to a particular assay) is proportional to the weight of
antibody in the MAb. This is not true for the PAb, as indicated above, since antibody populations can be affected differentially. Figure 1 illustrates some of the principles inherent in MAb and PAb populations. Figure 2 illustrates the principles of relating affinity and avidity on dilution of MAbs and PAbs. These are important considerations in assessing the usefulness of such reagents in assays.

One distinct disadvantage of MAbs is that they may be too specific. The ability to recognize a single determinant may be extremely useful in differentiating small differences between related disease agents but may be too limited in a general diagnostic capacity and may 'miss' reacting with strains with high antigenic variation in the determinant recognized by the MAb.

2. PRODUCING MAbs IN DEVELOPING COUNTRIES

The methodologies for inoculation regimes, mice, cloning methods, selection of fusion partners, etc., have been validated extensively in developed country laboratories. Although there are many technical innovations, protocols which work well have been established.

The decision to set up a MAb production facility has to be examined on a strict cost–benefit basis. There are many MAbs available that should be sought to allow exploitation in developing tests. Thus, where a MAb facility is envisaged, it should be to produce MAbs for national needs (which in turn will attract interest from other scientists with related problems seeking diagnostic reagents). There should be a clear problem oriented approach whereby exact needs are defined.

Setting up a laboratory of sufficient standard to support tissue culture and ensuring continuity of supplies (media, calf sera) can be a problem, since there are stages during the preparation of MAbs where antibiotics cannot be used. Thought has also to be given to liquid nitrogen facilities for storing cells, etc. This is an expensive exercise and therefore the needs have to justify the means. A MAb production facility can be beneficial to a wide range of other scientists and can act as a 'central supplier' of MAbs; however, this can cause infrastructure problems and requires significant organization when testing and selection of clones is needed. This is often complicated by the high competition from scientists anxious to obtain reagents. Animal facilities are also necessary, and methods of expanding clones and growing large quantities of MAb must be considered. Training of staff is paramount; they must have experience in laboratories where MAb production is 'routine'. In summary, setting up and sustaining a MAb production facility is expensive. There is no guarantee that suitable MAbs will be produced. This uncertainty is due partly to the inherent problems of the biology of the disease agent and partly due to production problems (e.g. contamination, loss of secretion). Even well equipped laboratories with well trained staff who have almost instant access to commercial reagents have problems. Any consid-
Polyclonal antibodies

Specific antibodies against different sites are mixtures of different antibodies with different affinities, classes and isotypes. Resulting mixture of all antibodies is a polyclonal antiserum.

\[ a,b,c,d \cdash n \] This represents a range of slightly different antibody populations recognizing the same site for antigenic sites X, Y and Z.

Monoclonal antibodies

Single population of antibody molecules with single affinity against epitope in antigenic site X

Single population of antibody molecules with single affinity against epitope in antigenic site Y

Single population of antibody molecules with single affinity against epitope in antigenic site Z

\[ \square \text{ Antigenic site A} \]
\[ \triangle \text{ Antigenic site B} \]
\[ \diamond \text{ Antigenic site C} \]

FIG. 1. Comparison of polyclonal and monoclonal antibodies.
FIG. 2. Comparison of polyclonal and monoclonal antibodies — effect on reaction with dilution. (a) Assume that the black antibody population has low affinity with the antigenic site and that the other two populations have high affinity. As serum is diluted the effect of high affinity antibodies is lost, leaving a serum containing predominantly low affinity antibodies against a specific antigenic site. Thus the quality of the serum has changed on dilution. (b) In the case of diluting the MAb, there is a single population with a single affinity for the antigenic site. Dilution merely decreases the weight of that antibody and has no effect on the affinity (quality) of the reagent in terms of its specificity.
eration given to setting up a laboratory must address this uncertainty. This factor has
to be viewed as applied research needed, routine production in a defined time frame
cannot be assumed.

3. USES OF MABs

3.1. Diagnosis

In the field of veterinary applications, MABs are the central reagent in many
immunoassays based on the enzyme linked immunosorbent assay (ELISA). The develop­
ment of specific tests for diagnosing diseases is dominated by MABs and has been
fuelled by a strong research base, mainly in developed countries allied to developing
countries through the study of related diseases. Many assays are dependent on MABs,
some of which form the basis of kits in the commercial sector. There must, however,
be a realistic pricing structure and some identification of the needs of developing coun­
tries to balance the cost of the kits, which are generally far too expensive. MABs have
a central role in the competitive ELISAs available for detection of antibodies against
rinderpest PPR, and brucellosis and for antigen detection for rinderpest, PPR and con­
tagious bovine pleuropneumonia. All these tests have been developed through the help
by the FAO/IAEA and developed country institutes and are used extensively as part of
disease control programmes. The use of competitive or inhibition ELISAs in conjunc­
tion with MABs merits attention, because this combination has solved many problems
inherent in indirect ELISAs, which were used for many years.

3.2. Differentiation of ‘strains’

The use of a single MAb, or more particularly the use of a panel of MABs, pro­
duced against one or more epitopes on a disease agent can be used to subtly differ­
entiate (identify) such agents rapidly. This approach requires a good understanding of
the disease agent and is usually centred at the research level. There are not too many
examples of defined panels of MABs for use by a wide group of scientists to allow a
standardized protocol. However, the potential of such an approach is enormous
whereby results can be obtained very rapidly on the identity of a strain. Moreover,
since the MAb reactivity can be characterized in terms of epitopic attributes, and
since the function of the epitopes can be elucidated, the detection or demonstration of
difference in particular epitopes can indicate specific properties of disease agents.
This process can be extended when sequencing of disease agents has been done.
Since neutralization escape mutants can be using the MABs, the epitopes involved in
neutralization mechanisms can be immediately identified at the sequence level. Thus,
for linear epitopes, the exact sequence involved for interaction of the disease agent
and the MAb can be determined. This capability has been exploited in the field of foot-and-mouth disease for the comparison of field strains and vaccine strains. MAbs link the chemical structure to the antigenic nature of disease agents.

The use of MAbs does create a difficulty common to all technology developments. The results obtained with MAbs often do not fully agree with existing results, necessitating a revision of ideas. This need to adjust to new data and assess its meaning in epidemiological terms is an important factor and must be addressed by diagnosticians, research workers and veterinarians in the field. A period of parallel study involving both pure and applied research is often needed to evaluate new reagents.

3.3. Novel assays

MAbs can be exploited for their specificity for antigen, their lack of cross reactivity with other products of infection, their possible high affinity, their subclass specificity (use of anti-mouse subclass reagents to detect different MAbs) and their physical properties. Many pen-side tests and biosensors use MAbs, some of which are illustrated in this symposium. The possibility of producing homogeneous assays (no separation steps) invariably resides with MAbs. Fully defined antigen and antibody assays are also possible. Genes defining the antigenic target for a given MAb can now be expressed in a variety of free systems, such as in baculovirus, E. coli, and yeasts, to provide a fully defined antigen. This, in combination with MAbs, forms the basis of an assay defined in terms of specificity and affinity. These offer the possibility of standardization between laboratories, even allowing individual laboratories to make the reagents if provided with the hybridoma and genetic material.

3.4. Standardization/harmonization tests

As already stated, MAbs offer advantages by allowing all laboratories to perform assays with the same reagent, freed from the constraints inherent with PAbs. Standardization is becoming increasingly important in terms of world trade, since results from laboratories become certificates to allow or disallow the export and import of animals and animal products. MAb based ELISAs have proved particularly amenable to standardization of protocols and kits are a distinct possibility. Kits (the provision of fully validated sets of reagents from a single source) have proved very useful in the rinderpest campaign in Africa and have allowed an external quality assurance programme to function well. It may not be possible to provide (sustain) complete kits for most diseases of veterinary interest. The use of a MAb based assay may offer a good compromise for standardization when used as the ‘dominating’ reagent in any assay. Thus the same MAb might be used in a variety of different functional ELISAs but provide the thread by which assays can be
harmonized, i.e. results can be compared and data adjusted in accordance with accepted criteria.

4. TECHNICAL ISSUES

Monoclonal and recombinant antibodies are broadly accepted for diagnosis of various diseases, purification of therapeutics and a general biochemical tool. EBV-transformation has been widely used for establishing cell lines for antibody production in the past. Comparing the yields of EBV-immortalized IgM and IgG producing B-cells, it seems that this virus preferentially immortalizes IgM producing ones. Besides this fact, the limited stability in antibody production of such cell lines is the reason why hybridoma technology based on cell fusion [1, 2] is superior for long term programmes. Various methods have been developed to bypass immunization.

Filamentous phage display libraries can be used to quickly identify antigen binding fragments. In a few cases, these fragments have been inserted successfully into the calcium dependent regulatory protein (CDR) regions of antibody chains. The selection and enrichment of phages expressing specific binding domains is carried out in vitro by so-called panning procedures. So far, this technology has not fulfilled all hopes.

Despite the great possibilities of recombinant antibodies, the correct folding, stability, especially stability of single chain antibodies, is still a problem. Currently, the hybridoma technology and the stability of the resulting antibodies is still the best and generally applicable method to produce in vitro.

Advances achieved in characterizing the diversity of cell surface proteins, in subtyping different pathogens, in the classification of lymphokines, etc., are mainly based on the availability of MAbs. Well established and stable hybridoma cell lines or other cell lines created by transfection for antibody production enable the production of antibodies of the same quality, specificity and avidity. In contrast, each additional immunization and each individual animal creates a different pattern of specific PAbs. This fact may limit the continuity of long term studies using only PAbs as diagnostic tools. Conversely, PAbs are less susceptible to single mutations of antigens; therefore, they are more properly used when a broad specificity is desired. Nevertheless, the question is not if MAbs or PAbs are generally better. Requirements and problems in diagnosis of livestock diseases are individually different, and it will be advantageous and helpful in all cases if both types of antibodies are available and combinations are used (e.g. a PAb may be used to capture antigen and an MAb used to describe the captured antigen). Comparing the generation and production, the need of more technology, equipment, laborious steps in screening, subcloning and the in vitro production for MAbs cause higher costs and consume much more time.
For the production of antibodies there are solutions for the production of milligram to kilogram amounts. A few litres of supernatant can be produced using cell culture dishes (Roux bottles, Roller bottles, Spinner flasks). For laboratory scale and large scale production, different types of scaleable fermenters are available. The trend in production within the last few years is to smaller fermenter volumes with higher cell densities. The successful stirred or airlift fermenters suitable for all scale sizes are more and more being replaced by systems using microcarriers to increase the cell densities/volume. For small scale production there are also a few systems for production with high cell densities on the market (e.g. Techno-mouse, hollowfiber systems). To reduce the costs for cell culture medium and the risks of viral and other possible contamination by foetal calf serum, the trend is to use protein free media. The low content of foreign proteins in the culture supernatants is also advantageous for the antibody purification.

In contrast to the generation and production of antibodies, procedures for the purification of mammalian IgG antibody can be applied, using the bacterial IgG binding proteins Protein A or Protein G [3] as ligands in affinity chromatography [4]. Alternative methods are epitope immunaffinity chromatography, ion exchange chromatography or hydrophobic interaction chromatography [5, 6]. In all cases it is advisable to first check the stability of the antibodies to the selected procedure. Purification of PAbs has usually been accomplished by salt precipitation using ammonium sulphate followed by ion exchange chromatography and gel permeation chromatography. The use of polyethylene glycol and caprylic acid precipitation has also been successfully applied for this purpose. Caprylic acid precipitation is a simple process [7], and albumin and IgM can be removed in a single step.

IgM purification is more difficult in small scale production. The large molecule is less stable to convenient methods, which require harsh elution conditions. The yield is usually much lower than with IgG purification. Often the avidity of the antibody is destroyed. In large scale production isoelectric precipitation of IgM antibodies is an inexpensive and reliable method resulting in high yields and purity [8].

Last but not least the stability of the purified product is of major interest. Optimal conditions for storage, suited stabilizers, conservatives, etc., should be tested well timed for liquid storage, or freezing as well as for lyophilization.

5. CONCLUSIONS

This paper has provided a short description of some points of interest on MAbs and their use in diagnosis. There is an enormous amount of literature about MAbs and their applications. Not mentioned here are the possibilities for their use in therapy or prophylactic prevention of diseases, and the development of anti-idiotypic vaccines. There is also little mention of engineered MAbs. Recombinant technologies can be
used to produce bispecific antibodies, chimeric antibodies and antibody fragments. These areas are being exploited in the human fields where, in terms of process biotechnology, kilograms of MAbs are produced routinely. It may well be feasible to think about such exploitation in the veterinary sphere whereby we protect animals by designer antibodies against defined epitopes. The main danger in the use of MAbs is that the research base necessary to understand the biology of host/pathogen relationships and the epidemiology of disease is being eroded constantly. There can be no application without knowledge.

REFERENCES

MOLECULAR ASPECTS

(Session 2)

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APPLICATION OF MOLECULAR TECHNIQUES TO DISEASE DIAGNOSIS
An overview

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Abstract
APPLICATION OF MOLECULAR TECHNIQUES TO DISEASE DIAGNOSIS: AN OVERVIEW.
Many nucleic acid based diagnostic tests have been developed since the beginning of the 1980s. Because of the large number of publications on that technology, the paper deals only with their different principles and also highlights the advantages and disadvantages of each kind of test.

1. INTRODUCTION

Molecular biology is a key component of pure research. More recently, techniques in this field have become important in disease management in the areas of diagnosis, epidemiology and pathogenesis and in protocols for prevention and treatment of disease [1, 2]. The traditional technique for disease diagnosis is the clinical observation. It is still important, but, in many cases, its result has to be confirmed by laboratory tests. These laboratory tests have been conventionally based on antibody detection, isolation of the aetiological agent and microscopic observations. Although isolation of the pathological agent is often the ‘gold standard’ technique for acquired disease diagnosis, it is sometimes difficult to achieve, for multiple reasons, including poor sample preservation and non-cultivation of pathogens. Molecular techniques have broadened the spectrum of diseases that can be diagnosed. Indeed, on the basis of the detection of the genetic material, these techniques do not need the presence of viable pathogens as do the isolation assays, nor high amounts in the specimen as required for microscopic observation.

In the past ten years, a revolution has been achieved in disease diagnosis with the advent of the in vitro polymerase chain reaction (PCR). However, other molecular technique based assays have been developed, and the principles of those different tests that have potential or immediate applications in the diagnosis of animal diseases
are examined. These tests can be divided into two main groups: restriction enzyme analysis and nucleic acid hybridization process.

2. RESTRICTION ENZYME ANALYSIS

For a long time, serological tests have been used to differentiate isolates of a microorganism (serotyping). Although the advent of monoclonal antibodies has brought an improvement in serotyping, the techniques are not sensitive enough to detect very closely related isolates if the changes in their genomes do not imply detectable modifications in the synthesized proteins. However, a single nucleotide modification in the genome can be detected if it occurs in a restriction enzyme cleavage site. Therefore, the use of a restriction enzyme, or a set of enzymes, to cleave

![Diagram of RAPD method]

**FIG. 1.** Schematic representation of the RAPD method. The DNAs from two isolates (A and B) of a pathogen are clipped with the restriction enzyme E2. The digested products are analysed by electrophoresis on agarose gel.
DNA of different isolates of a pathogen can be used to detect a natural variation (polymorphism). In practice, the purified DNA is digested to different fragments whose numbers and lengths vary according to the distribution of the enzyme cleavage sites within the genome. The digested products are separated by electrophoresis using agarose gel. The comparison of their electrophoretic profiles allows the detection of differences (Fig. 1). This technique, known as restriction fragment length polymorphism (RFLP), has been applied to the analysis of different isolates of *Mycoplasma mycoides* ssp. *mycoides* SC, the agent of contagious bovine pleuro-pneumonia (CBPP). While those isolates cannot be differentiated serologically, RFLP tests allow their classification into groups that reflect their geographical origins. This technique has also demonstrated that the vaccine strain KH3J could be differentiated clearly from T1/44, another vaccine strain that has been distributed in Africa for vaccine production in past years.

Since the RFLP test depends on the modification in restriction enzyme cleavage sites, it is sometimes necessary to use several enzymes to detect a difference between analysed samples. Moreover, any modification outside the known restriction enzyme cleavage sites will not be detected. Therefore, RFLP has little (if not no) diagnostic value, because it cannot be used routinely. However, it might be useful for epidemiological studies as demonstrated in the case of the CBPP causal agent.

3. DIAGNOSTIC ASSAYS BASED ON NUCLEIC ACID HYBRIDIZATION

Nucleic acid hybridization is based on the ability of two single nucleic acid strands, in favourable conditions, to associate and form a stable duplex. This association occurs by hydrogen bond formation in accordance with the base pairing rule defined by Watson and Crick in 1953 [3], and thus its strength depends on the sequence of each nucleic acid strand. The high specificity that can be achieved by nucleic acid hybridization has made this procedure an ideal technique for disease diagnosis, and many tests have been developed according to this principle.

3.1. Diagnosis by DNA probes

To detect the association of two nucleic acid strands, one must be labelled with an easily detectable molecule (Fig. 2). This labelled nucleic acid, the probe, is selected such that it will form a strong duplex with the target molecule to the exclusion of other nucleic acids present in the test sample. The strength of the association of the two molecules will vary according to the degree of the mismatch between their respective sequences. Conditions such as salt concentration, temperature and presence of formamide, which destabilize the hydrogen bonds, will increase the specificity of the hybridization process. There are three kinds of probe: double stranded
FIG. 2. Principle of the use of a nucleic acid probe. The nucleic acid of the test sample is extracted and denatured. It is allowed to hybridize with another nucleic acid (probe), which is labelled with a reporter molecule (label). The probe will form a duplex with a strand only when their sequences complement each other.
DNA (dsDNA), single stranded RNA and oligonucleotide. Before use, dsDNA probes have to be denatured into single strands. However, during the hybridization reaction, two competitive events occur – the self-annealing of the probe and its association to the target. This competition will lower the sensitivity of the test and also the kinetics of the desired reaction. Such competition is avoided by the use of single stranded probes: RNA and oligonucleotides. Because of the extreme sensitivity of RNA to RNase contamination, oligonucleotides are the probes of choice. Since they are short in length, their hybridization kinetics is higher than in the case of longer probes. They are synthesized chemically and could be obtained in large quantities with high quality and at low cost.

Hybridization is carried out according to two formats, liquid phase or solid phase. With the liquid phase format, the different reagents interact freely. In this case, the chance that the probe and the target molecules hybridize is increased. The hybrid can be detected using agarose gel electrophoresis after the digestion of the single stranded nucleic acids by nuclease S1.

Another method to monitor the liquid phase hybridization is the hybridization protection assay (HPA). In this method, the probe is labelled with an acridium ester [4]. After the hybridization reaction, the unreacted probe is destroyed by alkaline hydrolysis, while the hybridized one is protected against this destruction and can be revealed by chemiluminescence.

The solid phase format is the most popular format. In this procedure, the target nucleic acid is bound irreversibly onto a solid support: nylon or nitrocellulose membrane, microtitre plate, magnetic or polystyrene beads, dipstick, etc. This fixation eases the process of getting rid of the unreacted probe. A hybridization solid phase format is also observed in the in situ hybridization. This is performed on fixed tissue sections and allows the localization of the cellular site of the pathogen under test.

In order to detect the nucleic acid hybrid, the probe has to be labelled with a reporter molecule which was, in most cases in the past, radioactively labelled. This has the advantage of being very sensitive, with the possibility of detecting $10^4$ to $10^5$ target molecules. However, there are important drawbacks in using radioactive probes: short shelf-life of the most commonly used radioisotopes ($^{32}$P, $^{35}$S and $^{125}$I), disposal of waste, and health risks. These features hindered the move of radioactive probe technology from research laboratories to routine diagnostic laboratories. A significant development for the current use of probes in disease diagnosis occurred in 1981 when Langer et al. [5] introduced the non-radioactive probe technology. Its advantages are safe handling, no requirement for special equipment, and long shelf-life of the label probe (at least two years at $-20^\circ$C). Unfortunately, results obtained with non-radioactive probes have been disappointing because of lack of sensitivity. Indeed, despite claims that some non-radioactive probes have a sensitivity limit comparable to that of radioactive probes, the current general practice has demonstrated otherwise.
It is important to realize that many factors, in addition to the features of the detection system, influence the sensitivity of the DNA probe test. Some of these factors are: the efficiency in recovering the target nucleic acid from the test material; the number of copies of the target sequence within the nucleic acid under test; the efficiency of the different steps in the hybridization process; the proportion of the non-target sequences; and other products present in the sample contributing to the background of the assay. Thus, to increase the sensitivity of nucleic acid based tests, different approaches have been sought.

Although efforts have been made to address the different factors mentioned above, the dramatic improvements in nucleic acid based tests followed the introduction of methods which aim to increase the number of the target sequence. The tests based on the target amplification are the PCR, the self-sustained sequence replication (3SR), the strand displacement amplification (SDA) and the ligase chain reaction (LCR). For the detection signal, the number of attached reporters (the probe) are increased, e.g. by use of the Qβ replicase reaction and the branched DNA assay (bDNA).

3.2. Diagnosis by PCR

The large number of papers and books which have been published on the PCR technique since its first description by Saiki et al. in 1985 [6] is proof of the success of this method. It has brought a revolution in both basic and applied biology research by allowing the analysis of rare genes in a short time. The technique consists of an in vitro primer directed synthesis of a region of the target DNA. Two primers are used for this synthesis, and their respective positions on the target define the fragment to be amplified. The amplification is accomplished by successive repetitions of the following steps: denaturation, primer annealing and primer elongation (Fig. 3).

Two main events have contributed to the success of the PCR technique: (1) the advent of thermostable DNA polymerases and (2) the automation of the whole process.

The use of thermostable DNA polymerases has obviated the need of polymerase addition after each denaturation step, which, when carried out at high temperatures (94–95°C), leads to inactivation of the thermolabile enzymes.

The number of nucleic acid molecules will, in theory, double at each cycle because the amplified product will serve as template at the next round. In fact, the final quantity of nucleic acid obtained after the whole amplification process depends on the average efficiency of each cycle. The achieved factor of amplification can be expressed by the following formula [7]: \( Q = (1 + X)^n \), where \( Q \) is the factor of amplification at the end of the PCR, \( n \) the number of cycles and \( X \) is the average efficiency of each cycle. Ideally, \( X = 1 \) and \( Q = 2^n \). Theoretically, a 10^9-fold amplification of
FIG. 3. Schematic illustration of PCR method. It is composed of three successive steps: nucleic acid heat denaturation, primer annealing and primer elongation by a thermostable DNA polymerase. The newly synthesized DNA strands are shown by broken lines while the starting DNA is indicated by the solid lines.
input DNA is obtained after 30 cycles in PCR. In fact, the true efficiency is variable and depends on different parameters, as outlined below.

1. **The amount of template.** When the concentration of template is high, the chance of the denatured strands to re-anneal with each other instead of annealing to the primers will be high, and the efficiency of new DNA molecules synthesis will drop. This implies that the PCR is less efficient when starting with a high quantity of target molecules rather than with a low amount, and the efficiency varies along the whole amplification process and is reduced when the amplified products accumulate.

2. **The length of the target sequence to be amplified.** Amplifying long sequences is less efficient than amplifying short sequences.

3. **The sequence of the primers.** The PCR efficiency is decreased when there are possibilities of primer self-annealing, dimer formation or primer annealing to a non-intended area. This mispriming event could also be related (in addition to the specificity of the primer sequences) to PCR conditions: high salt concentrations or low annealing temperature. While correct PCR products accumulate in an exponential way, the mispriming ones increase in a linear fashion. The mispriming event is reduced by all factors that increase the stringency of the primer annealing to the template.

After the PCR, the amplified product (amplicon) is detected by UV visualization after an agarose gel electrophoresis or by hybridization of an internal probe to the amplicon after its blotting onto a membrane filter (Southern blot after gel electrophoresis or direct dot blotting).

Another format of such assays is the colorimetric detection of the amplicon in a microplate well onto which it is attached through an internal oligonucleotide [8, 9]. A non-radioactive label is introduced into the amplicon during the PCR process or is attached to an internal probe, and the detection is carried out as an enzyme linked immunosorbent assay (ELISA) test after nucleic acid hybridization.

Whatever the format of detection of the amplicon through a probe, this process increases both the specificity and the sensitivity of the PCR. Moreover, the colorimetric detection in a microplate allows an easy quantification of the assay. The sensitivity and specificity of the PCR can be highly enhanced by carrying out what is called nested PCR. This test consists of reamplifying an amplicon with another set of primers whose positions on the target nucleic acid are internal to those of the first ones. It is assumed that this double amplification is $10^3$ to $10^4$ times more sensitive than a single round PCR [10, 11].

The PCR technique provides epidemiological data when the amplicons from different isolates of a pathogen are compared by RFLP or after sequencing. This allows the drawing of a phylogenetic tree of a pathogen and also the tracing of a disease source. With the random amplified polymorphic DNA (RAPD) technique, a PCR in which random primers are used instead of specific ones, it might be possible also to classify different isolates of a pathogen just by the comparison of electrophoretic migration profiles of the amplicons.
The rapidity, specificity and high potential sensitivity of PCR make it potentially the ideal test for disease diagnosis. Unfortunately, the high sensitivity of the test also constitutes the main obstacle to its wide introduction as a routine test in diagnostic laboratories. Indeed, a minute positive contamination of a sample will lead to a false positive result. Therefore, precautions are to be rigorously taken to avoid such a contamination [12]. Precautions vary according to laboratories and are summarized as follows:

(1) Subdivision of the PCR laboratory into different, well separated working areas (reagent preparation, specimen preparation, mixing, amplification, PCR products analysis). Each area has its ‘own material’, and all PCR steps are performed in a one-directional procedure from the reagent preparation area to the PCR products analysis area.

(2) Use of aerosol resistant tips (ART tips) to avoid airborne contaminations.

(3) Decontamination of PCR reagents by UV illumination (this does not seem to be completely effective in inactivating DNA fragments shorter than 500 bp [11]).

(4) Decontamination of PCR reagents by DNase digestion (this treatment does not seem to be completely effective in all cases [11]).

(5) Use of uracil DNA glycosylase for controlling carry-over contamination from a previous PCR product [13]. For this technique, dTTP is replaced by dUTP in the dNTP mix for the PCR. The synthesized amplicon DNA, which has incorporated the abnormal base uracil (a component of RNA but not of DNA), is destroyed by uracil-N-glycosylase just before the thermal cycling process. This enzyme is heat inactivated during the first steps of the PCR.

(6) Reduction of the number of pipetting operations by making stock solutions that are aliquoted in a single use format.

(7) One tube reverse transcription-PCR (RT-PCR). Instead of using two tubes for RT-PCR for the amplification of RNA, the RNA transcription step to cDNA and the amplification are carried out successively in the same tube without opening it. This process reduces the number of handlings and thus reduces the risk of sample-to-sample contamination [14, 15].

(8) Use of an internal positive control through which the amplified product could be clearly distinguished from the test sample amplicon. Indeed, as for any other diagnostic test, negative and positive internal controls have to be introduced in the PCR assay to obtain reliable results. However, an internal positive control that would give the exact amplicon as the test sample could be a source of cross-contamination and, therefore, a source of a false positive result.

Another issue to be addressed when performing PCR is the false negative result. This might happen when the pathogen to be diagnosed exhibits a high degree of variation in the genome sequence from isolate to isolate, as is the case for many RNA viruses. Primers, designed according to the gene sequence of one or few isolates of a pathogen, might not be able to detect a genetic variant if the nucleotide changes are
located at their 3’ end. Therefore it is always sensible to check a negative PCR result by another PCR assay with primers located on another area of the genome. Some scientists address this problem by carrying out the multiplex PCR [16]. For this assay, at least two sets of primers are used in a single reaction for the detection of at least two different target sequences. The technical difficulty of multiplex PCR lies in the design of the primers to avoid dimer formation that will decrease the reaction efficiency.

3.3. The 3SR technique, or nucleic acid sequence based amplification (NASBA)

The 3SR technique is an isothermal enzymatic in vitro amplification technique based on the RNA transcription process [17, 18]. This first version of the method is based on the use of two specific primers, with a T7 promoter sequence attached to one of them, and three enzymes — a reverse transcriptase enzyme (the avian myeloblastosis virus reverse transcriptase, AMV-RT), the RNase H enzyme and the T7 RNA polymerase.

Figure 4 is a schematic representation of a 3SR reaction. From an RNA target, the assay is started by the AMV-RT action, which synthesizes a single stranded cDNA. This reaction is primed by the specific oligonucleotide A, which contains, at its 5’ end, the T7 RNA polymerase binding sequence. This reverse transcription is followed by the digestion of the RNA strand in the duplex by the E. coli RNase H enzyme. The AMV-RT completes the synthesis of a double cDNA. The T7 promoter is then used by the T7 RNA polymerase for RNA synthesis in the final step of an amplification cycle. It is assumed that 10–1000 RNA copies are obtained per DNA molecule. The new RNA strands serve as templates for reverse transcription in a second round of the amplification process. The system is self-sustained until the exhaustion or inactivation of one of the reagents. With the 3SR method, there is an exponential increase in the number of nucleic acid target molecules as in PCR, and a 10^7-fold amplification in 60 min has been claimed [18]. It seems that, with the optimization of different parameters of the system, a production of 10^8 copies of the target molecule could be expected in a 30 min reaction [19].

A two-enzyme 3SR reaction has also been described [19]. In this format, only the AMV-RT and the T7 RNA polymerase are used. The E. coli RNase H enzyme is replaced in the reaction mixture by the addition of 10% dimethyl sulfoxide and 15% sorbitol. These additives enhance the endogenous RNase activity of the AMV-RT. It was also observed that, with these two reagents, the quantity of AMV-RT and T7 RNA polymerase can be reduced, although target sequences that could be amplified are longer than for the three-enzyme reaction. In another optimized protocol, both primers have been designed to contain the T7 promoter sequence [19]. It should also be noted that the T7 promoter can be effectively replaced by SP6 or T3 promoters.
3.4. Strand displacement amplification (SDA)

SDA is a primer directed DNA synthesis amplification system like PCR (Fig. 5), but SDA is performed isothermally [20, 21]. After denaturation of the target
DNA, a primer containing a restriction enzyme sequence site is annealed to one strand. This directs the synthesis of the complementary strand by an exonuclease-free DNA polymerase in the presence of a mixture of three normal dNTPs and one thio-dNTP. The primer-opposite strand is filled in, so at the end of the reaction a blunt dsDNA is obtained, which contains an unusual nucleotide. This will introduce a hemi-modification of restriction enzyme sites on newly synthesized fragments of the DNA molecule, which prevents the fragments from complete cleavage by restriction enzymes. Their use results in the introduction of a nick on only the unmodified strand. This forms an initiation point for the polymerase to repair the DNA by synthesizing a new strand and, in this way, the enzyme restriction site is reconstituted. While the polymerase is synthesizing a new DNA strand, it replaces the downstream strand,
which will finally be ejected into the solution. The reconstituted restriction enzyme site, still hemi-modified, is nick digested again and will serve for the initiation of another cycle of DNA synthesis. The displaced DNA strand serves as template for the minus primer that has the same restriction enzyme site as for the plus primer. Therefore, the completion of the method will result in an exponential amplification of the target sequence. It is claimed that the use of polymerase and restriction enzyme, both heat stable, at 60°C could produce a $10^{10}$-fold amplification rate in 15–20 min [22]. Operating at higher temperature has the advantage of increasing the stringency of the primer hybridization and, therefore, the specificity of the reaction. The observation of the amplified product is made using ethidium bromide staining following agarose gel electrophoresis, or by use of a probe detection system.

3.5. LCR

LCR is an amplification method which is based on the ligation of two primers whose respective positions are adjacent on the target nucleic acid sequence. The primers can be designed to leave a gap between them once they are hybridized to the template. The gap must be filled in by a thermostable polymerase before the ligation event takes place [23]. This variant of the method is called gapped LCR (Gap-LCR). The LCR is processed cyclically as is PCR, but it uses a thermostable ligase and two sets of pair primers: each set is complementary to one strand of the target DNA (Fig. 6). The reaction is started by the DNA denaturation, followed by the primer annealing to the template. Then, in the last step, the ligation event is mediated by a thermostable enzyme if there is no base mismatch at the junction of the two adjacent primers [24, 25]. The ligated products serve as templates in subsequent cycles. Therefore the ligated products accumulate exponentially, making the assay a sensitive test; however, this sensitivity may be limited by the formation of undesired products, resulting in a target independent blunt-end ligation of duplex primers. The Gap-LCR resolves this shortcoming. The sensitivity can also be increased by coupling LCR to PCR. The main advantage of LCR is its high specificity because it allows an easy detection of a single base change. Coupling LCR to PCR could be seen as a target amplification technique for Gap-LCR, because the amplified product contains a small sequence not present in the initial probes, or as a signal amplification technique for simple LCR. The used primers could be tagged with reporter molecules: a ligand is carried by one primer while the detectable label is on the second primer [26]. In this way, the ligated molecule is captured and then detected.

3.6. Qβ replicase amplification

This signal amplification system is based on the use of the RNA dependent RNA polymerase, the replicase, with the fragment of the phage Qβ 5000 genome
FIG. 6. Schematic representation of the LCR. A cycle is composed of heat denaturation, primer annealing and ligation of neighbouring primers.
FIG. 7. $Q_\beta$ replicase amplification procedure. A hybrid RNA molecule is composed of a target-specific probe sequence (bold line) and the $MDV_1$ fragment that is recognized by the $Q_\beta$ replicase. This hybrid molecule is allowed to hybridize with the target nucleic acid. After a washing to remove unbound molecules, the $Q_\beta$ replicase is added to the reaction for amplifying the hybrid probe RNA molecule.

required for replication, and named the midivariant ($MDV_1$ RNA). For the assay, a sequence that complements the target is inserted into $MDV_1$ [27–30]. This chimeric molecule is hybridized to the target. After washing off the unbound probe, the hybridized probe is amplified by the addition of the $Q_\beta$ replicase (Fig. 7). It is claimed that a $10^9$-fold increase is obtained in 20 min without temperature cycling [31]. The
FIG. 8. Schematic representation of the bDNA technique (adapted from Ref. [22]). The process is a succession of different steps to hybridize the target nucleic acid, target probe, amplifier and probe to a solid support.
amplified product is detected in agarose gel after staining or by using a secondary labelled probe.

3.7. bDNA

This signal amplification method was developed in 1993 by Urdea et al. [32] and relies only on a succession of hybridization events (see Fig. 8). The nucleic acid under detection is allowed to hybridize with the target probe primer in a liquid phase. The resulting hybrid is captured onto a solid support coated with specific oligonucleotides. Then the amplifier, which has multiple binding sites for the label, is added to the hybridization solution. Finally, the label is added, then its substrate for the revelation. The test is performed as an ELISA on a microplate and allows the quantification of the target present in the test sample.

4. COMPARISON OF DIFFERENT MOLECULAR AMPLIFICATION TECHNIQUES

To address the lack of sensitivity of DNA probe assays, different systems have been developed to increase the number of target molecules or the detection signal. Except in the branched DNA assay, which is based on the use of probes with multiple reporter molecules, all other methods rely on in vitro amplification technologies. These amplification technologies employ natural processes such as DNA and RNA replication, transcription, DNA ligation and DNA repair. Each amplification method has its advantages and disadvantages. Generally, methods that amplify the detection signal also have the risk of increasing the background. Some advantages and disadvantages of each method are summarized in Table I.

5. CONCLUSIONS

An explosion of specific, sensitive and rapid disease diagnostic methods has taken place since the beginning of the 1980s. These diagnostic methods have influenced the approaches to implementing efficient disease control measures. The greatest potential has been realized through the advent of genetic recombinant technology that made available specific and highly pure reagents: monoclonal antibody technology and nucleic acid probes. The interest in the use of molecular biological approaches has centred on the relatively high specificity of the hybridization process. Furthermore, since the target of the diagnostic test is genetic material, the spectrum of diseases that could be easily diagnosed has been expanded beyond that of conventional approaches.
Despite the potential advantages of nucleic acid probes, early assays based on their use for disease diagnosis have not met with great success. Although the use of radiolabelled probes for routine methods can give sensitive assays, the potential health risk and problems with availability and shelf-life have restricted their exploitation. These drawbacks have occasioned the development of non-radioactive probes. Unfortunately, except with the presence of multiple copies of the target sequence, the sensitivity of non-radioactive probes has proved much lower than expected. Scientists have therefore looked for possibilities of increasing either the quantity of the nucleic acid target sequence or the detection signal. Their efforts have been successful through the development of several elegant in vitro amplification systems. Among these systems, the PCR is the most popular. No doubt all of them will have a large impact on animal disease management. The current enthusiasm for the use of PCR in veterinary medicine is due to its high sensitivity, specificity and rapidity; however,
despite the features that make PCR superior to conventional diagnostic methods, its use is not always justified. In some parasitic and bacterial diseases, the causal agents are present in such high quantities that there is no need to implement a sensitive diagnostic test. In these cases, a simple microscopic observation is appropriate, because it provides results in few minutes at low cost. When the objective is to unequivocally identify the aetiological agent from an epidemiological point of view, a test such as PCR is indicated and justified, and indeed may be the only test able to perform the required task. The PCR allows not only an accurate differentiation between species or subspecies of a same microorganism, but also allows easy screening of a large number of samples, which are required for efficient epidemiological surveys. An output of PCR for epidemiology is the determination of the phylogenetic relationship between different isolates of a pathogen. For that, PCR is associated either with DNA sequencing or to RFLP analysis of the amplicons. This result could also be obtained through the implementation of different formats of the PCR test such as RAPD or microsatellite amplification and their analysis [2].

It is obvious that diagnosticians, with nucleic acid based diagnostic tests such as PCR, have powerful tools in hand. For the transfer of this new technology to developing country laboratories, some issues have to be addressed:

(1) Technical capabilities in the recipient laboratory. Before the introduction of PCR or other nucleic acid in vitro amplification techniques in a laboratory, the personnel should be trained. Indeed, they should know the basics of molecular biology techniques and also all protocols that are needed for obtaining reliable results (avoiding false positive results or RNA contaminations by RNase).

(2) Sustainability of the technique in regard to its cost. The PCR technique is being introduced in several developing countries through projects funded by external donors. In many cases, the host countries have no funds to sustain the expenses of required reagents once projects are ended. PCR techniques demand the use of many disposable materials for obtaining reliable results.

(3) Patents. This last issue is relevant to the Hoffman-LaRoche patent that covers the PCR process. In the case of developing countries, who pays the end-user service licence if the test is implemented routinely? A correct answer to this question is of great importance.

Nucleic acid in vitro amplification techniques, because of their high sensitivity at a level never achieved thus far by any other diagnostic test, pose an interesting question that is relative to the significance of a positive result when all other tests are negative. Once the contamination possibility has been ruled out, an interpretation is needed regarding the outcome of the infection and the epidemiology of the disease. For example, bluetongue virus RNA has been detected in blood taken from a calf 16 to 20 weeks after infection. These samples were no longer infectious for either insect vector or sheep [33]. In this case, the PCR positive result is not correlated with the ability of the animal to transmit the disease to another and therefore has no
importance epidemiologically. This finding means that the predictive value of PCR has still to be established in some cases.

In conclusion, nucleic acid based assays are powerful techniques that should currently be regarded as a complement of other techniques, in particular, the pathogen isolation that is the 'gold standard' technique for the confirmation of disease diagnosis.

REFERENCES


DIAGNOSIS OF CONTAGIOUS CAPRINE AND CONTAGIOUS BOVINE PLEUROPNEUMONIA BY PCR AND RESTRICTION ENZYME ANALYSIS

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Abstract

DIAGNOSIS OF CONTAGIOUS CAPRINE AND CONTAGIOUS BOVINE PLEUROPNEUMONIA BY PCR AND RESTRICTION ENZYME ANALYSIS.

Contagious caprine pleuropneumonia (CCPP) and contagious bovine pleuropneumonia (CBPP) are caused by Mycoplasma capricolum ssp. capripneumoniae and Mycoplasma mycoides ssp. mycoides, small colony type (SC), respectively. These species belong to the group of six mycoplasmas referred to as the Mycoplasma mycoides cluster. The members of the M. mycoides cluster are closely related, and some of them are very difficult to grow. Diagnosis based on identification of the causative agents by cultivation, biochemical reactions, or serology is therefore difficult, and improved diagnostic methods are sorely needed. Identification methods for M. capricolum ssp. capripneumoniae and M. mycoides ssp. mycoides SC, based on detection of the 16S rRNA genes from all members of the M. mycoides cluster by polymerase chain reaction (PCR) and differentiation of species by restriction enzyme analysis with PstI and AluI, respectively, have been developed in the Department of Bacteriology, National Veterinary Institute Laboratory, Sweden. The methods are based on the fact that the members of the M. mycoides cluster have two rRNA operons and that there are sequence differences (polymorphisms) between the two 16S rRNA genes from the two operons. The majority of these polymorphisms are characteristic for each species, and some of them can be utilized for differentiation between the species. Techniques for extraction of DNA from lung tissue and from clinical material dried onto filter paper for preservation are described. Pleural fluid was found to be an ideal sample for direct analysis by PCR, but this material can also be dried onto filter paper for preservation and sent to another laboratory for the PCR analysis. These identification systems designed for M. capricolum ssp. capripneumoniae and M. mycoides ssp. mycoides SC have been used for diagnosis of CCPP and CBPP, respectively, by analysis of clinical samples brought to the laboratory as frozen lung tissue or preserved by drying onto a filter paper.
1. INTRODUCTION

Mycoplasma is the trivial name for a group of microorganisms that belongs to the class Mollicutes [1]. Mycoplasmas were earlier believed to be different from bacteria, but have later been shown to be very closely related to gram positive bacteria with a low G+C content in their genomes [2]. Mycoplasmas lack a cell wall and can be regarded as degenerated bacteria with a reduced genome size (0.58–1.7 Mbp). The complete genomes have been sequenced from the human mycoplasmas *Mycoplasma pneumoniae* [3] and *M. genitalium* [4]. Mycoplasmas represent the smallest free living organisms that can be grown in artificial media without host cells. Normally, however, they live in close contact with eucaryotic cells, where they are protected from too harsh conditions and have a rich supply of nutrients. They have limited capabilities to synthesize amino acids, nucleotides and lipids and are, therefore, dependent on exogenous supply of some of these substances. About 150 mycoplasmas have been described, and the class Mollicutes consists of eight genera [5]. At least ten new species are described each year, and their taxonomy will probably be revised again in the future. The clinically important species are found within the genera *Mycoplasma, Acholeplasma* and *Ureaplasma*. The mycoplasmas have also been divided into five phylogenetic groups based on sequence analysis of the 16S rRNA genes [2]. These groups (the hominis, the pneumoniae, the spiroplasma, the anaeroplasm and the asteroleplasma groups) have been further subdivided into sixteen clusters [2, 6]. Many species which are important in veterinary medicine are found in the so-called *M. mycoides* cluster within the phylogenetic spiroplasma group.

The classical *M. mycoides* cluster is composed of the following species: *M. capricolum* ssp. *capricolum*, *M. capricolum* ssp. *capripneumoniae*, *M. mycoides* ssp. *capri*, *M. mycoides* ssp. *mycoides* large colony type (LC), *M. mycoides* ssp. *mycoides* small colony type (SC) and *Mycoplasma* sp. bovine serogroup 7 (Table I). Sequence analysis of the 16S rRNA genes shows that *M. putrefaciens* can be included in the phylogenetic *M. mycoides* cluster [2]. However, *M. cottewii* and *M. yeatsii* [7] have been shown to be closely related to *M. putrefaciens* [8], and these three species are in fact sufficiently different from the members of the classical *M. mycoides* cluster to be arranged in a separate cluster, the *M. putrefaciens* cluster [9]. *M. cottewii* and *M. yeatsii* were originally isolated from the external ear canal of goats, but have not been shown to be associated with any disease condition [7]. The other species cause diseases in goats and cattle, but have also been isolated from other ruminants (see Table I). The globally most important diseases caused by mycoplasmas are contagious bovine pleuropneumonia (CBPP) and contagious caprine pleuropneumonia (CCPP), which are included in the A and the B list, respectively, from Office international des épizooties (OIE) of communicable animal diseases [10]. These diseases are caused by *M. capricolum* ssp. *capripneumoniae* and *M. mycoides* ssp. *mycoides* SC, respectively. *M. capricolum* ssp. *capripneumoniae* was earlier referred to as
TABLE I. MYCOPLASMAS BELONGING TO THE CLASSICAL *Mycoplasma mycoides* CLUSTER AND SOME CLOSELY RELATED SPECIES

<table>
<thead>
<tr>
<th>Species</th>
<th>Type strain</th>
<th>Disease</th>
<th>Main host</th>
<th>Other hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. capricolum</em> ssp. capricolum</td>
<td>Calif. kid</td>
<td>Arthritis, mastitis</td>
<td>Goats</td>
<td>Sheep, (cattle)</td>
</tr>
<tr>
<td><em>M. capricolum</em> ssp. capripneumoniae</td>
<td>F38</td>
<td>CCPP</td>
<td>Goats</td>
<td>(Sheep)</td>
</tr>
<tr>
<td><em>M. mycoides</em> ssp. capri</td>
<td>PG3</td>
<td>Pleuropneumonia, arthritis</td>
<td>Goats</td>
<td>—</td>
</tr>
<tr>
<td><em>M. mycoides</em> ssp. mycoides LC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Y-goat</td>
<td>Pleuropneumonia</td>
<td>Goats</td>
<td>Sheep, (cattle)</td>
</tr>
<tr>
<td><em>M. mycoides</em> ssp. mycoides SC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>PG1</td>
<td>CBPP</td>
<td>Cattle</td>
<td>Buffalo, goats, sheep</td>
</tr>
<tr>
<td><em>M. sp. bovine</em> serogroup 7</td>
<td>PG50</td>
<td>Arthritis</td>
<td>Cattle</td>
<td>(Goats, sheep)</td>
</tr>
</tbody>
</table>

The following species are closely related to the *M. mycoides* cluster:

<table>
<thead>
<tr>
<th>Species</th>
<th>Type strain</th>
<th>Disease</th>
<th>Main host</th>
<th>Other hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. cotewii</em></td>
<td>VIS</td>
<td>—</td>
<td>Goats</td>
<td>—</td>
</tr>
<tr>
<td><em>M. putrefaciens</em></td>
<td>KS-1</td>
<td>Mastitis, arthritis, polyarthritis</td>
<td>Goats</td>
<td>—</td>
</tr>
<tr>
<td><em>M. yeatsii</em></td>
<td>GIH</td>
<td>—</td>
<td>Goats</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> Large colony type.  
<sup>b</sup> Small colony type.
Mycoplasma sp. strain F38, but has recently been assigned to the above species [11]. CBPP [12–14] and CCPP [15–17] are of great concern in Africa and Asia since these diseases cause great economic losses. Europe is free of CCPP, but several outbreaks of CBPP are reported from Portugal and Spain every year. Two other mycoplasmal diseases are included in the B list of OIE, namely, contagious agalactia in goats, which is caused by \textit{M. agalactiae}, and mycoplasmosis in poultry, which is caused by \textit{M. gallisepticum}.

Many diagnostic methods for CBPP and CCPP are based on immunological reactions such as enzyme linked immunosorbent assay (ELISA), complement fixation, immunocytochemical tests and protein immunoblotting [12, 15]. Some of these methods are time consuming to perform and not sufficiently specific. Furthermore, recent reports on variable surface proteins of mycoplasmas [18] indicate that it could be risky to rely on monospecific antibodies in diagnostic systems based on antigen detection. Improved diagnostic procedures for these diseases are therefore needed, and many new possibilities appeared following the introduction of gene technology. Both DNA probe techniques and methods based on in vitro amplification by the polymerase chain reaction (PCR) have been designed for diagnosis of CCPP and CBPP, but for most clinical samples only PCR has sufficient sensitivity. Some of the PCR techniques are based on the so-called CAP gene that was originally found in \textit{M. mycoides} ssp. \textit{capri} [19]. The function of the CAP gene is unknown, but it has also been found in other members of the \textit{M. mycoides} cluster. The sequence variability of the CAP genes from different members of the \textit{M. mycoides} cluster is sufficient for development of species specific PCR systems [20–22]. A nested PCR system based on a 72 kD immunogenic protein of \textit{M. mycoides} ssp. \textit{mycoides} SC has been utilized for detection of this species, and it was suggested to be used for diagnosis of CBPP [23]. Another gene that has been used as a diagnostic tool is the 16S rRNA gene. Sequence analysis of the 16S rRNA molecule or its gene has also proved very useful for studies of the evolution [24] and the phylogeny [25] of bacteria. The first extensive molecular phylogeny of mycoplasmas was based on sequence analysis of 16S rRNA [2], and the phylogeny of the members of the \textit{M. mycoides} cluster has also been established by this method [26]. It was shown that \textit{M. capricolum} ssp. \textit{capricolum}, \textit{M. capricolum} ssp. \textit{capripneumoniae} and \textit{Mycoplasma} sp. bovine serogroup 7 forms one distinct cluster, the \textit{M. capricolum} subcluster. \textit{M. mycoides} ssp. \textit{mycoides} LC and \textit{M. mycoides} ssp. \textit{capri} form another distinct cluster, the \textit{M. capri} subcluster, whereas \textit{M. mycoides} ssp. \textit{mycoides} SC forms an intermediary branch between these two subclusters. The 16S rRNA molecule or its gene have also been utilized as targets for diagnostic probes [27] or PCR primers for mycoplasmas. One important reason for this is the huge amount of sequence information of bacterial 16S rRNA sequences that is available in the data banks. A diagnostic probe complementary to the 16S rRNA molecule will have the potential of being very sensitive because of the high copy number of rRNA in growing bacteria. However, for most clinical purposes the
sensitivity of an rDNA probe is still not sufficient, and in vitro amplification by PCR has to be used for adequate sensitivity.

Bacteria contain three forms of rRNA (5S, 16S and 23S rRNA) which are organized in the genome in the form of rRNA operons. Bacteria often have the following typical organization of the rRNA operon(s):

\[ 5' - 16S \text{ rRNA} - \text{spacer region} - 23S \text{ rRNA} - 5S \text{ rRNA} - \text{trailer region} - 3' \]

Bacteria can have from one to ten rRNA operons, and fast growing bacteria need a higher number of rRNA operons for efficient protein biosynthesis. The members of the *M. mycoides* cluster have two rRNA operons designated *rrnA* and *rrnB* [28]. Sequence differences (polymorphisms) between the 16S rRNA genes from the two operons have been identified by DNA sequencing for the members of the *M. mycoides* cluster [26, 29] and also for some other mycoplasmas [6, 8]. Such polymorphisms have been used to design identification systems for *M. capricolum* ssp. *capripneumoniae* [29] and for *M. mycoides* ssp. *mycoides* SC [30].

This paper describes how the PCR systems based on the 16S rRNA genes for *M. capricolum* ssp. *capripneumoniae* and *M. mycoides* ssp. *mycoides* SC can be applied to different kinds of clinical material. The purpose of the paper is also to describe these two diagnostic systems in such a way that researchers with only limited experience in PCR technology can use the methods successfully.

2. MATERIALS AND METHODS

2.1. Extraction of DNA from lung tissue

Lung tissue from cows or goats can be treated in the same way, and tissue should preferably be collected from regions with visible lesions.

(1) Begin with a piece (about 0.5 cm \( \times \) 0.5 cm \( \times \) 0.5 cm) of fresh or frozen lung tissue. Cut and mince the tissue in a petri dish with the aid of two scalpels. Cut the tissue carefully into pieces as small as possible, then add 500 \( \mu \)L of phosphate buffered saline (PBS) and continue cutting. Try to make the tissue homogeneous in PBS.

(2) Transfer 300 \( \mu \)L of the suspension (try to avoid any pieces of tissue) to a microcentrifuge tube and add 385 \( \mu \)L of STE buffer (100 mM NaCl, 50 mM Tris-HCl buffer (pH7.4) and 1 mM EDTA), 5 \( \mu \)L of proteinase K solution (20 mg/mL) and 10 \( \mu \)L of SDS (20%).

(3) Mix by vortexing and incubate for 4 h at 50°C. Vortex a few times during the incubation.
(4) Add 400 μL of buffer saturated phenol and mix by vortexing.
(5) Centrifuge for 3–5 min at about 12 000g.
(6) Transfer the aqueous (upper) phase to a clean tube and add 400 μL of chloroform.
(7) Mix by vortexing and centrifuge as above.
(8) Transfer the aqueous (upper) phase (about 300 μL) to a new tube and precipitate with 0.1 volumes of 3M sodium acetate and 2.5 volumes of cold (−20°C ethanol (99%). Mix carefully by hand (several times). It should be possible to see a precipitate of DNA from the lung cells even if there are no mycoplasmas present in the sample. Allow the precipitation to proceed at −20°C for 30–60 min or overnight.
(9) Centrifuge for 20 min at 12 000g to get a pellet of DNA.
(10) Remove the supernatant and wash the pellet once with 500 μL of 75% ethanol.
(11) Centrifuge as above for 5 min, remove the supernatant and dry the pellet under a lamp for 20–30 min or in a vacuum centrifuge (SpeedVac) for about 5 min. Avoid overdrying, which can make the pellet difficult to dissolve.
(12) Dissolve the pellet in a small volume (25–50 μL) of water or TE buffer (10 mM Tris-HCl buffer [pH7.4], 1 mM EDTA) and use 5 μL (neat and 10-fold dilution) in the PCR experiments.

2.2. Preparation of pleural fluid for direct analysis by PCR

Fresh pleural fluid from diseased goats or cattle is the ideal sample for PCR analysis, and it can be directly analysed by PCR since it is a clean material and does not seem to contain inhibitors to the Taq DNA polymerase. Furthermore, pleural fluid from diseased animals often contains a large number of organisms and is therefore relatively easy to analyse by PCR. Use 5 μL (neat and 10-fold dilution) in the PCR experiments.

2.3. Drying of samples onto filter paper

If the sample has to be sent to another laboratory for analysis by PCR, it can be dried onto filter paper for preservation. Pleural fluid, for instance, can easily be preserved by this procedure. A suspension culture of mycoplasmas can also be dried, and it is possible to use the homogenized lung tissue after step (1) in Section 3.1 for drying. Use Whatman 3MM chromatography paper, indicate the application spot with a circle and apply about 100 μL of the material (pleural fluid, lung tissue suspension or suspension culture) to be dried. Let the material air dry at ambient temperature. It is very important that the filter paper is dry before being put in a heat sealable plastic bag and sent to another laboratory for analysis.
2.4. Preparation of samples dried onto filter paper for PCR

(1) Cut out the area around the sample application spot from the filter paper and place it in a microcentrifuge tube. Add 0.5 mL of sterile PBS, mix by vortexing and leave the tube at room temperature for 30 min. Vortex every 5 min for a few seconds.

(2) Heat the sample in a boiling water bath for 10 min for efficient solubilization of DNA from the sample and cool the tube on ice.

(3) Centrifuge at about 12 000g for 1 min and transfer the liquid to a new tube.

(4) Analyse 5 µL (neat and 10-fold dilution) by PCR.

2.5 Preparation of mycoplasma cultures for direct analysis by PCR

(1) Centrifuge 1.0 mL of an outgrown suspension culture of mycoplasmas in a microcentrifuge for 20 min at about 12 000g.

(2) Resuspend the pellet twice in 0.5 mL of PBS and centrifuge as above.

(3) Resuspend the pellet in 50–200 µL of water (depending on the size of the pellet) and heat the suspension in a boiling water bath for 10 min to lyse the mycoplasmas. This material can be frozen and used as a positive control in the PCR experiments. Avoid too many freezing and thawing cycles. Use 5 µL (neat and 10-fold dilution) in the PCR reactions.

The pellet from step (2) can also be used for DNA preparation as described above. If control DNA is prepared from a large volume (>10 mL) of a suspension culture of mycoplasmas, the amount of DNA and its quality should be estimated by determining $A_{260}$ and $A_{280}$. $A_{260} = 1$ corresponds to about 50 µg of DNA per mL, and the ratio $A_{260}/A_{280}$ should be above 1.6.

2.6. In vitro amplification of the members of the M. mycoides cluster with the M. capricolum ssp. capripneumoniae primers MmF and MmR

Note that these primers are not specific for M. capricolum ssp. capripneumoniae, but will amplify a region containing a restriction site for PstI that can be used for differentiation (see Fig. 1) [26]. The sequences of the primers are given in Table II [26, 29, 30]. Prepare a suitable volume of the following master mix and distribute 43 µL into the number of reaction tubes required:

- $H_2O$: 30.2 µL
- Taq buffer (10 ×): 5.0 µL
- MgCl$_2$ (25 mM): 3.0 µL
- dNTP mix (2.5 mM of each): 4.0 µL
- MmF primer (50 pmol/µL): 0.4 µL
- MmR primer (50 pmol/µL): 0.4 µL
FIG. 1. Target regions (underlined) for the PCR primers for the members of the M. mycoides cluster in the total consensus sequence of the 16S rRNA genes from both rRNA operons [26]. The recognition sites for PstI and Alul within the amplified segment of M. capricolum ssp. capripneumoniae and M. mycoides ssp. mycoides SC, respectively, are also underlined. The discriminatory restriction sites for M. capricolum ssp. capripneumoniae (Mcacp) and M. mycoides ssp. mycoides SC (MmymySC) are indicated with arrows. Nucleotide positions that are not identical for all members of the M. mycoides cluster are denoted in boldface with the corresponding ambiguity code according to the International Union of Biochemistry.
TABLE II. PCR PRIMERS FOR IN VITRO AMPLIFICATION OF THE MEMBERS OF THE
*Mycoplasma mycoides* CLUSTER [29, 30]

<table>
<thead>
<tr>
<th>Designation</th>
<th>Nucleotide positions&lt;sup&gt;a&lt;/sup&gt;</th>
<th>To be used for</th>
<th>Sequence (from 5' to 3')</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>MmF</td>
<td>718–740</td>
<td><em>Mcacp</em></td>
<td>CGA AAG CGG CTT ACT GGC TTG TT</td>
<td>Forward</td>
</tr>
<tr>
<td>MmR</td>
<td>1243–1265</td>
<td><em>Mcacp</em></td>
<td>TTG AGA TTA GCT CCC CTT CAC AG</td>
<td>Reverse</td>
</tr>
<tr>
<td>F-REAP3</td>
<td>143–168</td>
<td><em>MmmySC</em>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>GAA ACG AAA GAT AAT ACC GCA TGT AG</td>
<td>Forward</td>
</tr>
<tr>
<td>R-REAP4</td>
<td>907–927</td>
<td><em>MmmySC</em></td>
<td>CCA CTT GTG CGG GTC CCC GTC</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

<sup>a</sup> According to the numbering of the 16S rRNA total consensus sequence obtained for the members of the *M. mycoides* cluster [26].

<sup>b</sup> *M. capricolum* ssp. *capri pneumoniae*.

<sup>c</sup> *M. mycoides* ssp. *mycoides* SC.
Add 5 μL of DNA preparations to the reaction tubes. Include positive and negative controls in some tubes. Overlay with two drops of mineral oil unless a thermocycler with a heated lid is used. Centrifuge the tubes for a few seconds to ensure that the reagents are mixed.

Dilute the Taq DNA polymerase according to the following scheme and prepare enzyme for one extra reaction to compensate for pipetting losses:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>1.6 μL</td>
</tr>
<tr>
<td>Taq buffer (10 ×)</td>
<td>0.2 μL</td>
</tr>
<tr>
<td>Taq DNA polymerase (1 U)</td>
<td>0.2 μL</td>
</tr>
</tbody>
</table>

Hot start: Place the tubes in the thermocycler at 94°C for 4 min, then transfer the tubes to a heating block (at about 85°C) and add 2 μL of the diluted enzyme to each tube. Place the tubes in the thermocycler and set the programme to: 95°C for 45 s, 62°C for 1 min, and 72°C for 2 min. Repeat for 30 cycles and finish with an elongation step at 72°C for 3 min. A final cooling step of 4°C (optional) can be used if the samples are left in the machine overnight.

Analyse the PCR products by electrophoresis in a 1.5% agarose gel in TBE buffer with the appropriate DNA size markers. Include 1 μL of a solution containing 10 mg/mL of ethidium bromide per 100 mL of agarose gel solution for staining of the DNA fragments. Note that ethidium bromide is a strong mutagen and should be handled accordingly. A stock solution of 10 × TBE buffer can be prepared by dissolving 121 g Tris, 51.3 g of boric acid and 3.72 g EDTA in H₂O to a final volume of 1000 mL. Mix 5 μL of the PCR product with 2 μL of gel loading solution (30% glycerol and 0.25% bromophenol blue). Include suitable size markers. The DNA bands in the gel can be visualized under UV light. Protect the eyes and the skin.

2.7. Identification of *M. capricolum* ssp. *capripneumoniae* by restriction enzyme analysis of the PCR products with *PstI*

The PCR products can be analysed directly without purification of DNA. The amount of PCR product to be used depends on the efficiency of the amplification and has to be judged from the result of the above agarose gel electrophoresis. Prepare the reaction mixture as follows: PCR product, 4–10 μL; Reaction buffer (10 ×), 2 μL; *PstI* (1.5–5 U), 0.1–0.5 μL. Add water to a final volume of 20 μL.

If the amount of PCR product estimated from the agarose gel electrophoresis is large, it may be necessary to use more enzyme. Perform the restriction enzyme analysis at 37°C for 1–2 h and analyse the product on a 1.5–2.0% agarose gel. The reaction buffer is usually supplied with the enzyme.
2.8. In vitro amplification of the members of the *M. mycoides* cluster with the *M. mycoides* ssp. *mycoides* SC primers F-REAP3 and R-REAP4

Note that the F-REAP3 and R-REAP4 primer pair is not specific for *M. mycoides* ssp. *mycoides* SC, but will amplify a region containing a restriction site for *AluI* that can be used for differentiation (see Fig. 1). The sequences of the primers are given in Table II. About 5 ng of genomic DNA is usually amplified with these primers in 50 μL of the reaction mixture containing 10 mM Tris-HCl buffer (pH8.3), 50 mM KCl, 4 mM MgCl₂, 0.2 mM of each dNTP, 5 pmol of each primer and 0.6 U of *Taq* DNA polymerase.

The reaction mixture should first be heated for 3 min at 96°C in the thermocycler and then for 33 cycles at 96°C for 30 s, at 60°C for 30 s and at 72°C for 1 min. A final extension at 72°C for 5 min is also used. Analyse the PCR products by agarose gel electrophoresis as described below.

2.9. Identification of *M. mycoides* ssp. *mycoides* SC by restriction enzyme analysis of the PCR products with *AluI*

The PCR products can, in principle, be analysed directly without purification of DNA, but for the best results it is sometimes preferable to purify the PCR product by, for instance, precipitation with ethanol as described in step (8) under Section 3.1. Dissolve the DNA preparation in 50 μL (or less if it has to be concentrated) of water. The amount of PCR product to be used for restriction enzyme analysis depends on the efficiency of the amplification and has to be judged from the result of the above agarose gel electrophoresis. The reaction mixtures should contain the following: 6 mM Tris-HCl buffer (pH7.5), 50 mM NaCl, 6 mM MgCl₂, 1 mM dithiotreitol, 0.1 mg/mL of bovine serum albumin and 5 U of *AluI*. Perform the restriction enzyme analysis in 20 μL at 37°C for 4 h, and stop the reaction by heating to 96°C for 5 min. The restriction cleavage can also be performed with the buffer supplied by the manufacturer. Analyse the products by gel electrophoresis (using 10 V/cm) in a 3% Metaphor agarose gel (FMC Bioproducts) in TBE buffer, with continuous cooling during the electrophoresis.

3. RESULTS

3.1. PCR with the *M. capricolum* ssp. *capripneumoniae* primers MmF and MmR

A segment of 548 bp of the 16S rRNA gene of all six members of the classical *M. mycoides* cluster is amplified with the primer pair MmF and MmR. A homologous
FIG. 2. Agarose gel electrophoresis of PCR products obtained with the primers for M. capricolum ssp. capripneumoniae from some mycoplasmas. The following samples were applied, from left: 1: molecular size markers (BglI-cleaved pBR 328 DNA and HindI-cleaved pBR 328 DNA); 2: M. capricolum ssp. capripneumoniae; 3: M. capricolum ssp. capricolum; 4: M. putrefaciens; 5: M. mycoides ssp. mycoides SC; 6: M. agalactiae; 7: M. capricolum ssp. capripneumoniae; 8: negative control; 9: molecular size markers.

Region in the 16S rRNA genes of both rRNA operons is amplified with these primers. We have analysed more than 50 strains representing all six members of the classical M. mycoides cluster from different parts of the world. The segment of the 16S rRNA genes from all these strains can be amplified with the primer pair MmF and MmR [29, 31]. The results of a PCR experiment with different mycoplasmas is shown in Fig. 2. Note that PCR products are not formed from M. agalactiae and M. putrefaciens. We have also analysed 17 other mycoplasma species or strains found in goats or that have been reported to give serological cross-reactions with members of the M. mycoides cluster. However, none of these strains were amplified with the primer pair MmF and MmR [29, 31].
3.2. Restriction enzyme analysis of the PCR products with PstI

The identification of *M. capricolum* ssp. *capripneumoniae* by restriction enzyme analysis with *PstI* is based on the fact that all other members of the classical *M. mycoides* cluster have a recognition site (CTGCAG) for this enzyme in the amplified segments of both rRNA operons. *M. capricolum* ssp. *capripneumoniae*, however, lacks this recognition site in the segment of the *rrnB* operon because of the presence of a nucleotide substitution (C→T) in position 844 [26]. Thus, after restriction enzyme analysis of the PCR product from *M. capricolum* ssp. *capripneumoniae*, three bands are obtained; one band represents intact PCR product of 548 bp from the *rrnB* operon, and the other two bands represent the two fragments of 420 bp and 128 bp from the *rrnA* operon. Two bands of 420 bp and 128 bp are obtained from all other members of the classical *M. mycoides* cluster. The strategy for differentiation between species is schematically outlined in Fig. 3. We have analysed more than 15 strains of *M. capricolum* ssp. *capripneumoniae* and more than 25 strains of the closely related species *M. capricolum* ssp. *capricolum* and *Mycoplasma* sp. bovine serogroup 7, and all gave the characteristic restriction patterns [29, 31]. However, one strain (4/2LC) of *M. capricolum* ssp. *capripneumoniae* gives slightly different proportions between the three bands as compared to a typical *M. capricolum* ssp. *capripneumoniae* strain [31]. This result was shown to be caused by one nucleotide substitution in position 1255 (G→A) in the target region for the reverse primer [26] (see Fig. 1). The results of a restriction enzyme analysis of *M. capricolum* ssp. *capripneumoniae* with *PstI* are shown in Fig. 4.

3.3. PCR with the *M. mycoides* ssp. *mycoides* SC primers F-REAP3 and R-REAP4

A segment of 786 bp of the 16S rRNA genes will be amplified from all six members of the classical *M. mycoides* cluster with the primer pair F-REAP3 and R-REAP4. These primers amplified homologous regions in the 16S rRNA genes of both rRNA operons. We have analysed representative strains of all six members of the classical *M. mycoides* cluster. The segments of the 16S rRNA genes from all these strains were amplified with the above primer pair [30]. We have also analysed other mycoplasma species or strains found in cattle. However, none of these strains were amplified with the primer pair F-REAP3 and R-REAP4 [30].

3.4. Restriction enzyme analysis with AluI

The identification of *M. mycoides* ssp. *mycoides* SC by restriction enzyme analysis with *AluI* is based on the fact that the PCR products obtained with the primer pair F-REAP3 and R-REAP4 from the members of the *M. mycoides* cluster have
different numbers of recognition sites (AGCT) for this enzyme. *M. mycoides* ssp. *mycoides* SC has four recognition sites in the amplified segment of the *rrnB* operon, but only three recognition sites in the amplified segment of the *rrnA* operon because of the presence of a nucleotide substitution (G→T) in position 426 [26] (see Fig. 1). All other members of the *M. mycoides* cluster have four recognition sites in the amplified segment of both rRNA operons. Thus, after restriction enzyme analysis of the PCR product from *M. mycoides* ssp. *mycoides* SC, six fragments are obtained. The sizes of these fragments are: 370, 236, 186, 184, 98 and 82 bp. The fragment of 370 bp originates from the *rrnA* operon and the fragments of 186 bp and 184 bp originate from the *rrnB* operon. It is not possible to resolve the fragments of 184 bp and 186 bp by agarose gel electrophoresis; therefore, five bands are obtained after the electrophoresis. Five fragments are also obtained from the other members of the *M. mycoides* cluster (236, 186, 184, 98 and 82 bp), which results in four bands after agarose gel electrophoresis. The strategy for differentiation between species is schematically outlined in Fig. 5. We have analysed 16 strains of *M. mycoides* ssp. *mycoides* SC from different parts of the world and representative strains of all members of the *M. mycoides* cluster, and all gave the characteristic cleavage patterns. Figure 6 shows the results of a PCR experiment of some mycoplasmas with the
FIG. 4. Agarose gel electrophoresis of PstI cleaved PCR products obtained with the primers for M. capricolum ssp. capripneumoniae from some members of the M. mycoides cluster. The following samples were applied, from left: 1: molecular size markers (as in Fig. 2); 2: M. capricolum ssp. capripneumoniae; 3: M. capricolum ssp. capricolum; 4: M. mycoides ssp. mycoides SC; 5: M. capricolum ssp. capripneumoniae; 6: molecular size markers.

primers for M. mycoides ssp. mycoides SC and a restriction enzyme cleavage of the PCR products with AluI. Note that M. putrefaciens is not amplified with the primers.

4. DISCUSSION

4.1. Can a diagnostic system based on a single polymorphism be robust?

It can always be argued that a diagnostic system based on group specific amplification and differentiation by restriction enzyme analysis based on one single nucleotide substitution in one of the two 16S rRNA genes cannot be very robust. However, it should be kept in mind that at least some regions in the rRNA genes are relatively conserved, and that a mutation in one of these genes is a comparatively rare event. We have also sequenced the 16S rRNA genes of the two rRNA operons from many different strains of M. mycoides ssp. mycoides SC originating from Africa,
FIG. 5. Schematic illustration of the restriction enzyme cleavage with Alul of the PCR products obtained from (a) M. mycoides ssp. mycoides SC and (b) another member of the M. mycoides cluster. The sizes of the fragments are given in bp.

Australia and Europe. There were few sequence variations between the strains, and only seven to eight polymorphisms were identified [32]. None of the few strain differences that were found affected the target regions for the primers or the polymorphism used for differentiation with Alul.

The evolution of mycoplasmas has been shown to be faster than for other bacteria [33]. M. capricolum ssp. capripneumoniae is different from the other members of the M. mycoides cluster in that it seems to have an unusually fast evolution in the 16S rRNA genes [26]. This species has an exceptionally large number of polymorphisms, and there is a great variation between strains [34]. It is therefore important to analyse as many strains as possible, both by sequencing of the 16S rRNA genes to get a general picture of the variations that can be expected and by the diagnostic PCR system to verify that it works. (A good diagnostic system should identify all strains within the species that cause the disease.) We have done both, and so far only one strain (4/2LC from Oman) has been found that has one nucleotide substitution in one of the 16S rRNA genes in the target region for the reverse primer [26]. This gives a slightly different proportion of the bands obtained after the restriction enzyme analysis because the segment from the rrnB operon is amplified with a
FIG. 6. Agarose gel electrophoresis of PCR products obtained with the primers for M. mycoides ssp. mycoides SC from some mycoplasmas before and after restriction enzyme cleavage with Alul. The following samples were applied from left: 1: molecular size markers (50 bp ladder); 2: negative control; 3: M. putrefaciens; 4: M. capricolum ssp. capripneumoniae; 5: M. mycoides ssp. mycoides SC; 6, M. capricolum ssp. capripneumoniae after cleavage with Alul; 7: M. mycoides ssp. mycoides SC after cleavage with Alul.

slightly better efficiency than the segment from the rRNA operon. The polymorphism in the restriction site for PstI is present in all M. capricolum ssp. capripneumoniae strains analysed. However, there is always a risk that M. capricolum ssp. capripneumoniae strains exist that lack the polymorphism in position 844 or that have an extra mutation in the restriction site, resulting in an unexpected band pattern upon restriction enzyme analysis.

4.2. Why develop several different PCR systems for the same species?

Several PCR systems have been developed for identification of M. mycoides ssp. mycoides SC and M. capricolum ssp. capripneumoniae. Some of these systems
are based on the so-called CAP gene, which was first identified in *Mycobacteria mycoides* ssp. *capri* after random cloning [19]. The function of the CAP gene is unknown, but homologous sequences have been found in the other members of the *M. mycoides* cluster. Sequence information from the CAP gene has been used to construct phylogenetic trees of the members of the *M. mycoides* cluster and to design DNA probes and PCR systems. One of the CAP gene systems is based on PCR primers specific for *M. mycoides* ssp. *capri*, *M. mycoides* ssp. *mycoides* SC and *M. mycoides* ssp. *mycoides* LC [19]. The PCR product is about 0.5 kb, and it is possible to differentiate *M. mycoides* ssp. *mycoides* SC from *M. mycoides* ssp. *mycoides* LC and *M. mycoides* ssp. *capri* by restriction enzyme analysis of the PCR product with *AsnI*. The sensitivity of this system has been improved by designing a nested PCR system with the first primer set amplifying all members of the *M. mycoides* cluster and the second set amplifying the subspecies of *M. mycoides*. Differentiation is then achieved by restriction enzyme analysis with *AsnI* [20]. Another nested PCR system also based on the CAP gene has been developed for identification of *M. mycoides* ssp. *mycoides* SC [21]. A segment of 460 bp of the CAP gene from all members of the *M. mycoides* cluster is first amplified with the outer primer pair. An inner primer pair that amplifies a segment of 275 bp from the PCR product of *M. mycoides* ssp. *mycoides* SC is then used for identification. The segment of 275 bp contains three specific restriction sites that can be used for confirmation of the result, and a digoxygenin labelled probe can be used to increase the sensitivity of the system. Another nested PCR system based on the CAP gene has been developed for identification of all members of the *M. mycoides* cluster [22]. A segment of 253–265 bp (depending upon species) from all members of the *M. mycoides* cluster is first amplified with a general outer primer pair. A set of at least six inner primer pairs are then used for species specific identification. Finally, a nested PCR system based on the immunogenic protein P72 has been designed for detection of *M. mycoides* ssp. *mycoides* SC in nasal fluids and bronchial lavage [23].

The above systems have advantages and drawbacks. For instance, nested PCR systems are claimed to be very sensitive, but can have contamination problems if the laboratory facilities are not optimal. PCR systems based on genes with unknown function have the drawback that it is impossible to foresee the sequence variation that could be expected within different taxon levels. Identification systems based on rRNA genes for closely related species, such as the members of the *M. mycoides* cluster, have to be based on rather few nucleotide differences because of the conserved nature of rRNA genes. On the other hand, if such sequence differences are present in the type strain of the species, they are probably also present in field isolates of that particular species. It is therefore valuable to have different systems based on unrelated genes from which to choose. If contamination problems appear with one system, another can be chosen, and if ambiguous results are obtained with one system, there is a possibility to confirm or reject the results with another system.
4.3. Preservation of samples

So far there are only limited possibilities to perform PCR experiments in countries where CCPP and CBPP are widely spread. It is therefore important to be able to collect the sample at one site, preserve it and send it to a laboratory with facilities for diagnostic PCR. The preservation procedure to dry different kind of samples on filter paper was developed to overcome difficulties in storage and transportation of samples in developing countries [31]. We have found this preservation method to be a useful alternative to sending frozen material between laboratories for analysis [31, 35].

ACKNOWLEDGEMENTS

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REFERENCES


USE OF THE POLYMERASE CHAIN REACTION IN EPIZOOTIOLOGICAL STUDIES OF VIRAL DISEASES

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Abstract

USE OF THE POLYMERASE CHAIN REACTION IN EPIZOOTIOLOGICAL STUDIES OF VIRAL DISEASES.

The polymerase chain reaction (PCR) has become a powerful diagnostic tool in veterinary virology. The research team at the Department of Virology of the National Veterinary Institute, Uppsala, Sweden, was among the first groups to develop and apply routine diagnostic PCR assays in veterinary virology, to develop PCR diagnostic kits and to introduce assays of molecular epizootiology, based on comparative nucleotide sequence analysis of the PCR products. In the paper the experiences of 10 years of application of these techniques are summarized, with special regard to technical developments, i.e. simplified methods of sample preparation, precautions to avoid false positive or negative results, comparison of standard and nested PCR and simple assays of visualization. The viruses involved in the routine PCR diagnostic work are listed in two tables. Examples are given concerning the problems of routine PCR diagnosis in veterinary virology. The use of the PCR as a basic method of 'molecular epizootiology' is discussed and illustrated with several examples. The approaches of molecular epizootiology are based on direct sequence analysis of the PCR products, comparative analysis of the sequences and construction of phylogenetic trees. By this approach the phylogenetic relations are determined and the viruses are rapidly identified and grouped. The accurate genetic identification of virus variants provides novel means to the epizootiologists to trace the geographic distribution of the viruses and to determine the origin of a given outbreak.

1. INTRODUCTION

The first reports on the polymerase chain reaction (PCR) method appeared in the middle of the 1980s [1, 2]. The PCR is a powerful method that can be used for the in vitro amplification of selected target DNA molecules, resulting in a several million-fold amplification of the target sequence within a few hours. The large amounts of PCR products can then be easily detected and identified [1–3]. None of the basic molecular biology techniques that have been developed in the last decade has had a greater impact than the PCR. Thousands of articles, published since 1985, indicate that PCR has been one of the most rapidly adopted techniques in biochemistry.
TABLE I. DETECTION OF DNA VIRUSES BY THE PCR

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amplified region</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mink parvovirus</td>
<td>L-ORF</td>
<td>Aleutian disease</td>
</tr>
<tr>
<td>Porcine parvovirus</td>
<td>VP2</td>
<td>Parvovirus infection of swine</td>
</tr>
<tr>
<td>Bovine adenovirus subgroup I</td>
<td>Hexon</td>
<td>Pneumoenteritis of calves and lambs</td>
</tr>
<tr>
<td>Bovine adenovirus subgroup II</td>
<td>Proteinase</td>
<td>Pneumoenteritis of calves</td>
</tr>
<tr>
<td>Pseudorabies virus (PRV)</td>
<td>gB, gE, gpD</td>
<td>Aujeszky’s disease of pigs, acute and latent</td>
</tr>
<tr>
<td>Bovine herpes type 1 (BHV-1)</td>
<td>gI</td>
<td>IBR/IPV disease complex</td>
</tr>
<tr>
<td>Bovine herpes type 4</td>
<td>gB, TK</td>
<td>Various syndromes</td>
</tr>
<tr>
<td>Equine herpes type 1 (EHV-1)</td>
<td>gp13</td>
<td>Equine abortion</td>
</tr>
<tr>
<td>Equine herpes type 2</td>
<td>ILT10, gpL</td>
<td>Respiratory disease, activation of R. equi</td>
</tr>
</tbody>
</table>

Reports on the application of PCR to the diagnosis of infectious diseases are also accumulating at an exponential rate [3–7].

Despite the large number of publications available today on PCR, the technique is performed as a clinical service in only a few laboratories. The main reason is that the routine diagnostic application of the new technique is hindered by the frequent cross-contamination of specimens (a consequence of the high sensitivity of the PCR).

Our team at the Department of Virology of the National Veterinary Institute, Uppsala, Sweden, was among the first groups to develop and apply routine diagnostic PCR assays in veterinary virology [8, 9]. Since 1989, PCR assays have been developed and used for the routine diagnosis of more than 20 viral diseases (Tables I and II; for review see Refs [6, 10]).

The aim of the present article is to summarize the experiences gained during nearly a decade on the application of various PCR assays in our routine diagnostic laboratory. The technical and biological aspects of the PCR are summarized, with special regard to simplified methods of sample preparation, precautions to avoid false positive or negative results, comparison of standard and nested PCR and development of simple assays of visualization.

2. EXPERIENCES IN THE PRACTICAL APPLICABILITY OF THE PCR IN ROUTINE DIAGNOSIS

2.1. Preparation of samples

Our aim was to develop PCR assays which are economical and easily applicable in sparsely equipped laboratories. Simple methods of DNA or RNA
### TABLE II. DETECTION OF RNA VIRUSES BY THE PCR

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amplified region</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>European brown hare syndrome virus</td>
<td>Caps</td>
<td>European brown hare syndrome</td>
</tr>
<tr>
<td>Rabbit haemorrhagic disease virus</td>
<td>VP60</td>
<td>Rabbit haemorrhagic disease</td>
</tr>
<tr>
<td>Bovine viral diarrhoea virus (BVDV)</td>
<td>gp48, p80</td>
<td>Bovine viral diarrhoea/mucosal disease, acute and persistentBorder disease of sheep</td>
</tr>
<tr>
<td>Classical swine fever virus (CSFV)</td>
<td>p80</td>
<td>Swine fever/hog cholera</td>
</tr>
<tr>
<td>General pestivirus detection kit</td>
<td>5'NC</td>
<td>Bovine viral diarrhoea/mucosal disease, acute and persistentHog cholera of swineBorder disease of sheep</td>
</tr>
<tr>
<td>Equine arteritis virus (EAV)</td>
<td>NP</td>
<td>Equine arteritis</td>
</tr>
<tr>
<td>Lactate dehydrogenase elevating virus</td>
<td>ORF7 (VP1)</td>
<td>Inapparent persistent infection in mice</td>
</tr>
<tr>
<td>Feline infectious peritonitis virus (FIPV)</td>
<td>NP</td>
<td>Infectious peritonitis of cats</td>
</tr>
<tr>
<td>Bovine leukaemia virus (BLV)</td>
<td>gp51, p24</td>
<td>Bovine leukosis</td>
</tr>
<tr>
<td>Respiratory syncytial virus (RSV)</td>
<td>F, G</td>
<td>Bovine RSV disease</td>
</tr>
<tr>
<td>Parainfluenza-3 virus (mouse)</td>
<td>NP</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>Sendai virus</td>
<td>NP</td>
<td>Sendai virus disease</td>
</tr>
<tr>
<td>Mouse hepatitis virus (MHV)</td>
<td>E1 protein/MP</td>
<td>Lethal intestinal disease in infant mice and hepatitis</td>
</tr>
<tr>
<td>Sialodacryoadenitis virus (SDAV)/Rat corona virus (RCV)</td>
<td>MP</td>
<td>Sialoadenitis and respiratory problems</td>
</tr>
<tr>
<td>Lymphocytic choriomeningitis virus (LCM)</td>
<td>NP</td>
<td>Various symptoms in rodents and persistent infection</td>
</tr>
<tr>
<td>Encephalomyocarditis virus (EMCV)</td>
<td>PP</td>
<td>Encephalomyelitis/myocarditis</td>
</tr>
</tbody>
</table>
purification were applied, for example, a guanidium thyocyanate method and a chelex procedure [11–14].

2.2. Construction of single and nested PCR assays

Initially, we developed single PCR assays with two primers. In some cases the sensitivity of these tests was not satisfactory because less than 10⁴ virus particles were not detected in the specimens. Subsequently, we developed PCR assays with nested primers. With nested PCR, single copies of viral genomes were detected [6, 15].

2.3. Simple methods of visualization

For visualization and identification of the PCR products, various simple methods were applied. The solid phase colorimetric method, Detect Immobilized Amplified DNA (DIANA), has been adapted to microplate. The other method, Colorimetric Detection Assay on Filter (CODAF), proved to be very rapid. However, despite the advantages of DIANA and CODAF, henceforward the nucleic acid hybridization methods were found most reliable for safe identification of PCR products (amplicons). In order to simplify the hybridization, various non-radioactive labelling methods of oligonucleotide probes were compared. Biotinylation at the 5' end by means of oligonucleotide synthesis was the most simple and practical labelling method in our laboratory. We found that hitherto a simplified filter hybridization provides the most rapid and safe diagnostic results [15].

2.4. Precautions to avoid false positive results

Various methods and tools have been tested to prevent or eliminate false positive results due to carry-over or cross-contamination. The ultraviolet exposure and the DNase treatments proved to be unsuitable for decontamination of PCR mixtures contaminated with amplicons shorter than 380 bp. By constructing special tube holders and openers, and by applying a simple technique of pipetting, the false positive PCR results were eliminated [15].

2.5. Precautions to avoid false negative results

The PCR laboratories may face not only the frequently documented false positive results, but also unexpected false negatives. The latter are mostly due to inhibitory effects of some ingredients and/or to pipetting errors. Therefore, in our laboratory, internal controls are used as indicators of amplification efficiency. The internal control molecules, termed 'mimics', were constructed to have the same primer binding nucleotide sequences as the viral nucleic acids but to flank a
heterologous DNA fragment of different size. As heterologous DNA, a part of human beta-actin gene was used for the mimic construction. The identical primer binding nucleotide sequences allowed co-amplification of the viral nucleic acid and the mimic in the same tube, and, simultaneously, the size differences allowed easy discrimination between the two PCR products. By running a rapid agarose gel electrophoresis after co-amplification, the presence or absence of the mimic PCR products provided proper information on the efficacy of the PCR in each reaction tube. We concluded that mimic molecules, co-amplified with the samples, significantly increased the reliability of the diagnostic PCR assays [16].

2.6. Correlation between the results of PCR assays and of other methods of virus detection

The achievements listed here resulted in economical, highly sensitive and safe PCR assays, which were tested on a large number of clinical specimens. Good correlation was found between the results of the nested PCR assays and of conventional diagnostic methods, e.g. virus isolation, enzyme linked immunosorbent assay (ELISA), Western blotting (for review see Refs [6, 7]).

2.7. Specific importance of the PCR in certain areas of viral diagnosis

According to our experience, the application of PCR is especially important in the following areas:

(1) To detect virus when the immunological response is weak (ELISA in the 'gray zone');

(2) To determine that young animals are infected or have only maternal immunity, e.g. young calves in a bovine leukaemia virus (BLV) eradication programme;

(3) To detect virus in toxic material, e.g. semen, mummified foetus;

(4) To detect viruses that do not grow or poorly propagate in cell cultures;

(5) At the end of the eradication programmes, when thorough investigation of single cases is necessary, e.g. herpesvirus latency and single reactor (SR) animals during the Aujeszky's disease eradication programmes in swine;

(6) To detect virus in fixed tissue samples, e.g. paraffin embedded blocks.

2.8. The application of PCR assays in routine diagnosis

The PCR systems provided a considerable improvement in our routine diagnostic service at the Department of Virology. For the time being, the PCR assays listed in Tables I and II are used in our routine diagnostic work.
2.9. Practical examples of the diagnostic applicability of the PCR assays

The PCR assays were applied not only for direct diagnostic purposes but also with other intentions, e.g. to estimate the potency of vaccines by studying the distribution of the virus in the animals.

In the present review several examples illustrate the wide applicability of the PCR as an assay of basic importance. These studies have been published in detail in various journals or have been submitted for publication. In the present review article we provide a short summary of the applications and of the results. In this summary we do not follow the list of various virus families (as in Tables I and II), but we start with the routine diagnosis and follow with other applications, such as tests of vaccines, classification of viruses, etc.

2.9.1. Direct detection of viruses by the PCR

2.9.1.1. Detection of proviral DNA of bovine leukaemia virus

A double PCR assay has been devised for the direct detection of BLV proviral DNA (Table II). The assay was directly applied on peripheral blood lymphocytes (PBL), avoiding the DNA purification procedures. When testing the sensitivity of PCR, less than eight genome copies of the provirus were detected in the background of two million negative lymphocytes [11].

In BLV infected herds, various age groups were examined by the indirect (serological) diagnostic tests of agar gel immunodiffusion and indirect ELISA as well as by the direct detection method of PCR. When examining the specimens from cows and heifers, close agreement was found between the results of the various methods. However, in young calves the indirect tests were not able to discriminate infection from colostral immunity. In such cases, the PCR proved to be a useful complementary assay for the safe diagnosis of BLV infection in young calves.

The early stage of BLV infection was studied in experimentally infected calves to assess the diagnostic applicability of the nested PCR. In addition, the kinetics of infection and virus distribution were evaluated. We have observed early cases when the BLV proviral DNA was detected in various organs (spleen, uterus, liver, kidneys, abomasum and lymph nodes) but not in PBL cells. These observations indicated that an animal may be infected without detectable levels of BLV proviral DNA in PBL and without circulating antibodies, further emphasizing the diagnostic importance of the PCR. This finding emphasizes that the PCR is the most reliable method for the early detection of BLV infection in cattle and a valuable tool for studying the tropism of the virus [12].
2.9.1.2. Detection of equine arteritis virus in semen, organs and swab specimens

Two pairs of primers were selected from the nucleocapsid gene of equine arteritis virus (EAV; Table II). To assess the detection range and clinical applicability of the nested PCR assay, a large number of EAV strains, semen samples and clinical and post-mortem specimens were tested in parallel by PCR assay and by attempted virus isolation in cell culture. The specimens were collected from various countries of Europe and from the United States. The primers recognized a highly conserved region of the virus genome, since all the geographically diverse specimens were amplified in the PCR [17].

2.9.1.3. Detection of 'single reactor pigs' during the Aujeszky's disease eradication programme

With a pseudorabies virus (PrV) gB ELISA, performed on 480 000 pigs on 8900 Swedish farms, approximately 1300 cases were observed with only a single animal reacting positive. These animals were termed SR. To find explanations for this peculiar phenomenon, the presence of PrV was investigated in organs of immunosuppressed and non-immunosuppressed SR animals. The virus was not detected by immunohistochemistry, virus isolation or co-cultivation. An in situ DNA hybridization test detected PrV gC gene sequences in the olfactory bulb of one sow. A nested PCR assay revealed gB, gE and gD gene sequences of PrV in the tissues of trigeminal ganglia, olfactory bulb, tonsils and brain. The nucleotide sequences of the amplicons revealed 98–100% homology with the corresponding sequences of PrV. The large latency transcript (LLT) was not detected in the organs of the SR pigs. Transmission of the SR phenomenon to animals in contact or to the next generation was not observed. Considering the present observations and because (1) PrV vaccination is not applied in Sweden, (2) the SR animals occur not only in southern Scandinavia, but also in northern Scandinavia (which has no history of PrV infection), and (3) viral reactivation was not observed under natural conditions or after experimental immunosuppression, it was concluded that the SR phenomenon can hardly be considered a typical PrV latency. The current findings show that certain herpesviral genomic sequences exist in apparently uninfected individuals [18].

2.9.2. Estimation of the potency of vaccines by using PCR

To estimate the potency of a porcine parvovirus (PPV) vaccine, vaccinated and non-vaccinated gilts were challenged, and the virus distribution was studied in the tissues of their foetuses. Virus detection in the foetuses was attempted by applying assays of haemagglutination (HA) and immunofluorescence (IF), as well as standard
(single) and nested PCR. By testing the presence of virus in organ suspensions of the foetuses from the vaccinated gilts, all detection methods yielded negative results. However, in the tissues of foetuses from non-vaccinated gilts, the virus was detected as follows: HA was positive in 69.5%, IF in 69.5%, standard PCR in 63.1% and the nested PCR in 82.6%. The results indicate that, compared to conventional methods of virus detection, the nested PCR was the most sensitive and reliable assay to detect PPV in fetal tissues, and it provided a novel approach both for the diagnosis of viral infections and for the potency tests of vaccines [19].

3. DIAGNOSTIC KITS BASED ON PCR DETECTION

In 1991–1992, four diagnostic PCR kits were developed in our laboratories for the detection of pseudorabies virus, equine herpesvirus type 1, BLV and pestiviruses. SVANOVA Biotech AB, Uppsala, Sweden, contributed to the design and distributed the kits. According to our knowledge, these are the first commercial PCR kits in veterinary virology. However, the experience of two years' careful distribution of the kits indicated that these systems were distributed too early in 1992. Certain laboratories reported good results but others had no experience with PCR and had technical problems. Considering these conditions and the limited size of the market, the production of kits has been suspended. For the time being, efforts are concentrated not on kit production but on the establishment of an international DNA diagnostic laboratory.

4. INTERNATIONAL DNA DIAGNOSTIC LABORATORY

An international DNA diagnostic laboratory will be opened at our institute. This laboratory will provide PCR diagnostic service for the direct detection of the viruses listed in Tables I and II. If needed, the laboratory will also perform analyses of molecular epizootiology (see below). The laboratory will receive samples from abroad and will also function as an international training centre of PCR diagnosis and molecular epizootiology.

5. PHYLOGENETIC ANALYSIS AND MOLECULAR EPIZOOTIOLOGY

A rapid method, based on direct sequence analysis of PCR products, has been applied for the fast and accurate identification of genetic variants of various viruses, i.e. caliciviruses, paramyxoviruses and pestiviruses (see below). By running PCR or
FIG. 1. Molecular epizootiology for the control of infectious diseases.
FIG. 2a. Phylogenetic tree based on a partial nucleotide sequence of the capsid protein gene of RHDV and EBHSV specimens.
FIG. 2b. Phylogenetic tree based on a partial nucleotide sequence of the capsid protein gene of two distant isolates of RHDV and EBHSV, respectively, and other caliciviruses. FCV: feline calicivirus strain F9 and CFI/68; SMSV: San Miguel sea lion virus serotypes 1 and 4; NV: Norwalk virus; SHV: Southampton virus.

reverse transcription-PCR (RT-PCR), the virus genomes are amplified from the samples of diseased animals or from tissue culture isolates. The nucleotide sequences of the amplified regions are rapidly determined and compared to the data of the computer gene banks. Rapid and precise identification of the virus will be made at the genetic level. These observations allow the construction of phylogenetic trees and the determination of evolutionary relations of various virus variants.

The method is also suitable for the rapid genetic identification of virus variants participating in various outbreaks. The rapid and accurate identification of the virus variants from various outbreaks is termed molecular epizootiology (Fig. 1). The rapid identification of the agents at the genomic level facilitates the immediate application of epizootiological control measures. By these means, the routes of virus spread are effectively traced and cut.

The following paragraphs present several examples of rapid phylogenetic identification and molecular epizootiology.
5.1. Identification and classification of rabbit caliciviruses

A 398 bp fragment of the capsid protein (VP60) gene of 39 clinical isolates of rabbit haemorrhagic disease virus (RHDV) and 17 isolates of European brown hare syndrome virus (EBHSV), collected between 1981 and 1995 from 17 countries, was amplified by RT-PCR and directly sequenced. The alignment of the nucleotide sequences and the subsequently constructed phylogenetic tree clearly separated RHDV from EBHSV (Fig. 2a). No intermediate isolates were found. The present results support the classification of RHDV and EBHSV as two distinct members of the Caliciviridae family. Nevertheless, a comparison with previously determined sequences of other caliciviruses shows that RHDV and EBHSV are more closely related to each other than to any other calicivirus (Fig. 2b) [20].

The large sequence data obtained were used to select highly specific diagnostic primers from a region of the VP60 gene to amplify a fragment of 316 nucleotides from the genome of RHDV and 265 nucleotides of EBHSV, respectively. In sensitivity studies, as few as ten copies of cloned viral genomic fragments were detected in each RT-PCR assay, and no cross-amplification was observed between the two viruses. The diagnostic value of the assays was confirmed on clinical material by testing fresh as well as formalin fixed, paraffin embedded liver and spleen specimens from a large number of geographically and temporally distant outbreaks. Thus, the two PCR assays provide highly specific and sensitive novel means of direct detection of the two caliciviruses. In addition, by detecting the viruses in formalin fixed, paraffin embedded tissues (PET), the RT-PCR assays facilitate retrospective virological and epidemiological studies. For example, the identification of EBHSV in PET specimens collected in the 1970s indicates that this virus appeared in the hare populations long before the first reports on EBHS during the 1980s [21].

5.2. Detection and grouping of bovine respiratory syncytial viruses

The genetic diversity of 20 strains of bovine respiratory syncytial virus (BRSV) was investigated by comparative nucleotide sequence analysis of a 406 bases part of the extracellular domain of the G protein gene, a region which is highly variable among human respiratory syncytial viruses (HRSV). The sequences of 12 BRSV strains, isolated during the last 27 years in Europe, Asia and North America, were determined by direct sequencing of RT-PCR amplicons. The corresponding data of eight further BRSV strains and three strains of HRSV were obtained from the GenBank. The comparative nucleotide sequence analysis revealed 84–100% homology among the BRSV genomes, whereas between BRSV and HRSV a low homology rate of only 34–38% was observed. The findings indicate that, in contrast to HRSV, which is divided into subgroups A and B on the basis of high antigenic and genetic variability, the extracellular domain of the G protein gene of BRSV is
apparently more homogenous. The results of the comparative nucleotide sequence analysis were not in agreement with the findings of previous antigenic studies, which suggested that BRSV, similarly to HRSV, could probably be divided into two subgroups. The moderate genetic diversity of BRSV, however, allowed the construction of a phylogenetic tree, which showed certain geographic clustering of the isolates, a phenomenon that is characteristic for HRSV. In the present studies, three lineages of BRSV were observed; the first lineage held one Japanese and eight American strains, the second involved ten European strains, while American strain A51908 represented the third lineage. From the results one can conclude that the examined region of the genome of BRSV is more homogenous (conservative?) than the corresponding part of the HRSV genome. Simultaneously, the genetic variants of BRSV are rather restricted to certain geographical areas.

5.3. Estimation of the genetic variability of classical swine fever virus

The genetic variability of classical swine fever virus (CSFV) was studied by comparative nucleotide sequence analysis of 76 virus isolates that were collected during a half-century from three continents. Parts of the E2 (gpS5) and the polymerase gene coding regions of the viral genome were amplified by RT-PCR, and DNA fragments of 254 bp and 207 bp, respectively, were sequenced. The comparative sequence analysis of the E2 region revealed two main phylogenetic groups of CSFV, indicating that the virus apparently evolved from two ancestor nodes. Group I (represented by the Brescia strain) consisted of old and recent American and Asian viruses as well as old English isolates from the 1950s. This group was subdivided into three subgroups, termed I.A to I.C. Group II (represented by the Alfort strain) and consisted of relatively recent isolates from Europe, together with the Osaka strain, which was isolated in Japan from a pig of European origin. Based on genetic distances, the group was divided into subgroups II.A and II.B. Malaysian isolates were branched into both groups, indicating multiple origins for contemporaneous outbreaks in that country. All ten vaccine strains tested were branched in group I, implying a common ancestor. The Japanese Kanagawa strain, isolated in 1974, and the British Congenital Tremor strain from 1964 were the most distinct variants of CSFV in our collection. The comparison of the nucleotide sequences of the polymerase coding region of 32 European strains distinguished subgroups II.A and II.B, which were similar to the corresponding subgroups of the E2 phylogenetic tree. Thus, the results revealed that the E2 region and the polymerase coding regions seem to be appropriate for the grouping of CSFV isolates from all over the world, distinguishing two major groups of the virus. The reliability of these regions for phylogenetic analysis is indicated by the similarity of the results obtained from the two separate parts of the CSFV genome [14].
5.4. Identification of a new pestivirus isolate from pigs as an ovine pestivirus

A pig pestivirus isolate, Frijters strain, was characterized using PCR-RFLP (restriction fragment length polymorphism) and rapid sequencing techniques. The PCR amplicons, prepared from the 5'-non-coding (5'-NC) genomic region, were cleaved by restriction endonuclease Ava I and Bgl I and the results indicated that Frijters strain is very probably a border disease virus (BDV). The sequencing and phylogenetic analysis of PCR amplicons prepared from the 5'-NC and from N\textsuperscript{pro} (p20) regions revealed that Frijters strain belongs to Moredun cp and ncp strains, the prototypes of BDV. Thus, it was concluded that the pestivirus strain, which circulates...
in large swine populations of Europe, is a BDV (Fig. 3). Since BDV was believed to represent agents that replicate in sheep, we obtained further evidence that certain pestiviruses are able to cross the host species barrier. Considering that the Frijters strain circulates in large swine populations in Europe, one can state that this is not an accidental breakage of the host species barrier but a common phenomenon. Natural infections of pestiviruses in various animal species are not only a taxonomical question but also a practical problem of great epizootiological importance. For example, the presence of BDV in swine herds might be a confusing factor during the CSFV eradication programmes, which are principally based on stamping out entire infected populations. Improper diagnosis in such cases can mislead the eradication strategy and may result in unnecessary stamping out of large numbers of CSFV-free animals. Considering these facts, there is a need to develop novel, highly specific laboratory diagnostic assays. The method presented here proved to be a practical novel approach for the rapid detection and reliable identification of various pestiviruses in nature [22].

5.5. A method to discriminate vaccine and wild strains of CSFV

A simple approach of viral genome identification was applied to discriminate between vaccine strains and field viruses of CSFV. A region of 284 nucleotides from the 5'-NC region of the viral genome was amplified by RT-PCR and the products were cleaved by restriction endonuclease BbrPI. When testing old English and certain American and Asian strains, as well as 11 vaccine strains from Europe, America and Asia, each PCR product was cleaved by BbrPI. The cleavage yielded two fragments, 195 bp and 89 bp in length, respectively. In contrast, the PCR products of 23 recent European field viruses, isolated in nine countries between 1977 and 1994, were not cleaved by the enzyme. Thus, the BbrPI cleavage of the PCR products provided a novel and simple approach to distinguish vaccine strains from field viruses of CSFV circulating today in Europe.

5.6. Detection and grouping of genetic variants of Newcastle virus

A region of the F gene (between nucleotides 334 and 1683) of Newcastle disease virus (NDV) RNA was amplified by RT-PCR. Restriction endonuclease enzymes HinfI, BstO1 and RsaI were used to cleave the PCR products, and fragment lengths were determined by agarose gel electrophoresis. The presence or absence of 30 cleavage sites was examined in more than 150 NDV strains, and physical maps of fragments were constructed. Six major lineages of NDV isolates were established. In addition, several putative subgroups of epizootiological interest were identified.

This grouping was verified by sequence comparison of a 378 nucleotides long region of the F genes of 24 representative strains.
Lineage I comprises lentogenic strains isolated mainly from waterfowl. ‘Old’ (prior to the 1960s) North American isolates of varying virulence, including lentogenic and mesogenic vaccine strains, belong to lineage II. Lineage III includes early isolates from the Far East. Vaccine strains Hertfordshire and Mukteswar are related to this group. Early European strains (Herts 33 and Italian) of the first panzootic (starting in the late 1920s) and their descendants were grouped in lineage IV. Lineage V includes strains originating from imported psittacines and epizootics of chickens in the early 1970s in Great Britain. Lineage VI comprises strains from the Middle East in the late 1960s, isolates from Hong Kong and China from the 1970s and from Hungary until the early 1980s. Pigeon paramyxovirus-1 strains, which are responsible for the third panzootic, form a distinct subgroup in this lineage.

The present grouping of NDV strains either overlaps antigenic groups based on binding to a panel of monoclonal antibodies [23] or makes union or further subdivision of them. It is concluded that restriction site analysis of F gene PCR amplicons is a relatively fast, simple and reliable method for the differentiation and identification of NDV strains. By this means, viruses involved in new outbreaks are rapidly identified at the genomic level. The rapid identification allows the application of immediate epizootiological measures, which prevents the disease from spreading to large geographical areas. Furthermore, the grouping presented here contributes to a better understanding of the molecular evolution of the NDV-F gene [24].

6. CONCLUDING REMARKS

Summarizing the experiences gained at a routine diagnostic laboratory during the last decade, we can conclude that PCR assays provide powerful novel means for the direct detection of a large variety of viruses. By taking precautions, false positive and false negative results can be eliminated, and the PCR laboratories do not have more technical problems than any other laboratory in virological diagnosis. Compared to conventional methods, the PCR provides novel alternatives of viral diagnosis. In certain cases, virus isolation is ineffective and may require several weeks, e.g. in the cases of latent herpesvirus infections, equine arteritis and certain strains of bovine viral diarrhoea virus (BVDV). Using PCR, a confirmed diagnosis is achieved within 24–48 h. In such cases, the PCR is a powerful alternative to the conventional methods; in other cases, it is considered as a good complementary technique.

The PCR amplification provides rapid means of phylogenetic analysis and contributes to the accurate classification of viruses. The use of the PCR in molecular epizootiology constitutes a novel tool for the rapid and effective application of epizootiological measures in the control of infectious diseases. Considering these points, the PCR methods have high scientific and economic impact in veterinary virology.
REFERENCES


APPLICATION OF THE POLYMERASE CHAIN REACTION FOR DETECTION OF TRYPANOSOMES

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Abstract
APPLICATION OF THE POLYMERASE CHAIN REACTION FOR DETECTION OF TRYPANOSOMES.

Diagnosis is an essential requirement in the management of disease both at the level of the individual when a decision has to be made whether to treat or not and at the epidemiological level for evaluating the performance of disease control strategies. A diagnostic test should be rapid, specific, highly sensitive and amenable to automation. The test should distinguish closely related species of infectious organisms if the disease syndromes they cause require different management approaches. Furthermore, it should be of use in studies that seek to elucidate the dynamics of the interaction of the infectious organism with its hosts, vectors and the environment. Recombinant DNA technology has given rise to DNA based diagnostics that offer new approaches to the development of novel assays for diagnosis of the African trypanosomiases, with the potential to fulfil a majority of these requirements. Highly repetitive DNA sequences that are specific for the different species or subspecies of the African trypanosomes have been identified and cloned. The nucleotide sequences of these fragments of DNA have been determined and oligonucleotide primers designed for highly sensitive detection of these parasites in the blood of mammalian hosts and in the saliva of the tsetse vector through the polymerase chain reaction (PCR). The use of these techniques in the detection of trypanosomes in mammalian hosts and the tsetse vector is presented and discussed in the context of their potential contribution to the epidemiology of diseases caused by trypanosomes.

1. INTRODUCTION

Parasite detection and specific identification are central to the study and eventual understanding of the epidemiology of the diseases they cause. Accurate identification of the parasites is necessary for several reasons, but particularly because of the parasite diversity and variability. African trypanosomes are among the parasites with an incredible potential for variation. The best studied variation in these organisms is antigenic variation involving the variant surface glycoprotein (VSG). In addition to antigenic variation, there are other forms of probably more subtle variations that are, however, relevant to the epidemiology of the diseases caused by the trypanosomes.
These include: mode of transmission, host and vector specificity, virulence and response to therapeutic drugs. In the phase of these variations, it is apparent that identification and characterization of pathogenic organisms, including the parasitic protozoa, will increasingly rely on the use of reagents derived from recombinant DNA [1–3]. Recombinant DNA based reagents are efficient and reliable and can be automated. They can be used to follow an infectious agent over time and space, to investigate disease incidence and transmission dynamics due to the infectious agents and to perform accurate diagnosis in hosts and vectors [4].

2. RECOMBINANT PLASMIDS FOR DETECTION OF TRYPANOSOMES

Over the past few years, we and others have identified several different DNA sequences that appear to be useful in the specific identification of different species of the African trypanosomes infective to man and livestock [5, 6]. These DNA probes, when used in hybridization assays, detect the parasites in both the mammalian host

<table>
<thead>
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<th>Parasite specificity</th>
<th>Recombinant plasmid</th>
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<tr>
<td><em>Trypanozoon</em></td>
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<tr>
<td><em>Trypanozoon</em></td>
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</tr>
<tr>
<td><em>T. evansi</em>, type A</td>
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</tr>
<tr>
<td><em>T. evansi</em>, type B</td>
<td>pKT700</td>
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<td><em>T. congolense</em></td>
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</tr>
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<td><em>T. congolense</em></td>
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</tr>
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<td><em>T. congolense</em></td>
<td>pgNgulia-11</td>
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<td><em>T. simiae</em></td>
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<td><em>T. vivax</em></td>
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</tr>
<tr>
<td><em>T. vivax</em></td>
<td>pgDSIL 800/3</td>
</tr>
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*a* Savannah type *T. congolense*.

*b* Kilifi type *T. congolense*.

*c* Tsavo type *T. congolense*.

*d* West African forest/riverine type *T. congolense*. 
blood and buffy coat samples, and in the vector gut and Proboscidea [7–9]. Additionally, they can distinguish the parasites at species, subspecies, antigenic repertoire and antigenic type levels.

Table I is a list of the recombinant plasmids containing different repetitive DNA sequences used in the identification of different trypanosome species or types. They are all repetitive, being arranged either in tandem arrays or as dispersed repeats in the genome. They hybridize specifically with DNA from the respective trypanosome species. Their capacity to detect trypanosomes present in naturally infected livestock or tsetse flies has been documented [8, 10, 11]. When labelled to high specific activity, the probes can detect the parasites both in the mouth parts and in the guts of a vector. Furthermore, they can detect mixed infections in both the mouth parts and the guts of naturally infected tsetse flies. These probes have ordinarily been used in radioactive format; however, with the availability of systems of non-radioactive labelling and detection of DNA, they have been converted to this format [12].

3. SENSITIVE DETECTION OF TRYPANOSOMES BY PCR

The nucleotide sequence composition has been determined for a majority of the DNA probes. It has therefore been possible to design oligonucleotide primers (Table II) [9, 12, 13] for polymerase chain reaction (PCR; [14]) amplification of DNA to detect low parasite numbers that may be present in the vector or host. Although the PCR can detect an extremely low number of parasites [15, 16], the sensitivity of this detection can be increased by hybridization of the PCR products with a specific probe. An additional advantage of this approach is that when primers specific for different trypanosome species are used in a single reaction tube to perform multiplex PCR, aliquots of the products obtained can be slot blotted and then separately hybridized with the different specific probes, facilitating the processing of numerous test samples. These reagents have made it possible to perform investigations which were impossible to do in the past, leading to a more accurate description of species prevalence [10, 11] in various locations, the discovery of new trypanosome genotypes [17] and the ability to demonstrate presence of trypanosomes in cattle in which such parasites could not be detected by microscopy or xenodiagnosis [12].

4. FUTURE PROSPECTS

Further refinements and improvements of these DNA based diagnostics will have to include conversion of the assays to ELISA format [18] and quantification of the parasites in infected hosts/vectors by quantitative PCR. These refinements will lead to their wider application, for better understanding of parasite population genetic
TABLE II. OLGONUCLEOTIDE PRIMERS FOR SPECIFIC AMPLIFICATION OF DNA FROM DIFFERENT TRYPANOSOMES

<table>
<thead>
<tr>
<th>Primer number or designation</th>
<th>Sequence of the primer</th>
<th>PCR product size</th>
<th>Trypanosome specificity</th>
<th>Reference</th>
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<td>ILO0342 IL0343</td>
<td>GAT CCG CAG CCG GCC CTG\n</td>
<td></td>
<td>CCG CCG TGG CTC CTT CCC</td>
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</tr>
<tr>
<td>ILO0344 IL0345</td>
<td>CGA GCG AGA ACG GCC AC\n</td>
<td></td>
<td>GGG ACA AAC AAA TCC CGC</td>
<td>370 bp</td>
</tr>
<tr>
<td>ILO0892 IL0893</td>
<td>CGA GCA TGC AGG ATG GCC G\n</td>
<td></td>
<td>GTC CTG CCA CCG AGT ATG C</td>
<td>400 bp</td>
</tr>
<tr>
<td>ILO0963 IL0968</td>
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<td></td>
<td>CCC TCG AGA ACG AGC A</td>
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</tr>
<tr>
<td>TCF1 TCF2</td>
<td>GGA CAC GCC AGA AGG TAC TT\n</td>
<td></td>
<td>GTT CTC GCA CCA AAT CCA AC</td>
<td>350 bp</td>
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<tr>
<td>TSM1 TSM2</td>
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<td></td>
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<tr>
<td>TVW1 TVW2</td>
<td>CTG AGT GCT CCA TGT GCC AC\n</td>
<td></td>
<td>CCA CCA GAA CAC CAA CCT GA</td>
<td>150 bp</td>
</tr>
<tr>
<td>ILO1264 ILO1265</td>
<td>CAG CTC GCC GAA GGC CAC TTG\n</td>
<td></td>
<td>GCT GGG\n</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Savannah type T. congolense.
\(^b\) Kilifi type T. congolense.
\(^c\) Tsavo type T. congolense.
\(^d\) West African forest/riverine type T. congolense.

structure and general population biology of the parasite, monitoring control efforts, i.e., tsetse control or livestock treatment campaigns, and conservation wildlife biodiversity [19].

It is envisaged that with the advent of genome analyses efforts DNA markers will be found that are linked to specific parasite phenotypes, such as drug resistance
and virulence. Nucleic acid diagnostics based upon such markers will be most useful in investigating the prevalence and direct consequences of these phenotypes.

As more information is gained regarding ways in which different parasites cause pathology in their respective hosts, it will be possible to devise nucleic acid based diagnostics which can provide an index of pathology or host morbidity resulting directly from the infection by a particular parasite type.

Because of future prospects of DNA diagnostics, many commercial companies have invested in the emerging field of genomics, with a hope of exploiting the information coming from the different genome analyses projects to develop novel diagnostic kits for detection of genetic mutations and infectious organisms. Although at present no kits are produced for the detection of parasitic protozoa, they will be relatively easy to develop once appropriate markers have been identified. The primary task remains that of identifying DNA markers, genes or gene mutations linked to specific phenotypes. Once such markers are at hand, they can be incorporated in novel diagnostics such as:

(a) Oligonucleotide ligation assay (OLA), employed in the detection and typing of polymorphisms arising from small deletions or insertions, often found in diallelic variants [20]. The assay can be effectively applied to the typing of infectious organisms or forensic samples.

(b) Nucleic acid sequence based amplification (NASBA™), which involves alternate steps in synthesis of DNA from RNA template and synthesis of RNA from the DNA template in a homogenous isothermal amplification process [21]. The method is effective in detecting viable organisms and has the advantage of high fidelity and rapidity.

(c) Systematic evolution of ligands by exponential enrichment (SELEX), which exploits combinatorial chemistry to identify an oligonucleotide with a high specificity to a particular molecule [20]. Once an oligonucleotide with the desired binding specificity has been identified, it is used in the enzyme linked oligonucleotide assay (ELONA) to quantify levels of the analyte molecule in the test sera. SELEX derived ligands are precise, accurate and specific.

5. CONCLUSIONS

In general, DNA based diagnostic reagents require no animals and are therefore not subject to animal variation during synthesis; they or detailed information about them are most often available in the public domain databases and from these they can be consistently resynthesized or recloned anywhere in the world. Additionally, the target molecule upon which they are based need not be immunogenic since the detection system will most often be based on invariant segments of
the genome. The reagents are normally stable during storage, and the assays can be automated.

REFERENCES


MOLECULAR TECHNIQUES IN FOOT-AND-MOUTH DISEASE EPIDEMIOLOGY

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Abstract

MOLECULAR TECHNIQUES IN FOOT-AND-MOUTH DISEASE EPIDEMIOLOGY.

The study of the epidemiology of foot-and-mouth disease (FMD) has been revolutionized by the introduction of molecular biological techniques that can establish genetic relationships between the causative viruses. Early biochemical techniques such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electrofocusing and ribonuclease T1 oligonucleotide mapping were used to augment traditional antigenic comparisons to relate different FMD virus isolates and strains. FMD epidemiology using nucleotide sequencing has been studied since 1987, and there is an accumulated database of nearly 1500 partial VP1 sequences representing all seven serotypes of the virus. This has created a unique position for the study of the global epidemiology of the disease. Studies have shown that FMD viruses may be grouped into genetic types that correlate with geographical location. It has been proposed that these geographically distinct genotypes be termed 'topotypes'. For FMD type O, at least six topotypes have been defined, one of which is probably now extinct; for type A, four topotypes have so far been identified; for type C, about six genotypes; and, for Asia 1, only one genotype. Studies on the SAT 1 and SAT 3 serotypes in southern Africa have shown the presence of three distinct topotypes for each serotype. These have probably arisen through the geographical isolation of wild buffalo herds and multiple introductions into domesticated cattle. The situation with the SAT 2 serotype was, however, different; only two genotypes were found, which did not correlate with the geographical origin.

1. INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious, economically devastating disease of cloven hoofed animals. Its host range is extremely wide, being capable of infecting nearly 70 species within 20 families of mammals [1]. Although rarely fatal, FMD can be an economically devastating disease because of serious loss of condition. In cattle, the disease is characterized by fever and vesicular lesions on the mouth, tongue, muzzle, hooves and udder. These lesions lead to salivation and
lameness. Secondary bacterial infections frequently occur, especially on the feet. Myocarditis may occur in young animals, sometimes resulting in death. The clinical picture in pigs is similar; however, sheep and goats are usually less severely affected. In the Indian buffalo, FMD closely follows the pattern of infection in cattle [2]. Although a number of wildlife species are known to acquire FMD infection in Africa [3–5], it is generally considered that the buffalo (Syncerus caffer) is the true free-living maintenance host of FMD on that continent [5]. Young buffalo are usually infected subclinically, generally with more than one serotype of FMD virus, in the first year of life [6–8]. Following infection, virus may persist in the pharynx of most buffalo for up to five years [8, 9]. For at least the first ten months of this period, virus may be transmitted to other susceptible buffalo [5, 7]. Despite repeated attempts to demonstrate transmission of FMD from African buffalo carrying virus to domestic cattle [6, 7, 10], this has only been reported on one occasion [11]. Under experimental conditions, transmission could only be effected during acute disease if contact between the two species was intimate [12]. Impala (Aepyceros melampus) and kudu (Tragelaphus strepsiceros) periodically develop lesions typical of FMD, and occasionally epizootics have been reported among these animals, presumably due to interspecies transmission [13, 14].

FMD has occurred in most areas of the world, except for Greenland, Iceland, Japan, New Zealand and the smaller islands of Oceania. In Australia, FMD last occurred in 1872. The United States of America experienced nine outbreaks of FMD between 1870 and 1929. In Canada, only two outbreaks have been recorded, in 1870 and in 1952. Mexico experienced outbreaks of FMD in 1926 and between 1946 and 1954 (types О and A). Apart from sporadic outbreaks, Europe is now free from FMD; since 1991, when routine FMD vaccination ceased, type О has been reported in Bulgaria (1991, 1993 and 1996), Italy (1993), Greece (1994 and 1996) and Turkish Thrace (1995 and 1996); type A appeared in 1996 in Albania, the Former Yugoslav Republic of Macedonia and Yugoslavia. In all these cases disease spread has been limited and control measures have been successful (Fig. 1).

Some South American countries have instigated rigorous control programmes based on vaccination and movement controls and have successfully eliminated the disease from either large areas (Brazil) or the whole country (Chile, Uruguay and Argentina).

FMD viruses belong to the genus Aphthovirus in the family Picornaviridae and are most closely related to members of the genus Cardiovirus (encephalomyocarditis virus, Theiler's murine encephalomyelitis virus and Vilyuisk human encephalomyelitis virus) and to the unclassified equine rhinovirus types 1 and 2.

Originally FMD virus strain relationships, and supposed epidemiological links, were based on antigenic comparisons; however, it is clear that significant antigenic changes may arise from limited nucleotide variation, and antigenic mimicry may also occur [15]. Therefore, there has been a shift in emphasis to studies that examine the
FIG. 1. Countries in which outbreaks were reported, 1992–1996.
virus genome. These have culminated with rapid reverse transcription–polymerase chain reaction (RT-PCR) and nucleotide sequence analysis.

2. BIOCHEMICAL/MOLECULAR METHODS USED TO DETERMINE VIRUS RELATIONSHIPS

2.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of the structural (and sometimes non-structural) polypeptides of FMD viruses on polyacrylamide gels has been used to determine possible relationships. Two techniques have been employed: SDS-PAGE, which separates proteins partly by their molecular weight and partly by their interaction with SDS [16], and electrofocusing (EF), which separates proteins by their isoelectric potential. Normally the viral proteins would be radiolabelled using $^{35}$S methionine to allow easy detection by autoradiography. A number of studies describing the relationships between FMD viruses have been published using SDS-PAGE [17–21] or EF [22]. However, only a limited amount of information can be gained from these methods.

2.2. RNase T1 oligonucleotide mapping

Direct comparison of the genomes of FMD viruses was first carried out by using T1 mapping [23, 24]. RNA extracted from virus grown in the presence of $^{32}$P-orthophosphate is digested with RNase T1, which is an endoribonuclease that preferentially cleaves 3' to G residues. The resulting oligonucleotides are separated by two dimensional PAGE. This technique has been used to establish genetic relationships between FMD virus strains [20, 25, 26]; however, the method has several drawbacks, including difficulties in comparing T1 maps produced at different times.

2.3. Direct RNA sequencing

Nucleotide sequencing was first applied to studying the epidemiology of FMD in the late 1980s. Beck and Strohmaier [27] used a base specific chemical cleavages method [28, 29] to ascertain the sequence of FMD virus cDNA fragments of the VP1 coding region. Most subsequent studies employed the more convenient and rapid primer extension (dideoxy) sequencing method [30–32]. The latter method uses a specific DNA oligonucleotide or primer to anneal to the RNA genome. This enables the enzyme, reverse transcriptase (RT), to make a complementary DNA strand using deoxynucleotide triphosphates (dNTPs), usually at 42°C. Four identical reactions are carried out, except that in each tube a different dideoxynucleotide (ddNTP) is added.
in a balanced proportion with the corresponding dNTP. As the reactions progress, cDNA is made, but a proportion of the cDNA in, for example, the ddGTP/dGTP tube, incorporates ddGTP instead of dGTP; this stops any further extension on that particular RNA genome. Eventually each tube consists of a number of RNA/cDNA hybrids that end at every occurrence of the base in question. Separation of the cDNA fragments by PAGE reveals a ladder showing the position of every corresponding base in each reaction. Since one of the dNTPs is radiolabelled (e.g. $^{32}\text{P}-\alpha\text{dATP}$ or $^{35}\text{S}-\alpha\text{dATP}$), the fragments can be visualized by autoradiography. Alternatively, the primer may be labelled with $^{32}\text{P}-\gamma\text{ATP}$ using polynucleotide kinase.

2.4. RT-PCR/cycle sequencing

The advent of RT-PCR amplification of RNA made the analysis of FMD virus isolates more rapid [33-35]. The virus RNA is copied into DNA using either specific or random primers plus RT and dNTPs. The RNA/cDNA hybrid is broken apart by heating at 94°C (this also inactivates the RT), a second specific primer anneals (annealing temperatures are dependent on the primers used), and the single stranded DNA (ssDNA) is copied into double stranded DNA (dsDNA) using a thermostable polymerase (e.g. Taq) and dNTPs at 72°C. Upon heating again, the DNA strands part, the two specific primers are able to anneal to each of the ssDNAs, and copying proceeds. This last step can be repeated 25-40 times to produce a large quantity of dsDNA. Cycle sequencing follows the same principle as primer extension (dideoxy) sequencing except that a thermostable polymerase is used. This enables unidirectional amplification of the ddNTP terminated fragments, thus increasing the signal.

3. FOOT-AND-MOUTH DISEASE MOLECULAR EPIDEMIOLOGY

3.1. Application of nucleotide sequencing to FMD epidemiology

Nucleotide sequencing was first used in the study of the epidemiology of FMD by Beck and Strohmaier [27], who investigated the origin of outbreaks of types O and A in Europe over a 20 year period. They concluded that the majority of these outbreaks had been caused by improperly inactivated vaccines or by virus escape from laboratories. Since that time, a number of other studies have used this technique for similar purposes; serotypes O [35-46], A [41, 47-51], C [52-56], Asia 1 [57, 58], SAT 1 [59, 60], SAT 2 [60, 61] and SAT 3 [60] have all been studied.
TABLE 1. NUMBER OF PARTIAL OR COMPLETE VP1 SEQUENCES IN THE WRLFMD DATABASE

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Number of sequences</th>
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<tbody>
<tr>
<td>O</td>
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</tr>
<tr>
<td>A</td>
<td>237</td>
</tr>
<tr>
<td>C</td>
<td>91</td>
</tr>
<tr>
<td>Asia 1</td>
<td>159</td>
</tr>
<tr>
<td>SAT 1</td>
<td>129</td>
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<tr>
<td>SAT 2</td>
<td>143</td>
</tr>
<tr>
<td>SAT 3</td>
<td>88</td>
</tr>
<tr>
<td>Total</td>
<td>1432</td>
</tr>
</tbody>
</table>

3.2. Definition of topotypes

In the World Reference Laboratory for FMD (WRLFMD), we have accumulated a database of nearly 1500 partial or complete FMD virus VP1 sequences (Table I). This database includes sequences which we have determined and those from published sources; also included are unpublished sequences given to us by a number of laboratories. Our analysis has shown that viruses belonging to each serotype cluster together phylogenetically (Fig. 2). Comparison of the sequences within each serotype has also allowed us to phylogenetically cluster related viruses into groups or genotypes, for example, in serotype О (Fig. 3). These genotypes appear to be geographically restricted, and thus we refer to them as topotypes. For the Eurasian serotypes (О, А, С and Asia 1), the level of nucleotide difference defining each topotype is 15%; however, for the South African Territories (SAT) serotypes, the level is 20% (a greater degree of variation is seen in these viruses). The former level (15%) has also been used to define poliovirus genotypes [62] using the same area of the genome. Our studies suggest that viruses from one topotype may spread to other geographical regions, but do not normally become established if the same serotype is already present. Thus the geographical restriction of the topotype can be maintained.

3.3. Serotype О

Most of our sequence studies have concentrated on FMD virus О since it is the most common and widespread serotype. So far six type О topotypes have been discovered: (1) Europe/South America; (2) South Asia; (3) South-East Asia;
FIG. 2. Genetic relationships between the seven serotypes of FMD virus.
FIG. 3. Definition of FMD virus type O genotypes (topotypes).
FIG. 4. Geographic distribution of FMD virus type O genotypes (topotypes).
(4) China/Hong Kong; (5) East Africa; and (6) Indonesia. Figure 4 shows the distribution of these six topotypes and the recent spread of the China/Hong Kong topotype to the Philippines in 1994, to the Russian Federation in 1995 and to Taiwan in 1997.

3.4. Serotype A

For FMD virus A we have found five topotypes: (1) Europe/South America; (2) South Asia; (3) Turkey; (4) Cameroon; and (5) Zambia. However, these studies are far from complete and more may be discovered. The recent outbreaks of FMD type A in the Baltic States are of the South Asia topotype and probably originated from the Indian subcontinent (Fig. 5).

3.5. Serotype C

FMD virus type C has a markedly restricted distribution compared with that of the other Eurasian serotypes. A number of topotypes have historically existed, including: (1) Europe/South America; (2) East Africa; (3) Angola; and (4) India. The introduction of C3/Resende into the Philippines in 1976 has led to a rapid evolution of the virus and the formation of a new topotype. Many of the type C outbreaks occurring sporadically in the Middle East were introduced from other geographical regions and did not persist. In recent years FMD virus type C has only been present in parts of South America, India, Bhutan and Nepal, Kenya and the Philippines. Some of these outbreaks may be due to the reintroduction of the virus into the field via improperly inactivated vaccines.

3.6. Serotype Asia 1

FMD virus Asia 1 is normally only found in Southern Asia from the Middle East to Viet Nam. The molecular epidemiology of the Asia 1 serotype has been well studied [57, 62] (see Fig. 6). From these studies we concluded that, by the 15% nucleotide difference criterion, all Asia 1 viruses fell into a single topotype. However, it was noticed that a group of genetically distinct viruses from South-East Asia was rarely (only one case in India) seen outside this region. Representative isolates of the other group were distributed throughout Southern and South-East Asia. Thus it may be worthwhile to regard Asia 1 viruses as belonging to two topotypes: South Asian and South-East Asian. In FMD virus type O the South Asian topotype (ranging from North Africa to India) is distinct from, but most closely related to, the South-East Asian topotype.
FIG. 5. Dendrogram depicting the genetic relationships between FMD type A viruses.
FIG. 6. Dendrogram depicting the genetic relationships between FMD type Asia 1 viruses.
4. CONCLUSIONS

Nucleotide sequencing is a powerful technique for the study of FMD epidemiology. Despite having a considerable database of FMD virus sequences (nearly 1500), much more work will be necessary to understand the complex spread and evolution of new virus strains.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the many staff and visitors involved in these sequencing studies at Pirbright, including D. Ansell, K. Marchant, B. Armstrong, Lin Fengsheng, E. Woodbury, V. Drygin, A. Scherbakov, A. Sambula and W. Linchongsubonkoch.

REFERENCES


Abstract

MOLECULAR APPROACH TO UNDERSTANDING AND CONTROLLING MORBILLIVIRUS INFECTIONS.

Rapid and accurate diagnosis is the most important factor which will determine the success of the current efforts to achieve global eradication of rinderpest. Improved diagnostic capabilities have great relevance for this programme, and molecular biology has provided many valuable new techniques which have been adapted for use in diagnostic and epidemiological studies on viruses. Nucleic acid probe hybridization and monoclonal antibody based ELISAs, which are more sensitive and more specific than classic diagnostic techniques such as agar gel immunodiffusion (AGID) and complement fixation, have proved their usefulness in recent years. However, the most significant advance has been the introduction of the polymerase chain reaction for the detection and identification of disease causing pathogens of medical and veterinary importance. This technique has revolutionized understanding of the epidemiology of rinderpest and other morbilliviruses. Molecular biology has also produced a new generation of recombinant vaccines which will be of use in controlling rinderpest and other morbillivirus diseases, and the molecular basis of morbillivirus pathogenicity is being studied using newly perfected techniques to obtain live virus from DNA copies of their genomes.

1. INTRODUCTION

Diseases caused by morbillivirus infections are of major importance in both human and veterinary medicine. The plagues of rinderpest which swept across Europe during the 18th and 19th centuries [1] and the great African pandemic, following its introduction into that continent at the end of the last century [2], had major economic and social consequences and resulted in the establishment of effective veterinary services to control animal diseases. However, rinderpest virus continues to cause major losses in cattle, buffalo and wildlife species in parts of Africa and Asia. Peste des petits ruminants virus has caused devastating losses of sheep and goats across the Asian continent from Israel to Bangladesh over the past four years. Canine distemper, although largely controlled by vaccination in domestic dogs, is responsible for epizootics in wild carnivores and continues to pose a threat to
some endangered species such as the giant panda. Measles virus remains one of the most significant causes of death in young children in the less developed parts of the world. In the past decade two new species of morbillivirus have been identified which were responsible for severe epizootic diseases in marine mammals [3]. These epizootics highlight the fact that 'new viruses' are constantly emerging, and molecular techniques have played a crucial role in the rapid identification and characterization of these pathogens. The other significant development resulting from molecular biology has been the production of recombinant rinderpest vaccines, although these have yet to be exploited in the battle to control rinderpest. The most recent scientific advance has been the ability to rescue live virus from DNA copies of morbillivirus genomes [4, 5] and this technology will enable us to identify the molecular determinants of pathogenicity and to produce genetically defined and marked vaccines.

2. MOLECULAR DIAGNOSTIC TECHNIQUES

The key to the successful eradication of any disease is the ability to identify the causative agent rapidly and with a very high degree of certainty, thus enabling the source of infection to be contained and dealt with in an appropriate manner. The process of rapid diagnosis began a quarter of a century ago with the development of the ELISA [6]. Later the development of monoclonal antibodies and their use in the ELISA greatly improved our ability to identify specific antigens and antibodies in clinical specimens [7, 8]. Modern molecular techniques are highly specific and are more sensitive than immunological techniques with the added advantage that they can be used to characterize pathogens at the genetic level. The most powerful diagnostic tool now available is the polymerase chain reaction (PCR). This was first described by Saiki and colleagues in 1985 [9] and there is now a very long list of pathogenic organisms for which a specific PCR diagnostic test is available [10]. The principle of the PCR is the repeated copying of a chosen region of DNA using specific forward and reverse primers. With the discovery of thermostable DNA polymerases derived from thermophilic bacteria [11], this repetitive copying of the DNA could be done in a single tube by repeatedly heating the DNA to high temperature (94°C) to dissociate the DNA duplex, cooling to allow annealing of the primers (37–60°C, depending on the primers used) and finally heating to the optimum temperature (72°C) for the polymerase to copy new DNA. The cycles are repeated 25–35 times (25 cycles theoretically increases the concentration of starting DNA 10^7 times) to produce a DNA product which can be visualised by ethidium bromide staining on an agarose gel. The size of the amplified DNA product is exactly defined by the location of the two primers on the target DNA, usually separated by 200–400 nucleotides for diagnostic purposes.
The PCR technique can only be used to amplify DNA. Since the genome of all morbilliviruses consists of a single strand of RNA, their genomes cannot be amplified directly by PCR but must first be copied into DNA using reverse transcriptase in a two step reaction known as reverse transcription/polymerase chain reaction (RT-PCR). RT-PCR has been shown to be useful for the rapid detection of rinderpest specific RNA in diagnostic samples and can be used for differentiating rinderpest from other morbillivirus infections, particularly peste des petits ruminants [12]. It is best to use both 'specific' and 'universal' primer sets for the detection of morbillivirus genomes since some host species are susceptible to infection with more than one morbillivirus, e.g. cattle, sheep and goats can be infected with either rinderpest or PPR and seals (pinnipeds) can be infected with either CDV or phocid distemper virus. This should also enable the detection of any unknown morbilliviruses which may exist. For this second category of primers, sequences from a gene which is highly conserved across the genus should be chosen. However, owing to the redundancy of the genetic code such sequences are not easy to find. The phosphoprotein (P) gene has some short, highly conserved regions where overlapping reading frames are used to encode the non-structural proteins, and these sequences are well conserved across the genus and can be used to produce a 'universal' primer set. Such a primer set enabled amplification of nucleic acid sequences from the newly discovered infections in dolphins in the Mediterranean in 1990 and enabled the virus to be characterized as a new virus long before conventional cloning methods could produce results [13]. Because of the great potential of RNA genomes to vary it is essential to have several alternative sets of primers for detection of RNA virus genomes, and we have also developed a second 'universal' primer set based on sequences in the highly conserved central region of the N protein gene of the morbilliviruses. Since these two universal primer sets detect different parts of the morbillivirus genome they can be used in one reaction tube, and this saves both time and valuable reagents. Similarly, a set of primers specific for a conserved, constitutively expressed cellular gene such as β-actin can be included to control for RNA quality (see Fig. 1). Poor preservation of clinical specimens leading to degradation of the RNA could result in a false negative, however; since the virus RNA is protected to some extent from RNase degradation by the nucleocapsid protein it is more stable than the naked actin mRNA, and some poorly preserved specimens which are negative with actin primers can prove positive for virus specific RNA (see Fig. 2). Samples which are negative with both actin specific and virus specific primers should be regarded as uninterpretable.

The size of the DNA product is determined by the distance by which the primer sequences are separated on the primary sequence of the genome being analysed. The position of the correct sized product can be determined by reference to the positive controls in the reactions and commercially produced DNA marker ladders. Non-specific DNA products can sometimes be produced and care is needed in the interpretation of results. Usually these non-specific products are of the wrong size and
FIG. 1. Use of the morbillivirus 'universal' primer sets, derived from conserved sequences in the phosphoprotein and nucleocapsid protein genes, to amplify cDNA derived from the RNA of various morbilliviruses. M: DNA marker (100 base pair product) ladder. 1: RPV cDNA amplified with N (234 base pair product) and P (429 base pair product) universal primers. 2: RPV cDNA amplified with RPV specific primers (452 base pair product). 3: cDNA used in tracks 1 and 2 amplified with actin specific primers. In this case the actin specific product (275 base pairs) was not produced. 4: PPRV cDNA amplified with N (234 base pair product) and P (429 base pair product) universal primers. 5: PPRV cDNA amplified with PPRV specific primers (452 base pair product). 6: cDNA used in tracks 4 and 5 amplified with actin specific primers. In this case the actin-specific product (275 base pair product) was produced. 7: Positive control (measles virus) cDNA amplified with the N and P universal primer sets. 8: Negative control (water replacing cDNA).

are more often found in negative samples or in samples where there is a very low concentration of the target nucleic acid. The specificity of the DNA product, particularly if it is a weak signal, should always be checked by some independent method. For this it is possible to use nested primer sets, restriction enzyme digestion of the product or nucleic acid hybridization. However, regular use of reamplification with nested primer sets to confirm product specificity increases the risk of sample contamination. The high potential variability of RNA genomes means that restriction enzyme sites may be altered and so this also is not an ideal method for confirming product specificity in the case of morbillivirus RT-PCR products. Hybridization analysis using
FIG. 2. Relative degradation of virus specific and β-actin specific RNA on incubation at 4°C or 30°C in ‘Trizol’ solution. Tissue pieces (T: tonsil; S: spleen; L: lymph node) were stored in Trizol denaturing solution for various times before the RNA was extracted. RNA was stable at 4°C for up to 10 days in all tissues (top panel) while only RNA in tonsils and lymph node was stable for the same period at 30°C (bottom panel). The positions of the virus specific and actin specific amplified DNA products are shown as DNAs A and B, respectively.
labelled oligonucleotide probes has proved the most convenient method for confirming product specificity. We have developed a very rapid procedure based on specific digoxygenin (DIG) labelled internal primers and a commercially available anti-DIG antibody to confirm the specificity of the amplified DNA products (Fig. 3). Because of the sensitivity of the technique great care should be taken to avoid cross-contamination of samples with previously amplified DNA products, and guidelines for good laboratory practice are well documented to ensure this problem can be avoided.

3. USE OF MOLECULAR TECHNIQUES FOR EPIDEMIOLOGICAL STUDIES

The great advantage that RT-PCR has over DNA probe analysis is that the resulting DNA product can be sequenced, either directly or after cloning in a suitable vector. From this data the virus can be analysed at the genetic level and the phylogenetic relationship of the new virus to other virus isolates can be determined using computer programs. Previously, this type of analysis was impractical on morbilli-viruses since it is difficult to isolate and grow field viruses in tissue culture and to

![Diagram](attachment:fig3.png)

FIG. 3. Use of digoxygenin (DIG) labelled primers to confirm the specificity of the PCR amplified DNA products. RNA from rinderpest and PPR viruses of different lineages were copied into DNA and amplified with specific F gene primers and the product DNAs probed using rinderpest and PPR specific internal primers labelled with DIG.
obtain sufficiently large amounts of pure virus RNA either for fingerprinting or direct sequence analysis.

Initial studies showed that rinderpest virus isolates could be separated into lineages which coincided with the continent from which the viruses originated [14, 15]. When historic strains were added to the database a total of five lineages could be distinguished but only two were represented in samples collected after 1983, a distinct African and a distinct Asian lineage [12, 16]. The recent outbreaks of rinderpest in wildlife (buffalo, eland and kudu) in the game parks in Kenya were unusual in that they first appeared in wildlife. The same virus was found in all three wildlife species and it fell into a different lineage from African viruses isolated during the preceding ten years, being phylogenetically most closely related to an older giraffe rinderpest isolate (RGK/1) from the same part of Kenya from 1962. Rinderpest virus strains currently in circulation can therefore be divided into three distinct genotypes, an Asian lineage and two distinct African lineages (Fig. 4) [17].

FIG. 4. Phylogenetic analysis of Asian and African strains of rinderpest virus based on sequence data derived from the fusion protein gene. The unrooted tree was derived using the Phylip Dnadist and Kitsch programmes [17].
Similar studies have been carried out on PPR virus isolates, and here we have identified four distinct lineages [18]. Only three of these virus lineages have been detected since the beginning of the 1990s and the fourth may have disappeared or evolved to produce one of the other three lineages. It is clear from these studies that the outbreaks of PPR which have occurred across Asia from Israel to Bangladesh in the past four years have all been caused by one virus. In contrast, a virus isolate from Ethiopia in 1996 was different and phylogenetically most closely related to a 1972 virus from Sudan (see Fig. 5).

In the case of recent fatal CDV infections of lions and other big cats in the USA and in Africa, it has been shown that the most likely source of infection for these cats is contact with local carnivores infected with CDV. There does not appear to be a specially adapted feline strain of CDV [19, 20] which could explain this unusual mortality in large cats infected with CDV. Serological and molecular analyses have confirmed that CDV continues to affect seals in Lake Baikal [21] and there is increasing serological evidence of widespread morbillivirus infection in cetaceans [22].

**FIG. 5.** Phylogenetic analysis of Asian and African strains of peste des petits ruminants virus using sequence data derived from the fusion protein gene. Analysis was carried out as described in Fig. 4.
The control of infectious disease is made easier if there is an effective vaccine available. Although the currently used tissue culture attenuated rinderpest vaccine is very effective in protecting animals from rinderpest infection it has some disadvantages which could be overcome using either recombinant vaccines or DNA based vaccines. These disadvantages are thermolability and the inability to distinguish vaccinated from naturally infected animals. The most commonly used vectors for the production of recombinant vaccines are poxviruses.

The most successful vaccine of this type is the vaccinia/rabies recombinant vaccine which is now widely used in Europe and the USA to control this disease [23]. In the case of rinderpest virus, both vaccinia and capripox recombinant vaccines have been produced which effectively protect animals against challenge with virulent virus in short term immunity trials. One long term immunity trial has been carried out using the vaccinia recombinant developed by Yamanouchi and colleagues which proved that it was effective for at least one year after a single shot vaccination [24]. Longer term trials are now in progress, and it is anticipated that the duration of immunity will at least be as long as that which vaccinia affords against smallpox. In addition it was shown that pre-exposure of the animals to wild type vaccinia virus did not abrogate its ability to generate an anti-rinderpest immunity [25], proving that cross-reacting poxvirus infections in the target species would not interfere with the vaccine take. The issue of human safety while handling these recombinant viruses has also been considered since vaccinia has the potential to cause human disease. The vaccinia vector used to produce the rinderpest recombinant vaccines is one of the safest vaccines and it was extensively used in the smallpox eradication campaigns in the 1970s. Extensive safety and efficacy tests have been carried out on this vaccine and it was shown to conform to all the safety requirements stipulated by the OIE expert committee on rinderpest recombinant vaccines [26].

The other vector, capripox virus, which has been used to produce rinderpest recombinant vaccines, has two major advantages over vaccinia when it comes to developing novel veterinary vaccines. Firstly, it is not a human pathogen and safety problems do not arise. Secondly, it is already used as a veterinary vaccine to protect against sheep and goat pox infections and so the recombinant will act as a dual vaccine. Trials on the vaccine at the Pirbright Laboratory and at the Kenya Agriculture Research Institute at Muguga have shown that the capripox–rinderpest recombinant vaccine will protect cattle against lumpy skin disease as well as rinderpest, thus making it an effective dual vaccine [27–29]. It has also been shown to cross-protect sheep and goats from PPR virus infection [30]. One drawback, however, is the reduced protection given when animals are prevaccinated with the wild type virus, indicating an interference with vaccine take by pre-existing antibodies [29]. This problem may be due to the use of a late rather than an early promoter to drive the
rinderpest genes and new recombinants have been produced which are driven by an early/late promoter and which are currently being tested in cattle.

5. FUTURE PROSPECTS

The most significant advance in the past two years has been the development of methods to rescue negative strand viruses from DNA copies of their genomes [31]. This has great potential for future improvements in vaccine design to combat diseases caused by these viruses. For example, marker epitopes could be introduced which would enable vaccinated animals to be readily distinguished serologically from naturally infected ones. We have already introduced a small open reading frame into the rinderpest genome and shown that the virus remains viable and expresses the foreign gene (Baron, unpublished results). Another role for this technology will be the production of reliably attenuated vaccines for use in wildlife species, possibly by deletion of one or both of the non-structural protein genes. This is needed in the case of CDV where the current vaccine is known to be virulent in some of the threatened wildlife species (e.g. large cats and the lesser panda) and less effective subunit vaccine must be used to protect them from infection. Similarly, no effective vaccine exists to protect pinnipeds from phocid distemper virus or cetacean species from the dolphin morbillivirus. Dual or multivalent vaccines could also be produced by incorporating other reading frames into the genome copy. The potential to confer the lifelong immunity generated by vaccination with rinderpest on the foreign immunogen could prove useful if, as in the case of foot-and-mouth disease, only short term immunity is conferred using the currently available vaccines.

DNA based vaccines also hold great potential benefits in terms of generating an effective cell mediated immune response on vaccination and in terms of vaccine purity and stability. However, their safety and potential to protect large animals have yet to be established.

6. CONCLUSIONS

The development of improved diagnostic tests is important for control programmes which are aimed at global eradication of rinderpest and other important veterinary diseases, since rapid and accurate detection of the pathogen is the most important factor which will determine their success. Molecular techniques are faster and easier to perform than the classic method of agent isolation followed by neutralization tests and animal inoculation studies. In addition they can be used to characterize viruses down to the nucleotide sequence level. This results in data which
are very useful for epidemiological studies and enable accurate tracing of virus outbreaks to be carried out.

Biotechnology has also produced a new generation of recombinant rinderpest vaccines but these have yet to be used in field situations, despite the proven success of similar recombinant vaccines in controlling rabies in Europe and in the USA [23]. These new vaccines have great potential usefulness for the control and eventual eradication of rinderpest since they are more stable and can enable vaccinated animals to be distinguished from naturally infected ones. The capripox–rinderpest virus recombinants have the added benefit of being able to protect animals against two important veterinary diseases.

The future holds the exciting prospect of finally being able to use virus rescue systems to understand the molecular basis of morbillivirus pathogenicity. This research should explain why some strains of the virus cause very mild clinical disease while others manifest all the classical signs of cattle plague with the consequent high mortality in affected herds. We will then be able to use that knowledge to design safer and better vaccines.

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REFERENCES


TICKS/VACCINES/EPIDEMIOLOGY
(Session 3)

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TICKBORNE DISEASES CONTROL

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Abstract

TICKBORNE DISEASES CONTROL.

Control of tickborne diseases overlaps with control of the tick vectors. It has often relied to a considerable extent on the use of chemical acaricides and curative drugs. The accent has shifted in recent years to a flexible approach, integrating various control measures. Reasons for this are acaricide resistance, economic factors and public health concerns. Cost effectiveness and sustainability in all respects are of primary importance. Control of tickborne diseases may integrate chemical and biological tick control methods, drug treatment of tickborne diseases, acquired and innate host resistance to tickborne diseases and to ticks, as well as ecological, sanitary and regulatory procedures.

1. INTRODUCTION

Ticks and tickborne diseases (TBDs) count among the most formidable obstacles to the great increase in animal production needed to feed the burgeoning human population of the developing world. Their economic impact is greatest in ruminants, particularly cattle and small ruminants, to a lesser extent in the domestic buffalo, while little is as yet conclusively known on the importance of ticks and TBD for camels. Their importance in a given situation depends on a number of factors, of which a major one is the type of livestock, particularly its degree of genetically determined susceptibility and its monetary value.

Ticks not only transmit numerous important protozoan, rickettsial and viral diseases, but also inflict great economic damage in other ways. They reduce weight gain and milk production, create abscesses and predispose for screwworm, decrease the value of hides and skins, and adult African Amblyomma ticks are associated with severe dermatophilosis. Some ticks inject toxins which can cause paralysis or other conditions such as sweating sickness. TBD control cannot really be considered in separation from that of the tick vectors. Reviews (see, e.g., Refs [1, 2]) give details of TBD and other tick associated damage and include various figures that have been advanced concerning their economic importance.

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To a large extent, control of ticks and TBDs is still based on the use of chemical acaricides. Intensive chemical tick control will only in exceptional circumstances achieve tick eradication and eradication of the diseases associated with the tick in question. Chemical tick control is normally unending, and when applied intensively is often used to prevent transmission of tickborne diseases, without aiming at their eradication.

Immunization against some of the TBDs, in particular of cattle, has been practised since the beginning of this century, especially where tick control is not sufficiently intensive. Fully virulent organisms, often with the help of specific curative drugs ('infection and treatment' method), were initially used, but at present attenuated vaccines are available for some of the TBDs, while various groups of scientists attempt to develop inactivated and recombinant vaccines.

Curative drugs are used to treat individual clinical disease cases, but in many circumstances should only be considered as a last resort when prevention has failed.

In recent years, there has been a tendency to try to rely less on acaricides and drugs, for various reasons which are set forth in the following sections.

1.1. Acaricide resistance

Resistance to acaricides is believed to develop by the selection of mutants occurring spontaneously in tick populations. Mutants which confer resistance against all present and future antiparasitic compounds may well be naturally present. Parasitic arthropods have been confronted with natural pesticides for millions of years, especially those produced by plants, and could not have survived without mutations.

When chemical tick control is intensive, resistance develops sooner or later against the acaricide used as well as against related molecules, and many important tick species of domestic animals have developed resistance to one or several groups of chemicals. Certain strains of the tick *Boophilus microplus* in Australia are at present resistant to all acaricides available on the market. The situation in many Latin American countries is similar. Some fairly recent reviews are, for example, Refs [3, 4] and several papers in the proceedings of a recent seminar [5].

A degree of resistance to drugs used against TBDs has been reported occasionally (e.g. [6, 7]), but may not yet be a major problem.

In the past the pharmaceutical industry used to come up with new acaricides to replace those against which resistance had developed. However, at present, the development of new acaricides and drugs is almost at a standstill, while several compounds have even been taken off the market. The reasons are of an economic nature.

1.2. Financial cost

Private industry has to live by the cost–benefit principle. Strict regulations, in particular on chronic toxicity and residues, make the registration of new acaricides
and drugs ever more difficult and costly, and each new compound is more expensive than previous ones. At the same time, the market for acaricides and drugs against TBDs of livestock, particularly in developing countries but even on a worldwide scale, is too limited in purchasing power for the industry to warrant the huge financial investments needed for the development of new compounds. (However, there may be the possibility of a spin-off from new developments in acaricides and drugs against ticks and TBDs of pet animals, a more lucrative market in richer countries.)

In many developing countries not only the cost, but also the scarcity of foreign exchange is a major factor. Acaricides and drugs are mainly manufactured in the industrialized world, and the foreign exchange that governments are prepared to use for their importation is often very limited.

Recovery, by the pharmaceutical industry, of the enormous sums needed for the development of new acaricides and drugs against TBDs thus becomes more and more problematic. Hence, the industry is unwilling to invest in research on new chemicals. Another major reason is that the development of resistance against acaricides appears to be inevitable, so that the life expectancy of a new compound is likely to be limited. We may have to rely on what is available at present and try to find the best strategy for delaying and managing acaricide resistance.

The discovery, towards the end of the seventies, that certain antimalarial compounds could be used for the treatment of the major theilerioses (by *Theileria parva* and *T. annulata*) created great excitement and led to the development of parvaquone and its successor buparvaquone, and of halofuginone lactate. Unfortunately, the cost of parvaquone and buparvaquone is high, certainly too high for the African market, and in spite of the high price it appears that the company concerned may not be able to recoup its investment in a limited market. The price of halofuginone lactate might possibly have been reasonable, as halofuginone was already on the market against coccidiosis, but problems associated with its toxicity and application appear to have prevented market penetration.

The use of babesicidal drugs is also limited, even in ‘developed’ countries. Since imidocarb dipropionate came on the market over 20 years ago, no new drugs against animal babesiosis have been developed, and some compounds have been taken off the market for economic reasons, some completely, others in certain countries.

New drugs are also unlikely to be developed specifically against the tickborne rickettsioses of livestock, but these benefit from the efficacy of some of the large spectrum antibiotics developed and used against many other bacterial and rickettsial diseases.

1.3. Environmental and toxicological aspects

The use of acaricides on animals entails a certain degree of pollution, especially when they are applied regularly and frequently. A major problem is how to dispose of
discarded dip or spray wash. Long term pollution of old dip sites by arsenic and chlorinated hydrocarbons is a problem, for example, in the USA and Australia. Most acaricides are also harmful to aquatic life, even in very low concentrations.

Endectocides such as ivermectin are known to affect the arthropod fauna in cow pats and to kill dung beetles, so that the dung is not quickly recycled. The problem may not be a major one if these drugs are used only occasionally as anthelmintics. Australia, where specific beetles for ruminant dung were originally absent and are imported, is particularly concerned.

Residues from acaricides in animal products (meat, milk, wool, etc.) constitute a universal problem of major public health importance; the worst compounds in this respect are found in the group of the organochlorines. Residue problems are also associated with some of the drugs used in the treatment of TBDs and tick associated diseases, such as dermatophilosis. For example, imidocarb diphosphate, one of the few remaining effective drugs against babesiosis and also applied in the treatment of anaplasmosis, should not be used on lactating animals and animals destined for human consumption, because of residue problems. Long acting formulations of tetracyclines, used in the treatment of rickettsial diseases such as anaplasmosis, heartwater and ehrlichiosis, as well as dermatophilosis, create residues, especially at the site of injection.

1.4. 

**Vulnerability of animal populations under intensive and regular antiparasitic treatment**

Cattle treated regularly and effectively since birth against ticks remain susceptible to all the local tickborne diseases. When acaricidal control is suspended for one reason or another (acaricide resistance, interruption in the supply of acaricide, breakdown of the control facility, civil war, etc.), very great losses may occur. The situation in Zimbabwe during the civil war is a classical example [8]. A major problem also occurs when animals from farms with intensive tick control are sold to owners who do not practise such control.

2. 

**CONTROL OF TICKS AND TBDs**

It is obvious that the cost of control should be less than the financial benefit that the owner will derive from it. The more valuable the animals and their products are, the more can be spent on control. Intensive tick control in indigenous cattle is generally not cost effective, because of their innate tolerance to TBD and tick infestation, and their relatively low monetary value. Exceptional situations are those where control is intended to prevent the introduction or spread of a particular disease or tick, to forestall important potential losses. The campaign for the eradication of *Amblyomma variegatum* in the Caribbean is an example: tremendous potential losses will be prevented in non-infested
islands and particularly in continental Central, South and even North America, if the campaign is a success. This African tick, which transmits heartwater and is associated with severe bovine dermatophilosis, has been introduced accidentally to the islands of the Caribbean, from where it constitutes a constant menace for the American continent.

Sustainability of control, which can be financial or ecological, is another basic concern. Because of financial and foreign exchange problems, but also because of other factors such as resistance and pollution, intensive acaricidal tick control and drug treatment are not sustainable in many circumstances.

For the various reasons mentioned, the emphasis is at present less on the use of acaricides and drugs and more on a multidisciplinary approach (see, e.g., Ref. [9]). There is a tendency towards integrated and sustainable control of ticks and TBDs, making optimal use of all available methods in a flexible way, adapted to local circumstances and keeping in mind economic and practical realities. Integrated control may include various components, such as the use of innate tolerance, of chemical, immunological, biological and ecological methods, while sanitary measures and surveillance may be very useful in certain conditions. The approach is not fundamentally new; there are numerous long standing examples of the control of ticks and TBDs by measures other than the use of acaricides and drugs.

A list of available and potential components of integrated control of ticks and TBDs follows. The literature is not intended to be exhaustive.

2.1. Chemical tick control

Acaricides are used intensively to eliminate ticks or to prevent disease transmission. The necessary frequency depends on the type of tick (multihost ticks spend less time on the host so that treatment has to be more frequent than that against one host ticks) as well as on the duration of the residual effect of the compound on the animal. In other cases, where there is a state of endemic stability for TBDs and the animals are of low monetary value, acaricides may be used strategically, only to keep ticks below the nuisance threshold. The cost–benefit principle always applies.

Apart from the classical use of acaricides in dips or sprays, other methods of applying acaricides offer advantages in certain conditions:

Application of acaricides by 'pour-on' offers the advantage that one can do away with specific tick control installations and their maintenance, such as spray races and dips. It is also more practical for small herds. One disadvantage is that the formulations offered for this purpose are more expensive. It has also been suggested, on theoretical grounds, that the method might select more quickly for resistance, especially when acaricides with a long residual activity are used.

Ticks in and on the ears can be controlled by plastic ear tags impregnated with a suitable acaricide. This procedure should be of considerable help in reducing the numbers of adults of the ‘brown ear ticks’ (*Rhipicephalus appendiculatus*) and other
ticks with preference for feeding on or in the ears. However, ear wax might offer a degree of protection to tick stages that feed deep inside the ears, such as larvae and nymphs of the spinose ear tick (Otobius megnini) and of Rhipicephalus evertsi.

Acaricides have been applied to pastures, but this would appear to be more wasteful, more polluting and less efficient than treating the host, which acts as a natural collector of ticks.

The host animal can be turned into a live trap for certain ticks by using natural or synthetic pheromones. A tick decoy on the tail of cattle, containing specific pheromones and an acaricide, has been tested with promising results against African Amblyomma ticks [10].

Flexibility and adaptation to local conditions remain of primary importance. For example, the pour-on method of control of ticks (and tsetse flies) leads to high concentrations of the product at the level of the skin; the skin is a favourite dish in certain areas of West Africa.

As mentioned before, the development of acaricide resistance is a major problem. It is important to try to postpone its emergence, particularly as there is so little development of new acaricides. Classically, it was believed that the best strategy was to stick to one group of acaricides until resistance is well established and then switch to another class of compounds with a different biochemical mechanism, and no automatic cross-resistance with the first group. However, this belief is not based on sound experimental grounds; it is not possible to come back to previously used groups to which resistance has developed, as the resistance factor persists in the tick population for long periods (at least 20 years in Boophilus microplus against arsenic and organochlorines).

In some cases it is possible to temporarily postpone the switch to another acaricide group by increasing the concentration of the compound to which resistance has developed. Once resistance has been shown to occur, it may still be possible to isolate and eradicate the resistant strain by quarantine and the use of acaricides which are effective on the strain in question.

In recent years it has been suggested that other strategies might be more effective, such as rotation of different acaricides, mixtures of acaricides with different biochemical mechanisms, and as much moderation as possible in applying acaricides. Contrary to the common belief that understrength dip wash or inefficient spraying will cause acaricide resistance to emerge more rapidly, it is likely that resistant mutants, already existing in the tick population, will be selected out faster when acaricide treatment is intensive. The success of such approaches has not yet been proved experimentally for acaricides, and it is urgent that field experiments be carried out.

2.2. Immunological control

Most of the immunization procedures used against TBDs hardly interest private industry because the market is too limited and the procedures are often complicated,
difficult to standardize and entail too many risks. Long standing methods against haemoparasitic diseases such as the bovine babesioses, bovine anaplasmosis and heartwater consist in the inoculation of the live virulent organisms in blood, followed by specific treatment. In some cases the organisms are attenuated. Immunization against tropical theileriosis is carried out by the injection of attenuated schizonts in lymphoid cell culture, and, on a smaller scale, against East Coast fever, by injecting virulent sporozoites derived from ticks, together with a tetracycline to limit the reaction. There is a real danger of contamination of such vaccines by unwanted pathogens, particularly viruses, even of vaccines based on cell cultures using serum in the medium. The use of virulent organisms entails other obvious risks. The use of such methods is mainly limited to government laboratories.

Several teams are working on the development of more modern vaccines against TBDs and their vectors (e.g. Refs [11–13]). Practical results are still few, but there is considerable promise. A French commercial vaccine is available against a babesiosis (of dogs), based on soluble antigens from culture. Of more importance to developing countries, two recombinant vaccines based on a ‘concealed antigen’ have been commercialized in recent years against the tick *Boophilus microplus*; more prolonged use on a larger scale and against a wide variety of strains of this tick species is still necessary to allow a final judgement on their real value.

A parasite is, of course, a much more complicated organism than a bacterium or a virus, but even against viruses such as those of influenza and AIDS, immunization is notoriously difficult. Nevertheless, progress in biotechnology is fast and we may reasonably expect extraordinary advances, but also spectacular disappointments, in the future.

2.3. Use of genetically resistant animals

Apart from artificial immunization, control can make use of the immune response to natural infection or infestation. Some individual animals react better to infection by a given TBD or to infestation by ticks than others belonging to the same population, and certain populations and breeds tolerate a given TBD or tick infestation better on average than others. Indigenous cattle populations in regions where a particular TBD is endemic are far more tolerant of the infection than imported animals. They also tolerate infestation by the local tick species much better. Such tolerance or resistance\(^1\) appears to be mainly the result of natural selection in endemic areas, and is hereditary.

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\(^1\) The term tolerance may be preferable to resistance, as there is no resistance to infection but the infection is better tolerated.
Unfortunately, most of the tolerant breeds are not very productive. The great increase in the human population of developing countries requires a rapid and drastic augmentation of the production of animal proteins, but this cannot be provided by the local resistant livestock. Cross-bred populations have a higher potential production than local livestock and are better adapted to adverse climatic and nutritional conditions than pure imported livestock, but their tolerance to TBDs and ticks is intermediate at best, which is often not good enough.

If the tolerance to a given parasite is based on one or a few genes only, it may be possible to transfer those genes from local to more productive but highly susceptible livestock. This could be done by classic selective cross-breeding programmes, or perhaps eventually by genetic engineering. It is also possible to select for productivity in a tolerant population or breed, although each breed has its genetic limits. None of these three approaches is rapid! Moreover, they require reliable criteria and genetic markers for tolerance, which are as yet hardly available for any TBD or tick [14].

Things may be complicated by the fact that the stress of high production is immunodepressive, so that genes responsible for tolerance may not be able to express themselves fully in highly productive animals. In addition to this innate tolerance, there is also an age linked tolerance to TBDs in young animals, which exists even in those born to non-infected dams of exotic susceptible breeds. This has been studied mainly in cattle. The average duration of age linked tolerance depends on the parasite concerned (several months at least for bovine babesiosis and anaplasmosis, only weeks for heartwater in susceptible breeds). The effects of innate tolerance and age linked tolerance are cumulated, so that young calves of local cattle populations in endemic areas tolerate the infection even better than those of susceptible breeds. Calves born to infected dams might moreover benefit from a degree of passive immunity, although cellular immunity generally appears to be the major component of the immune response in most TBDs. Congenitally transmitted infection might also be responsible for immunity to heartwater in calves [15].

Use of genetical resistance is especially useful in situations where financial and management restraints and/or an insufficient veterinary infrastructure prevent the application of expensive control measures. It is best used in conditions of endemic stability, i.e. where the the inoculation rate is sufficiently high to infect all animals early in life, so that innate and age linked tolerance can prevent clinical disease, and an immune population results. Depending on the type of animals, it may be necessary to apply limited chemical tick control, aimed at keeping tick infestation below the damage threshold but sufficiently high to maintain endemic stability. In certain cases the innate tolerance in local animals is so high that the incidence of clinical disease is negligible even in regions where serological surveys would indicate an absence of endemic stability (for instance, in the cases of Maure cattle and tropical theileriosis in Mauritania [16], and of local zebu and heartwater in Malawi [17]).
2.4. Biological control

This consists in using predators and diseases of parasites. Examples of biological control methods being successfully applied against ticks and TBDs are still rare. Most of the theoretical possibilities have either not been followed up or have not led to practical results. Predation by chickens may be applicable in certain conditions [18]. Promising experimental results have been obtained with a parasitoid against the tick *Amblyomma variegatum* [19]. The use of entomophagous nematodes against ticks is being investigated, promising results being reported by certain authors [20] while others are less enthusiastic [21]. Exploration of the potential use of fungi against the ticks *Rhipicephalus appendiculatus*, *A. variegatum* and *Boophilus microplus* also gives hope [22-23].

Nevertheless, the prospects for practical application of biological control methods do not appear to be very promising for the near future, but continuation of research in this field should be encouraged.

2.5. Ecological control

Some examples are:

— the use of toxic plants against ticks on pastures;
— pasture rotation to deprive (larval) ticks of their hosts;
— control of weeds, bushes and trees on pastures;
— grazing non-susceptible animal species on contaminated land, for example, horses or small ruminants to cleanse the vector ticks of infection with bovine theileriosis;
— bush fires to destroy ticks in the environment;
— zero grazing of the animals, in order to avoid contact with ticks on the pastures.

Some of the proposed methods are theoretically attractive, but not always practical. When given the choice, ticks tend to avoid toxic plants. Grazing management is not a realistic approach where grazing is communal, or in the case of transhumance. The period during which ticks (even larvae) can survive is often too long to allow pasture rotation to be incorporated into a rational management of the farm. Bush fires in the dry season may not reach most of the ticks, which often hide in microclimatic shelters such as soil cracks until better conditions allow them to ascend the vegetation.

2.6. Reproductive control

This is meant to break the reproductive cycle by irradiation or genetic manipulation. It is a very specific and non-polluting approach (although there are certainly
pollution problems associated with a radiation source). The sterile male technique has been and still is used successfully in the eradication of the New World screw-worm fly (Cochliomyia hominivorax) and in the control of tsetse flies (Glossina spp.). Male ticks can also be sterilized by irradiation, but it is difficult to imagine how sterile males can be applied in the field, and separation of males and females could be expensive. Also, female tsetse flies produce at most some 10 descendants, but female ticks lay thousands of eggs. The crossing of closely related species, such as Boophilus annulatus and B. microplus, may produce sterile offspring, and can also (at least theoretically) be used in control; the same practical problems, such as how to apply the ticks and the necessary separation of male and female ticks, are valid and are likely to prevent this approach from being practical and cost effective.

2.7. Drug treatment

In the field, in conditions where the veterinary infrastructure is weak, drug treatment of TBDs is often a last resort when prevention has failed. Nevertheless, it can be very effective, but in order to successfully apply curative treatment, the disease agent should be correctly diagnosed and the drug should be available and correctly applied in a timely fashion. This obviously requires the existence of an adequate veterinary infrastructure. Furthermore, the cost effectiveness of curative treatment depends to a large extent on the value of the livestock concerned.

2.8. Sanitary control and surveillance

Surveillance and quarantine measures, combined with appropriate legislation, can be useful or even indispensable in preventing the spread or importation of parasite species, or of pesticide resistant strains. For example, surveillance measures based on judicious legislation have to be an integral part of the programme for the eradication of the tick Amblyomma variegatum from the Antilles.

3. CONCLUSIONS

The classical approach to controlling tickborne diseases by chemical control of the vectors and curative drug treatment is running into many problems, as explained above, and is not sustainable. Integrated control seeks to make use of every possible method available and applicable in any particular circumstances. Control has to be cost effective and sustainable from a financial as well as an ecological point of view. Several of the possible approaches need further evaluation as to their practicality and sustainability.
REFERENCES


[21] MAULEÓN, H., BARRÉ, N., PANOMA, S., Pathogenicity of 17 isolates of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) for the ticks *Amblyomma variegatum* (Fabricius), *Boophilus microplus* (Canestrini) and *Boophilus annulatus* (Say), Exp. Appl. Acarol. 17 (1993) 831–838.


VACCINE DELIVERY IN THE 21st CENTURY

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Abstract

VACCINE DELIVERY IN THE 21st CENTURY.

A major problem in many vaccination programmes aimed at the effective immunization of human and agricultural animal populations is the logistics of sustained vaccine delivery. These problems stem from the formulation of the vaccine antigen, the selection of adjuvants, the cost of production, quality control, the shelf-life of the vaccine itself, delivery to the target population and ensuring an appropriate programme of injections so that full immunity develops. The authors wish to explore the idea of an essentially passive system for the delivery of vaccines. Over a number of years, they have focused their attention on the development of transgenic technology in insect vectors of animal and human disease. One essential stage in the life-cycle of these insects, and the reason why they act as vectors of disease causing agents, is the necessity for a bloodmeal. The authors have been exploring the concept of using these haematophagous insects as a vehicle to deliver antigens directly to human and animal populations as one part of an overall immunization programme. The concept essentially involves creating transgenic haematophagous insects which are capable of expressing the desired antigens in their saliva, so that when they take a bloodmeal a small amount of antigen is delivered to the host. Current data strongly suggest that over a prolonged period of time this repeated low level exposure to the vaccine antigen could potentially lead to immunity in the human or animal host. The progress which has been made in developing this approach, together with its potential advantages and disadvantages, is discussed.
1. INTRODUCTION

It is now over 200 years since Jenner introduced the concept of vaccination as a strategy to combat infectious diseases of humans and their livestock. Throughout this time, vaccination has been a central pillar in our attempts to improve humanity’s ‘quality of life’. With the advent of recombinant DNA technologies and more recent techniques for cell manipulation, many people had assumed that novel vaccines would rapidly flood the market, but surprisingly few new and successful vaccines have emerged to date. We examine here some of the underlying difficulties in vaccine technology and raise some alternative strategies for consideration.

In retrospect, the most successful vaccines used to date have been based upon undefined antigens, often live, naturally or artificially attenuated, organisms. Additionally, the targets of the immune responses engendered have often (though not invariably) been simple (prokaryotic) organisms with direct life-cycles. Looking forward, e.g. in the children’s vaccine initiative, we recognize that essential criteria for the design of future vaccines require that they be safe, heat stable, effective when given by the oral route, and requiring few inocula.

2. CURRENT PROBLEMS IN VACCINE DESIGN AND DELIVERY

With the advent of recombinant DNA technology and modern peptide and polysaccharide biochemistry, people enthusiastically embraced the idea that, particularly in the case of blood borne pathogens, both safe and stable vaccines could rapidly be developed. However, successful attempts to develop such vaccines have been disappointingly infrequent, and many highly publicized trials have done little to promote confidence in the overall objective. The reasons for the slow progress achieved are varied and include those shown in Table I.

Recognizing that recombinant vaccines will present only a small fraction (sometimes only one) of the potential targets of an effective immune response, such immunogens are highly at risk if the target immunogen is capable of change; if it lacks appropriate immunogenicity; if it cannot be targeted in the tissues of the immunized host or if it cannot be delivered effectively within a community.

2.1. Changes in target antigen

Amongst many other immune evasion mechanisms, pathogens and parasites have evolved two major molecular strategies by which they vary the protein antigens presented to their hosts, namely antigenic polymorphism and antigenic variation. In the former, each parasite in a population has the potential to express one of a diverse range of different alleles encoding the ‘offending’ immunogen. Hence, in a
TABLE I. SOME PROBLEMS ENCOUNTERED WITH VACCINES

<table>
<thead>
<tr>
<th>Target antigen changes</th>
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<tr>
<td>(a) Antigenic polymorphism or variation in target antigen</td>
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<th>Vaccine lacks immunogenicity</th>
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<tr>
<td>(a) Antigens lack immunogenicity</td>
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<td>(b) Immune response induced is not appropriate</td>
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<th>Delivery</th>
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<tr>
<td>(a) Difficulties in identification and targeting of the individuals/population at risk (especially for zoonotic infections, e.g. leishmaniasis)</td>
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<tr>
<td>(b) Logistics of vaccine delivery (storage, inoculation)</td>
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<tr>
<td>(c) Costs and sustainability (manufacture, safety, delivery to target population)</td>
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population of hosts, within which individual hosts display a restricted immune response, different clones of parasite are capable of surviving in different hosts, thereby ensuring survival of a diverse range of parasite genotypes in the population. Examples of this variation are readily found in a wide range of parasites of both medical and veterinary importance, e.g. MSP1 antigen in *Plasmodium falciparum*. Alternatively, every parasite may have the ability to switch over a period of time between the expression of one of a wide range of alleles within every genome, with a frequency that exceeds the ability of the host to mount a sterile immune response, e.g. the variant surface glycoprotein (VSG) of *Trypanosoma* or Pfemp1 in *Plasmodium*. In both of these situations, an effective recombinant vaccine would be required to encode enough of the most frequently expressed antigens to be effective in the majority of situations.

Current concepts to overcome/circumvent such problems include: use of killed pathogens, e.g. papilloma virus immunization in turtles; the use of naturally or, with increasing frequency, genetically engineered attenuated pathogens, e.g. *Toxoplasmae* (Toxovax) or *Babesia*; the use of related non-pathogenic organisms that have been engineered to express critical immunogens from related or unrelated hosts. Such use of live carriers is widespread in experimental models and includes the use of prokaryotic, e.g. *Vaccinia, Salmonella, Vibrio*, or eukaryotic organisms, e.g. *Plasmodium, Leishmania* and helminths. All such living systems for vaccine delivery, of course, remain to varying degrees subject to the constraints of safety, ensuring there is no possibility of reverting to a pathogenic phenotype, and of delivery (requirement for refrigeration and a short shelf-life). Methods that avoid these complications by use of recombinant DNA vectors and/or the use of peptide/glycoconjugate chemistry are diverse and lie half-way between the use of a single
immunogenic molecule and an attenuated vaccine. Mixed antigen presentation attempts to induce an immune response to a number of different entities simultaneously. The more complex each entity, the lower the probability that the parasite will rapidly develop (through mutation or antigenic variation/polymorphism) resistance to the host's specific immune response. Thus, potentially the least effective of these strategies is to use multiple antigenic peptides (MAPs), where selected B and T cell epitopes from one or more proteins are linked in a single construct, usually to a branched polylysine backbone. Such approaches have been examined in malaria, hepatitis and HIV.

Mixtures of whole immunogens should theoretically be preferred. Such mixtures could be achieved by the use of large DNA vectors, or transformed live vectors (e.g. Vaccinia:NYVAC-Pf7, which can express seven different malaria proteins) encoding a range of proteins. Alternatively, simple mixtures of the immunogens can be made; this permits inclusion of glycoconjugates. Such mixtures can be formulated at the time of manufacture, e.g. protein cochelates (proteins/phospholipids/calcium) carrying influenza, parainfluenza or HIV, or at the time of administration (e.g. diphtheria, tetanus, cellular pertussis and Haemophilus influenzae B). The latter option clearly presents the most complicated delivery mechanism and is therefore the most expensive to administer.

2.2. Targeting/inducing an effective and appropriate immune response

The very rapid advances in the understanding of the vertebrate immune system has revolutionized our appreciation of the difficulties to be encountered in the induction of an effective immune response. Again, this understanding is more critical in the design of a vaccine using a limited range of immunogens compared to those using a multifaceted challenge by live attenuated vaccines.

Recognition which of the diverse immune effector mechanisms that a host uses to kill invading pathogens is critical to the development of sterile immunity and a prerequisite to the use of vaccines with limited immunogenicity. Broadly speaking, the responses can be defined as specific, e.g. antibody or cytotoxic T cell mediated, or non-specific, e.g. cytokine complement or phagocyte mediated, though the latter can also be employed within specific killing mechanisms. Different pathogens are variously susceptible to these different mechanisms. Intracellular pathogens can be attached by cytotoxic T cells, cytokines and, to a lesser extent, by complement mediated lytic mechanisms; extracellular pathogens are more at risk from antibody mediated mechanisms, the antibodies responsible being of a range of immunoglobulin classes. Induction of the appropriate effector mechanisms has been shown to be carefully regulated, a major dichotomy in the induction process being the selection of a Th1 or a Th2 response. The former leads to a cytotoxic response, and the latter tends to induce an antibody mediated response. Thus, the ability to direct the uncommitted
T cell following its contact with the antigen presenting cell to one or an other pathway is critical to the eventual efficacy of the vaccine. The critical role of the rapidly expanding family of interleukins in this regulation has resulted in the selective inclusion of, e.g., IL12 in the formulation of vaccines that successfully stimulated a protective Th1 response in mice.

Current approaches to influencing the direction of the immune response include the use of DNA vaccines to transform the host directly such that the expression of the parasite antigen within the host cells leads to a good CD8 cytotoxic T cell response. Such vaccines have additionally been shown to be capable of inducing good antibody response. The benefit of this approach could, in the long term, be a 'single hit' immunization which achieves a sustained presentation of antigen; the counterpoint of this could be that if the immunogen were found to be allergenic there would be no simple way to block presentation of the antigen. Secondly, safety issues as to whether the encoding DNA could be permanently integrated into the host genome and the outcome of such integration need to be addressed.

The increasing understanding of adjuvant technology has led to less dramatic methods of directing the immune response. These include the use of detergent or oil based adjuvants, e.g. QS21 and MF59, to stimulate CD8 responses preferentially. In the same vein, 'complexing' the immunogen with attenuated Salmonella or with the B subunit of cholera toxin has very clear applications in the selective targeting of the immunogen to the mucosal epithelium in mice.

The second component of developing an effective immune response is the question of both raising and sustaining an effective response. This again is a question of the design of an appropriately complex molecular construct that will interact with both appropriate B and T cells for a sufficient time to induce an effective response, and/or the design of an appropriate delivery mechanism. Techniques for immunogen construction include both peptide and glycoconjugate chemistries and adjuvant design. Complexing the antigen(s) of choice with 'helper/carrier' proteins, e.g. tetanus toxoid or Hepatitis B antigen, is now commonplace, and the use of antigens linked to monoclonal antibodies targeted to the macrophage Fc receptors for IgG to enhance presentation has been assessed in model experiments. There is now considerable expertise in combining proteins into 'particulate' delivery systems, e.g. proteosomes (hydrophobic meningococcal outer membrane proteins), liposomes and ISCOMS (immunostimulatory complexes), and on a larger molecular scale the microencapsulation of antigens into particulate reservoirs. Lactide/glycoside microcapsules have been very widely used to deliver a diverse range of antigens (proteins, polysaccharides and even prokaryote lysates, from >20 pathogens). These have the particular advantage that they can be stored dry and are simple to deliver, but the current need to use organic solvents in their preparation has some limitations on the antigens that can be delivered by this otherwise attractive mechanism for sustained antigen release.
2.3. Vaccine delivery

There is no doubt that rapid advances are being made in vaccine technology. The introduction of genetic transformation technology of either host or delivery vector is, however, of significant future importance, particularly with respect to the need to obtain sustained delivery to widely dispersed host communities, which remains a major logistic and financial problem. Nowhere is this more critical than in the case of diseases in developing nations where the costs of vaccination for a single disease can exceed the annual per capita health budget and where diseases have a reservoir other than the host of interest, e.g. zoonotic diseases of humans such as leishmaniasis or toxoplasmosis, or of cattle, e.g. trypanosomiasis and piroplasmosis. In the latter case, the possibility of having to vaccinate a reclusive reservoir host is particularly problematic.

3. THE CONCEPT — BLOOD SUCKING INSECTS AS VACCINE DELIVERY MECHANISMS

As indicated above, a major problem in the effective immunization of human and animal populations is the logistics of vaccine delivery. This could be addressed if it were possible to find mechanisms by which humans, livestock and animal reservoirs could be constantly immunized/boosted by the bite of haematophagous insects. Simply stated, we wish to consider the possibility of constructing a haematophagous insect (here the mosquito) that will secrete into its saliva a vaccine antigen which will induce, sustain or boost an immune response in the bitten person/animal. We have chosen to examine this question in a model system, i.e. using the anopheline and aedine mosquitoes to express a well studied transmission blocking antigen (Pbs21) of the rodent malarial parasite *Plasmodium berghei*.

4. SELECTION OF THE MODEL TO ASSESS THE CONCEPT

4.1. The antigen

Pbs21 is an ookinete antigen; it is a member of the highly conserved gene family that also encodes Pfs25 and Pfs28; the former is a human vaccine candidate about to undergo field trials and the latter is the homologue of Pbs21. None of these antigens is subject to natural immunization or boost by infection; therefore, the availability of a repeated ‘challenge’ may be of practical importance in the field. Pbs21 is a highly immunogenic protein; as little as 5 μg has induced a significant transmission blocking potential. The entire protein has been expressed in insect cells (using
baculovirus vectors) and the recombinant protein is as immunogenic as the native molecule [1, 2]. Constructs of the gene are available expressing all, or selected, TB epitopes of the molecule, with a signal sequence that ensures secretion by insect cells [3], and without a membrane anchor motif at the carboxyl terminus, which results in the release of a soluble immunogen from insect cells [1].

A wide range of high titre antibodies recognizing both conformation dependent and/or independent epitopes are available, together with ELISA, ELISPOT, IFAT and immunogold techniques to detect and measure antigen expression. Parasite antigen (ookinetes), native protein and recombinant protein are all available to determine the immune response induced by the antigenic bite, together with an extensive database on the immune response of mice to Pbs21 proteins (recombinant and native). Transmission blocking assays for monitoring the impact of immunization on parasite transmission are also routine (both from membrane feeds and intact mice).

4.2. The insect

Ideally, the haematophagous insect chosen for transgenesis should: be widely distributed; be of catholic bloodfeeding habit; deliver substantial quantities of saliva at the time of biting; be easily reared in the laboratory; be one whose eggs may be stored for long periods; have been the subject of significant molecular biological studies; and be one for which there should exist techniques for either transitory or permanent genetic transformation. The mosquito is clearly the organism of choice. Recognizing that in areas where malaria is endemic humans receive between 5 and 200 mosquito bites per night in the rainy season, clearly the potential for delivery of proteins in the saliva could be very significant. Wide ranging studies on the allergic reaction to mosquito bites attest to the immunogenic potential of immunogens in insect saliva [4]. In Aedes, it has been shown that sera from naturally exposed adults recognized up to four saliva proteins [5]. The dominant responses were of the IgG4, IgE and IgG1 isotypes and directed against a protein of ‘36kDa’. The Aedes gene encoding a major saliva protein of ‘37kDa’ has been cloned and characterized [6]. Clearly, therefore, if an immunogen of choice is placed under the control of a strong promoter from one of the naturally immunogenic saliva proteins, e.g. the 36/7kDa protein, it is possible that enough protein is expressed to be immunogenic by bite. Aedes mosquitoes are also an ideal choice in that they are easy to maintain and are not malaria vectors.

4.3. Insect molecular biology

Over the past few years, considerable effort has been directed towards developing a much deeper understanding of the molecular biology of insect vectors [7]. One aspect of this has focused on the idea of using transgenic techniques to create
mosquitoes or other vectors which are incapable of transmitting pathogens, such as the malaria parasite [8–10]. The concept being proposed here is to use the insects and their biting behaviour in a creative fashion to induce or boost immunity in the recipient host to interrupt pathogen life-cycles. We, and others, have developed methods and DNA vectors which may be used to introduce and express DNA in cultured mosquito cells [11] as well as in mosquito embryos [12–14]. In the cell culture, system cells can be transfected with DNA either transiently or stably and the introduced DNA expressed under the control of appropriate promoters [11]. Similar experiments have recently proved successful in the transient transfection of both mosquito salivary glands [15] and midgut [16]. Finally, the techniques have been developed for introducing DNA into mosquito embryos and, although integration does not occur at high frequency, the introduced DNA does occasionally integrate into the recipient genome so that it is passed on to subsequent generations of the mosquito [12–14]. The current problem with this technology is the lack of a suitable, high efficiency transformation vector to allow frequent integration of novel genes into insect genomes.

A number of genes have also been identified which are expressed in a salivary gland specific manner in *Ae. aegypti* [6]. One of the major objectives of this research has been to characterize a fully functional salivary gland specific promoter for the mosquito. The upstream regions of these genes are now available and contain the appropriate control sequences. We are therefore in a position to combine the 5' control regions of salivary gland specific genes with specific domains of the gene coding for Pbs21 which are immunogenic. These combined cassettes can then be introduced into transfection vectors to undertake the transgenic manipulations desired.

5. PROGRESS TO DATE TO ASSESS THE CONCEPT

To determine whether recombinant Pbs21 may be expressed in excised *Ae. aegypti* salivary glands, we placed the entire coding region of the pbs21 gene under the control of the *Bombyx mori* baculovirus immediate early gene promoter, in the expression vector pVJ12-IEGprom [17, 18]. This construct was then used to transf ect cultured salivary glands, using the transfection reagent DOTAP.

In situ hybridization using a 370 nucleotide antisense RNA probe corresponding to the internal region of the pbs21 gene [19] first revealed the presence of Pbs21 mRNA within transfected glands 12 h post-transfection. Control glands did not show this expression. Recombinant Pbs21 was first detected immunocytochemically within transfected glands 24 h after transfection using the conformation independent monoclonal antibody 13.1.15, known to recognize a linear epitope of Pbs21. High levels of gene expression were observed in the single layer of epithelial cells which form the periphery of the glands, but expression could also be observed throughout the entire gland.
These results indicate that the gene encoding Pbs21 is transcribed within excised *Ae. aegypti* salivary glands to produce a recombinant protein that is recognizable immunologically. It has been suggested [20] that expression of exogenous DNA within cultured salivary glands may result from the highly active metabolic state of the glands. Between the ages of three to five days post-emergence, the glands are synthesizing a few proteins in large quantities, and this activity may facilitate the expression of foreign DNA. Alternatively, the experimental procedure may affect a small population of cells such that they gain the ability to regulate and replicate in culture, and it is these cells which take up and express the DNA.

The pVJ12-IEGprom/pbs21 construct was also used to transfect an *Ae. aegypti* cell line, Mos20, using the transfection reagent polybrene. Immunocytochemical detection and western blot analysis revealed the presence of recombinant Pbs21, detectable by using MoAb.13.1.15. When the recombinant Pbs21 from this system was used to immunize groups of BALB/c mice, a sustainable IgG response was induced, first detectable after the second subcutaneous immunization. No response was seen in sera from control mice. Sera from experimental mice were found to recognize native Pbs21 under both reducing and non-reducing conditions. In a transmission blocking assay in which pooled immune sera from either experimental or control groups of mice were mixed with whole blood infected with *P. berghei* parasites and administered to *An. stephensi* mosquitoes via a membrane feed, a transmission blockade of 71.8% was induced (Table II).

These results suggest that recombinant Pbs21 produced by a mosquito cell system is an effective immunogen capable of inducing a significant transmission blocking immunity in the mouse. The recombinant product from this system appears to be a considerably more effective immunogen than the recombinant Pbs21 produced in the *E. coli* system which induced an average transmission blockade of just 33% [21]. This has been attributed to loss of conformation dependent epitopes, which is a consequence of expression in a prokaryotic system. It appears that recombinant Pbs21 expressed in mosquito cells is considerably more effective than that expressed in

<table>
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<th>TABLE II. RESULTS OF TRANSMISSION BLOCKING ASSAY USING ANTIGEN PRODUCED IN TRANSFECTED MOSQUITO CELLS</th>
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<tr>
<td>Control</td>
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<tr>
<td>Mosquito +ve/No. dissected</td>
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<tr>
<td>Prevalence of infection (%)</td>
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<tr>
<td>Mean oocysts/mosquitos (±SE)</td>
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E. coli. It is, however, difficult to compare the mosquito recombinant Pbs21 with that produced in the baculovirus system because we have not quantified the amount of immunogen used for immunization, nor was the assay for transmission blocking identical.

Possible suggestions as to this difference include variation in post-translational modifications of the protein such as glycosylation or expression of the hydrophobic membrane anchor which may be important for the generation of transmission blocking antibodies. However, there is evidence [22] demonstrating that vaccination of both mice and *Aotus* monkeys with an unglycosylated Pfs25 expressed in yeast, which lacked the hydrophobic anchor region, generated complete transmission blocking immunity, but only when administered together with adjuvants. Alternatively, one type of epitope may favour the induction of transmission blocking immunity over another. Both recombinant Pbs21 expressed in the baculovirus insect system and the native immunogen induce almost exclusively conformation dependent antibodies in the rodent. However, the fact that the conformation independent MoAb.13.1.15 is a powerful blocker of parasite development indicates that the induction of transmission blocking immunity is not confined to conformational epitopes.

The construct employed in this set of experiments contained the entire coding region of the pbs21 gene including the putative carboxy-terminal anchor encoding region. This required the use of adjuvants for immunization of mice. Future work will use an 'anchorless' construct, thereby obviating the need for adjuvants and more fully assessing the possibility of delivering vaccines directly through the bite of a transgenic mosquito.

This is the first successful demonstration that the Pbs21 gene, when expressed in mosquito cells and salivary glands, produces a product which is both antigenic and induces transmission blocking in the mouse model. This paves the way for experiments which aim at introducing this gene into live mosquitoes via embryo microinjection [23]. We will then be able to assess how much antigen is produced in mosquito saliva and hence how effective this blood sucking insect may be as a delivery vehicle for this and other vaccines.

6. FUTURE PROSPECTS

The research we are undertaking aims at applying transgenic technology to haematophagous insects, in this case the mosquito, for use as an immunogen delivery system. This is an exciting concept but requires that we objectively establish the fundamental aspects of the approach. We already know that the Pbs21 encoding gene can be expressed in insect cells to produce an immunogenic recombinant product, which confers immunity on the recipient if used in an appropriate regime. It is also
clear from a number of studies, including our own research, that people living in areas such as Finland and Tanzania, where they are constantly exposed to mosquito bites, will develop immunity to mosquito saliva antigens. This immunity clearly shows that the mosquito is capable of delivering sufficient antigen, given a relatively high biting rate per person and per day, to induce immunity to saliva antigens. These observations by themselves are sufficient to indicate that, if a transgenic mosquito expressing a highly immunogenic parasite protein could be produced and delivered in sufficient numbers to recipient populations, immunity to the target of interest would be induced.

Although this present study focuses on the use of mosquitoes as the delivering insect, there are a range of other haematophagous insects with different feeding habits which may be more appropriate in particular instances. It is also possible to envisage the localized release of large numbers of laboratory propagated and sterilized transgenic insects in specific well defined localities. In the case of *Ae. aegypti*, this is particularly feasible because the eggs of this mosquito may be stored for very prolonged periods. Large numbers of insects may therefore be produced to order. Additionally, if the insects themselves are sterilized this will overcome the problem of releasing fertile mosquitoes which are capable of interbreeding with natural populations and hence raising the possibility of spreading the heterologous genes to non-target populations. The utilization of sterile insect technology also highlights the potential involvement of the Joint FAO/IAEA Division because of its long standing commitment to the utilization of nuclear and related techniques in the sterile insect technique (SIT). It is also clear that in the case of human vector borne diseases the approach may be used to vaccinate not only the human population but also the animal reservoir populations.

The concept is not limited to using insect vectors of disease as ‘flying vaccinators’. Any insect that takes a blood meal from the organism which is the target for immunization may be used as a vaccine vector. Indeed, the use of non-vector species of catholic feeding habits would be particularly attractive. In addition, we have focused our attention on introducing the gene for only one antigen into the mosquito system as our model. Clearly, however, once the concept has been established, a number of genes coding for a vaccine cocktail which could provide protection against measles, polio, HIV, malaria, etc., could all be incorporated into a transgenic mosquito.

Finally, such an approach may find particular application in the veterinary field where, for example, transgenic ticks could be developed, capable of delivering a cocktail of vaccines providing protection against cattle pathogens.

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REFERENCES


REGULATION OF GENE EXPRESSION IN MAMMALIAN CELLS USING THE LAC REPRESSOR


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Abstract

REGULATION OF GENE EXPRESSION IN MAMMALIAN CELLS USING THE LAC REPRESSOR.

The study describes the construction of a one step inducible lac repressor/operator mammalian expression system within the context of DNA mediated vaccination (naked DNA). It is novel in that it contains, on a single vector (pSO1), all the cis and trans controlling elements necessary to manipulate (switch on/off) and control the expression of a reporter gene in a mammalian cell. The enhanced green fluorescent protein (EGFP) gene was cloned downstream of the cytomegalovirus immediate–early enhancer promoter (PCMV IE) and a lac operator (LacO), which was in turn regulated by the expression of the lac repressor under the control of a second PCMV IE. The number of cells fluorescing were greatly increased following isopropyl B-D thiogalactoside (IPTG) induction. In the repressed state, fewer pSO1 transfected cells expressed the EGFP, and the fluorescence intensity was also lower than that observed for the induced pSO1 transfected cells. Observation by microscopy was quantified by FACScan analysis on the different populations of cells. There was always a significant difference between the induced and repressed pSO1 transfected cells in terms of the percentage of the population of cells fluorescing and the intensity of the fluorescence. Of the repressed pSO1 transfected HeLa cells, only about 10% showed fluorescence at 507 nm wavelength. However, of the induced pSO1 transfected HeLa cells, 68% showed fluorescence at 24 h, 51% at 48 h and 46% at 72 h post-transfection. To demonstrate this system’s ability to be manipulated, two experiments were conducted in parallel. Almost 40% of the pSO1 transfected HeLa cells (24 h repressed, followed by 24 h induced) responded to the induction and expressed EGFP. A similar result was obtained for pSO1 transfected HeLa cells (48 h repressed, followed by 24 h induced); 36% of the cells responded and expressed EGFP. Alternatively, there was a decrease in the number of EGFP expressing cells when pSO1 transfected HeLa cells were induced for 24 h, followed by 24 h of repression (i.e. 24% at 24H+/24H– as compared to 51% at 48H+). Furthermore, for pSO1 transfected cells kept in the presence of IPTG for 24 h and then for 48 h in the absence of IPTG, a significant decrease was observed of the number of EGFP expressing
cells (3% at 24H+/48H− as compared to 46% at 72H+). The plasmon surface resonance results confirmed the ability of the different extracts to prevent or reinstate transcription. We were able to determine dissociation rate constants (kd_{lacO} = 9 \times 10^{-4} \text{ s}^{-1} and kd_{PCMV-LacO} = 6 \times 10^{-4} \text{ s}^{-1}) for the interaction of lac repressor (from the heterologous protein extract) with its lac operator and with the PCMV IE-Lac operator. The data were similar to those obtained for purified lac repressor/LacO interactions by plasmon surface resonance studies (kd = 3.4 \times 10^{-4} \text{ s}^{-1}) and by classical enzyme kinetic studies (kd = 6.5 \times 10^{-4} \text{ s}^{-1}). Furthermore, the dissociation rate constants for the interaction of the cellular transcription machinery with the minimal cytomegalovirus immediate–early promoter sequence (without the enhancer sequence) present in both the constructs PCMV and the PCMV-LacO (the latter in the presence of IPTG) were similar (kd_{PCMV} = 2.68 \times 10^{-4} \text{ s}^{-1} and kd_{PCMV-LacO} = 5.6 \times 10^{-4} \text{ s}^{-1}).

1. INTRODUCTION

In 1990, Philip Felgner and co-workers (Vical, San Diego) demonstrated that muscles of living mice produced foreign proteins if injected with naked genes. Since 1992, the concept of nucleic acid or DNA mediated immunization (also called DNA vaccines or genetic immunization) [1, 2] as a simple method for eliciting an immune response has been receiving ever increasing support.

Nucleic acid vaccines, or the use of antigen encoding DNAs for vaccination purposes, represent a new approach to the development of subunit vaccines. A subunit vaccine presents only selected components of a virus or protein to the immune system. Previous methods of subunit vaccination have used purified proteins or proteins expressed by viral vectors. Each of these methods has substantial limitations that could be overcome if the immunizing protein, or parts thereof, could be expressed directly in host cells.

Nucleic acid vaccines offer this opportunity, with immunization (protection) accomplished following uptake and expression of inoculated DNA. DNA mediated immunization is a potential method of stimulating cellular and humoral immunity. The expressed protein is expected to enter the cytosolic pathway; thus, specific peptides will become associated with the major histocompatibility complex class I (MHC I) proteins. As with a replicating intracellular organism, a cytotoxic T cell response can be observed. Furthermore, since an antibody response is also seen, the endosomal processing pathway in antigen presenting cells is probably also involved. This approach is particularly suited for producing antigen/peptide specific antibodies, especially where a protein is difficult to purify or where its structure is destroyed during purification, or when the protein is unknown but the gene has been obtained. In addition, multigene constructs or multiple plasmid constructs could be used (encoding secretory tags, immunomodulators such as cytokines, and immunogenic carrier proteins).
The technique of DNA mediated vaccines has recently been described as an alternative to recombinant proteins for eliciting an immune response to a particular antigen. DNA cloned into suitable vectors can be directly introduced into mammalian tissue in vivo, resulting in expression of the foreign gene [3] and a specific immune response in the target animal [1]. Such DNA mediated immunization may in fact mimic live attenuated vaccines by producing both humoral and cell mediated responses [4-8, 2]. As with subunit vaccines, however, the response can be targeted to a particular component of the immunogen, thereby providing a way of avoiding potentially unfavourable clinical reactions which may occur with live vaccines.

The study of gene function in complex genetic environments such as mammalian cells would greatly profit from systems that would allow stringent control of the expression of individual genes. Ideally, such systems would not only mediate an ‘on/off’ situation of gene activity but would also permit limited expression at a defined level. Attempts to control gene activity by various inducible eukaryotic promoters responsive to, for example, heavy metal ions [9], heat shock [10] or hormones [11, 12] have generally suffered from leakiness of the inactive state or from pleiotropic effects caused by the inducing principles themselves, such as elevated temperature or glucocorticoid hormone action [13]. Recently, a tetracycline regulated system has been described in which gene activity is induced in the absence of the antibiotic and is repressed in its presence [14]. The main disadvantages to this system include the continuous treatment of tetracycline to repress expression and the slow clearance of antibiotic from bone, which interferes with quick and precise induction. No and collaborators [15] have explored an alternative, using the insect molting hormone ecdysone as a potential inducer. In their system, a pulse of the steroid hormone ecdysone, or the synthetic analog muristerone A, triggers the expression of the gene of interest. They reported some good dose response and induction rate characteristics advocating control of both the amount and the time period for which a desired gene is induced. Their two step system, however, relies on the fact that a particular cell is transfected with two plasmid vectors, one carrying the ecdysone receptor and the other carrying the ecdysone responsive promoter plus the heterologous gene. The fact that both the tetracycline regulated and the ecdysone regulated systems are based on two step control (i.e. two different plasmids carry interdependent controlling elements) makes them undesirable as potential DNA mediated vaccines.

In search of a regulatory one step system that could serve as a model not relying on endogenous control elements and with the notion of potential of DNA mediated vaccines, we have used the well described lac repressor/lac operator/inducer (LacI/LacO) system of Escherichia coli in mammalian cells. Here, we describe a control system that in HeLa cells allows tight control and regulation of expression of the enhanced green fluorescent protein (EGFP) from Aequorea victoria located on the same plasmid construct.
FIG. 1. Construction of the repressible/inducible one step plasmid construct pSO1.

2. MATERIALS AND METHODS

2.1 Construction of eukaryotic DNA vectors

pZBl: The 1082 bp *E. coli* LacI gene was amplified by the polymerase chain reaction (PCR) from plasmid pNM52 (kindly provided by Dr. N. Minton [16]), which contains the optimized LacI gene fragment [17] (Fig. 1). The PCR conditions were: denaturation at 94°C/15 s, annealing at 66°C/15 s and elongation at 72°C/1 min and primer set PZF3/PZF4 (Table I). The PCR step enabled us to substitute the original LacI initiator codon by ATG and to introduce an improved Kozak sequence [18] at the site of translation initiation (..AATGTGA44.. for ...GCCATGA44...). The PCR product was digested for 2 h with restriction enzymes NheI and Xmal and ligated into the prepared sites of plasmid pS65T-C1 (Clontech). After digestion with NheI and Xmal, pS65T-C1 was treated with alkaline phosphatase (calf intestine, CIP) for 1 h, after which the CIP was inactivated by heating to 65°C for 15 min. This allowed cloning of the LacI fragment in an orientation where it was placed downstream and under the control of the cytomegalovirus immediate–early promoter enhancer (PCMV IE) and upstream of the SV40 polyadenylation 3' end processing signals (SV40 PolyA) in the construct named pZOFl (Fig. 1). To destroy the BamHI site in
**TABLE I. PRIMER SEQUENCE COMPOSITION**

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Sequence composition (5’–3’)</th>
<th>Feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>PZ3b</td>
<td>GCTTGTCCACGCTAGCGGAAGAGAGTCAATTCAGGGTGGTG CCCCATGA</td>
<td>NheIa</td>
</tr>
<tr>
<td>PZF4b</td>
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<td>Xmala</td>
</tr>
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<td>BamHIa</td>
</tr>
<tr>
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<td>GATTCGATTGGATCCGATACATTGAGTGGTGAC</td>
<td>BamHIa</td>
</tr>
<tr>
<td>PZF7d</td>
<td>GTTACGAAATTCATTGTGAGCGGATAACAATTACCATGGTGAGGCAAGGGCAGGACTGTCCACCG</td>
<td>EcoRIa</td>
</tr>
<tr>
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<td>GTTCGGCTACCTAGGATCTACTTGTACAGCTCGTCCATGCCAGAGTGTACCC</td>
<td>KpnId</td>
</tr>
<tr>
<td>BBC1e</td>
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<td>biotinylated 5’ end</td>
</tr>
<tr>
<td>BC2e</td>
<td>GTGATCTGAGCAGTTCACCTAAACGC</td>
<td>—</td>
</tr>
<tr>
<td>BBA1f</td>
<td>AATTGTGAGCGGATAAATT</td>
<td>biotinylated 5’ end</td>
</tr>
<tr>
<td>BA2f</td>
<td>AATTGTATCCGCTACAAATT</td>
<td>—</td>
</tr>
<tr>
<td>BBX1g</td>
<td>CAGGATGCAAATGTGGAAG</td>
<td>biotinylated 5’ end</td>
</tr>
<tr>
<td>BX2g</td>
<td>GTACCGTCTTCATGGGAGAAAATAATAC</td>
<td>—</td>
</tr>
</tbody>
</table>

a The restriction enzyme sequence included in the primer design.

b Primer set to amplify LacI (a Kozak and ATG sequence were introduced).

c Primer set to amplify the PCMV IE/LacI/Poly A cassette.

d Primer set to amplify the LacO-EGFP fragment (the minimal LacO operator sequences were introduced upstream of the EGFP gene and stop codons in all three reading frames downstream).

e Primer set to amplify the minimal cytomegalovirus immediate–early promoter (PCMV).

f Oligonucleotide set used to form the double stranded minimal lac operator core.

g Primer set to amplify an internal fragment of the LacI gene.

the MCS of pZOF1, the construct was digested with BamHI, treated with T4 DNA polymerase in the presence of dNTPs and religated [19]. This construct, pZB1, was used as template to amplify the 1905 bp PCMV IE/LacI/SV40/PolyA fragment as a BamHI fragment cassette for subcloning into pCI (Promega). After PCR amplification (denaturation at 94°C/15 s, annealing at 64°C/15 s and elongation at 72°C/90 s and primer set PZF5/PZF6), the LacI fragment was digested with BamHI for subcloning into the mammalian expression backbone vector (pCI).
pSO1: Plasmid pCI was used as the parental vector (Fig. 1) in our construction of an inducible one step expression plasmid. Vector pCI was EcoRI/KpnI digested and CIP treated. The enhanced green fluorescent protein (EGFP) gene (719 bp) [20, 21] was amplified by PCR (denaturation at 94°C/15 s, annealing and elongation at 72°C/60 s) with primer set PZF7/PZF8 (Table I), from plasmid pEGFP-C1 (Clontech). This allowed us to introduce directly upstream of the EGFP initiation codon a minimal E.coli lac operator (LacO, 21 bp) core sequence (5' AATTGTGAGCGGATAACAATT3' [22]) and translational stop codons (12 bp) in all three reading frames directly downstream of the EGFP sequence. The 750 bp PCR amplicon was digested with EcoRI and KpnI before an overnight ligation at 15°C with the EcoRI/KpnI digested and alkaline phosphatase treated pCI vector. In the resulting construct (pCE1), the EGFP gene is placed downstream of the PCMV IE/Intron and upstream of the SV40 PolyA signals. pCE1 was subsequently digested with BamHI, CIP treated and ligated overnight at 15°C with the BamHI released fragment containing PCMV IE/LacI/SV40 PolyA from pZB1. In pSO1, the EGFP cassette (PCMV IE/intron/LacO-EGFP/SV40 PolyA) was selected in the opposite orientation to the LacI cassette (PCMV IE/LacI/PolyA) (Fig. 1).

A typical 25 cycle 50 μL PCR reaction (Perkin Elmer 9600 thermocycler) consisted of 10 ng DNA template, 2.5 mM MgCl₂, 2.5 mM dNTPs (Bohringer Mannheim), 250 ng of each primer and 1 unit bio-X-act polymerase (Bioline). The PCR conditions are indicated in the text. All enzymes were from Promega unless otherwise stated. During plasmid construction the relevant products (after PCR amplifications or restriction enzyme digestion) were purified with the Wizard PCR Preps DNA purification system from Promega and checked on an ethidium bromide stained agarose gel and handled according to standard procedures (Sambrook, et al., 1989). The DNA used for subsequent cloning or for transfection experiments was purified by Qiagen anion exchange chromatography according to the manufacturers’ instructions (Qiagen). All the primers used in this study were synthesized in-house on a Millipore Expedite™ nucleic acid synthesis system.

2.2. In vitro transcription and translation

The HeLa nuclear extract (HNE) from Promega, a transcription preparation based on the method of Dignam [23], was used according to the Promega manual. A basic HNE transcription reaction (25 μL) consisted of 10.5 μL 1 x HeLa transcription nuclear extract buffer, 1.5 μL MgCl₂ (50 mM), 1 μL 25 x rNTPs, 50–100 ng template DNA and two units (0.5 μL) of nuclear extract. In some cases, 2 μL of the relevant translation reaction was added (see the legend of Fig. 2). The reaction was gently mixed by tapping and incubated at 30°C for 60 min. The reaction was stopped by direct precipitation with 95% ethanol (two volumes) in the presence of 0.3 M
FIG. 2. In vitro transcription and translation analysis on 8% SDS-PAGE. Lane 1: pSO1 as template, with the addition of 2 μL unlabelled HNE/RRL (−) IPTG to the transcription reaction. Lane 2: no DNA template. Lane 3: pZBl as template. Lane 4: pNM52 as template. Lane 5: pSO1 as template in a normal HNE/RRL reaction. Lane 6: pCE1 as template. Lane 7: pSO1 as template, with the addition of 2 μL cold HNE/RRL (+) IPTG to the transcription reaction. Lane 8: pCI as control template.

sodium acetate (pH5) at −70°C for 15 min. The RNA was centrifuged for 10 min at 12 000 g, and the supernatant was aspirated. The RNA pellet was washed once with 85% ethanol, centrifuged for further 5 min at 12 000 g, the supernatant again aspirated and the pellet dried in a vacuum desiccator. The RNA pellet was resuspended in 10 μL nuclease free water and stored at −20°C.

For the translation of the RNA, the RNA template was heated to 65°C for 10 min and cooled on ice before use. The standard 50 μL translation reaction (Promega manual) consisted of 35 μL rabbit reticulocyte lysate (RRL) (Promega), 1 μL RNasinR ribonuclease inhibitor (40 U/μL), 1 μL amino acid mixture (1 mM, minus methionine), 2 μL [35S]methionine (>1000 Ci/mmol, Amersham, catalogue No. SJ1015) and 2 μL RNA template. The translation reaction was incubated at 30°C for 2 h, and 5 μL of the HNE/RRL reaction was analysed [19] on SDS-PAGE (8% gel) to determine radioactive incorporation. After electrophoresis, the gel was dried under vacuum and exposed to X-OMATRRA Kodak film at −70°C.
2.3. Cells and transfections

HeLa cells were grown at 37°C in a CO₂ incubator in the presence of Dulbecco's modified Eagle medium (DMEM) with GlutaMAX™ (DMEM, Gibco-BRL), 2 µg/mL Ciproxin® (Bayer) and 7.5% foetal bovine serum (GibcoBRL) on plastic (Nunc) until they were approximately 80% confluent. The LipofectAMINE™ transfection procedure was as described in the manufacturer's manual (GibcoBRL). Briefly, the monolayer of cells was washed twice with serum free DMEM before the transfection mix was added. The transfection mix was made up as follows: solution A: 1.5 µg of DNA into 100 µL serum free DMEM and solution B: 12 µL LipofectAMINE reagent into 100 µL serum free DMEM. The two solutions were gently mixed and incubated at room temperature (RT) for 55–60 min to allow the DNA liposome complexes to form. Before adding the DNA liposome complex mix to the cells, 800 µL serum free DMEM (with (+) or without (−) 5 mM isopropyl β-D-thiogalactoside (IPTG) was added, and the transfection mix was gently added to the cells and incubated at 37°C. After six hours, the transfection solution was removed, and complete medium was added ((+)//(−) 5 mM IPTG) and incubated for a further 24–72 h.

2.4. Optical fluorescent detection of the enhanced green fluorescent protein expression in HeLa cells

EGFP expression in HeLa cell monolayers was detected by using a Leitz DMRB fluorescent microscope fitted with a Leica Wild MP52 camera. EGFP encodes the green fluorescent protein (GFP) GFPmut1 variant (Clontech), which contains the double-amino-acid substitution of the Phe-64 to Leu and Ser-65 to Thr. excitation maxima = 488 nm, emission maxima = 507 nm. This red shifted variant of wild type GFP has been optimized for brighter fluorescence and increased expression in mammalian cells.

2.5. FACScan analysis

The transfected cells were incubated with Trypsin-EDTA (1 x) (GibcoBRL) at room temperature for 5 min to detach the cells from the plastic and disrupt the monolayer. The cells were washed twice with ice cold 1 x HBSS buffer (100 mL 10 x HBSS stock is; 0.4 g KCl, 0.06 g KH₂PO₄, 8 g NaCl, 0.35 g NaHCO₃, 0.06 g Na₂HPO₄·2H₂O, 1 g D-glucose, pH7.4) by centrifugation (500g_av for 5 min) and made up to 250 µL with 1 x HBSS buffer. The cells were kept on ice until analysed. Between 4000 and 6000 cells were scanned for the presence of EGFP from a
2.6. **Surface plasmon resonance**

Surface plasmon resonance measurements [24–26] were conducted by using a BIAcore 2000 instrument from Biosensor Pharmacia at 25°C. The 5' biotinylated PCR amplified or annealed oligonucleotides in 35 μL were injected across a streptavidin pre-treated dextran sensor (Sensor Chip SA5; Biosensor Pharmacia) surface in the BIAcore apparatus at 5 μL/min in HBS buffer (10 mM Hepes (pH 7.4), 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20 (v/v)) in the presence of 10 mM MgCl₂. Hybridization of the HeLa nuclear cell extract (protein concentration of 1 mg/mL) was carried out in the same buffer (with the further addition of 0.5 μg/μL Poly (dl-dC) (Sigma)(w/v)) by flowing 95 μL at 15 μL/min across the immobilized ligand. The biotinylated ligands were prepared as follows: fragments of plasmid pSO1 were PCR amplified to represent the 91 bp minimal cytomegalovirus immediate–early promoter [27] (PCMV; biotinylated primer BBC1/primer BC2 set, denaturation at 94°C/15 s, annealing at 68°C/15 s and elongation at 72°C/15 s), the 112 bp PCMV-LacO core sequence (PCMV-LacO; biotinylated primer BBC1/primer BA2 set, denaturation at 94°C/15 s, annealing at 64°C/15 s and elongation at 72°C/15 s) and a promoter non-related sequence of 110 bp from an internal part of the LacI fragment (LacI; biotinylated primer BBX1/primer BX1 set, denaturation at 94°C/15 s, annealing at 64°C/15 s and elongation at 72°C/15 s). The 21 bp minimal lac operator core sequence (LacO; biotinylated synthetic oligonucleotide BBA1 was annealed with synthetic oligonucleotide BA2 by heating equimolar amounts to 75°C followed by gradual cooling) (see Table I for the sequences of the primers). After PCR or oligonucleotide annealing, the fragments were purified using the Wizard PCR Preps DNA purification system from Promega followed by agarose gel purification (Geneclean, BIO 101) to separate the amplified fragments from free primers. Ligands were bound in equimolar ratios to the sensor chip at between 150 and 250 resonance units (RU). The various HeLa cell extracts (analytes) were prepared essentially as described by Hu & Davidson [28] and Dignam [23]. In short, HeLa cell monolayers were transfected as previously described with pSO1 and incubated in the presence or absence of IPTG for 24 h. The cells were washed twice with PBS [19] and treated with Trypsin-EDTA. The individual cells were collected by low speed centrifugation (500 × g) and resuspended in 50 mM Hepes (pH 7.4) containing 150 mM NaCl (2 × 10⁶ cells/0.5 mL). The cells were broken by rapid freezing/thawing (3 ×) at −70°C and the cytosolic and nuclear fractions separated by centrifugation (500g). The nuclear fraction (sediment) was resuspended in 20 mM Hepes (pH 7.4), containing 15% glycerol, 0.2 mM EDTA and 150 mM NaCl, and disrupted by using a dounce homogenizer. The homogenate was centrifuged for 20 min.
FIG. 3. Optical fluorescence detection of EGFP expression. (a): pZB1 transfected HeLa cell cultures (mock transfected) (10 mm = 23 μm). (b): pSO1 transfected HeLa cell cultures in the absence of 5 mM IPTG (10 mm = 23 μm). (c): pSO1 transfected HeLa cell cultures in the presence of 5 mM IPTG (10 mm = 24 μm). (d): pSO1 transfected HeLa cell cultures in the presence of 5 mM IPTG (10 mm = 11 μm).
at 25 000g and the resulting sediment was discarded. The supernatant was frozen in 25 µL aliquots (protein concentration of 10 mg/mL).

The reaction between the immobilized DNA and the transcription factors or repressor protein were assumed to follow pseudo-first-order kinetics [29], where the dissociation rate constant kd is obtained from the slope of the ln (R/R₀) versus (tₙ - t₀) plot.

3. RESULTS

3.1. Construction of pSO1

The final repressible/inducible one step eukaryotic DNA vector pSO1 contained the EGFP cassette (PCMV IE-Intron/LacO-EGFP/SV40 PolyA) in opposite orientation to the LacI cassette (PCMV IE/LacI/PolyA) (Fig. 1). The orientation and presence of the relevant genes were confirmed with restriction enzyme digestion (data not shown).

3.2. In vitro translation and transcription

After each cloning step the resulting construct was transcribed and translated in vitro to validate the PCR amplified gene (Fig. 2). The HNE/RRL experiment confirmed that the genes were intact after PCR amplification and did not contain internal termination codons generated by the bio-X-act polymerase. For pCE1, ³⁵S-Met labelled EGFP was detected at the predicted size of 28 kDa (Fig. 2, lane 6). In the case of pZBl, we were able to detect a radiolabelled protein band (38 kDa) (Fig. 2, lane 3) similar to the predicted size of the lac repressor. In the presence of 2 µL of unlabelled (without [³⁵S]Met) pSO1 expressed HNE/RRL reaction (with 5 mM IPTG) solution, two bands were detected with sizes corresponding to the LacI and EGFP gene products (Fig. 2, lane 7). In the presence of 2 µL of the unlabelled pSO1 expressed HNE/RRL reaction (without 5 mM IPTG) solution, only the LacI protein band was detected (Fig. 2, lane 1). In the absence of an aliquot of the HNE/RRL solution, two bands, corresponding to the 28 kDa and 38 kDa protein bands, were always seen (Fig. 2, lane 5).

3.3. EGFP expression in HeLa cell cultures

We were able to maintain the HeLa cell cultures up to a maximum of 80 h post-transfection in the presence of 5 mM IPTG. The cell culture transfection experiments were conducted over 72 h at 24 h intervals. No fluorescence was observed for either the naive cells (normal cells) or the mock infected cells (pZBl transfected cells) (Fig. 3(a)).
FIG. 4. FACScan analysis of EGFP expression in HeLa cell cultures.
A: LipofectAMINE transfection of pSO1 (24 h post-transfection).
B: LipofectAMINE transfection of pSO1 (48 h post-transfection).
C: LipofectAMINE transfection of pSO1 (72 h post-transfection). EGFP fluorescence peak channels are indicated for the presence of 5 mM IPTG (+IPTG) and for the absence of 5 mM IPTG (−IPTG) as well as the endogenous fluorescence in the cells when transfected with either pZBl or no plasmid (mock and naive controls) in the presence of 5 mM IPTG.
D: EGFP expression in the presence of 5 mM IPTG 48 h post-transfection (48H+). EGFP expression in the presence of 5 mM IPTG for 24 h followed by 24 h in the absence of IPTG (24H+/24H−). EGFP expression in the absence of 5 mM IPTG for 24 h followed by 24 h in the presence of IPTG (24H−/24H+) at a peak channel of 50.
E: EGFP expression in the presence of 5 mM IPTG 72 h post-transfection (72H+). EGFP expression in the presence of 5 mM IPTG for 24 h followed by 48 h in the absence of IPTG (24H+/24H−) and EGFP expression in the absence of 5 mM IPTG for 48 h followed by 24 h in the presence of IPTG (48H−/48H+) at a peak channel of 50.
The HeLa cells transfected with pSO1 expressed EGFP (green fluorescence). We examined parallel cultures (in the presence and in the absence of IPTG) to analyse the control of the inducible one step LacI system. In the pSO1 transfected HeLa cell cultures (+) IPTG, high levels of EGFP were detected by fluorescence microscopy (Figs 3(c) and (d)) compared to the pSO1 transfected HeLa cell cultures in the absence of IPTG (Fig. 3(b)). In these samples fewer cells were expressing EGFP and at a lower intensity. The number of cells fluorescing was approximately the same for 24, 48 or 72 h post-transfection; only the 24 h post-transfection results are shown in Fig. 3.

3.4. FACScan analysis

To quantify the regulation (i.e. the switching on/off of expression of the EGFP gene) of the one step repressible/inducible system, the transfected HeLa cells were subjected to a FACScan analysis (Fig. 4). For each analysis, more than 4000 cells were examined from a scanned population of 20 000. No difference was observed between the naive cells (no DNA transfected) or the mock transfected cells (pZB1 transfected) at 24, 48 or 72 h post-transfection and the observations were, therefore, considered as the background noise (peak channel (PkCl) = 1.5, standard deviation (SD) = 2.15) (results not shown). There was always a significant difference between the IPTG induced and the uninduced pSO1 transfected cells in terms of the number of cells fluorescing and the intensity of fluorescence (Fig. 4). At 24, 48 and 72 h post-transfection, only about 10% of the approximately 4000 cells scanned of pSO1/(-) IPTG showed fluorescence at 507 nm (mean peak channel (PkChl) = 11.14, SD of 16.56). In parallel, for pSO1/(+) IPTG, for 24 h post-transfection, 68% (PkChl = 50.48, SD = 20.09) (Fig. 4A); for 48 h post-transfection, 51% (PkChl = 45.32, SD = 13.17) (Fig. 4B) and for 72 h post-transfection, 46% (PkChl = 52.33, SD = 14.44) (Fig. 4C) showed fluorescence of the cells scanned.

Almost 40% of the pSO1 transfected HeLa cells, incubated for 24 h in the absence of IPTG followed by 24 h in the presence of IPTG, showed fluorescence (PkChl = 45.32, SD = 15.48) (Fig. 4D). A similar result was obtained for pSO1 transfected HeLa cells kept for 48 h in the absence of IPTG followed by 24 h in the presence of IPTG: 36% of the cells showed fluorescence (PkChl = 48.70, SD = 14.14) (Fig. 4E). In parallel, pSO1 transfected cells kept in the presence of IPTG for 24 h and then for 24 h in the absence of IPTG showed a decrease in the number of fluorescent cells as well as in the intensity of the fluorescence, 24% of the cells showed fluorescence with a PkChl = 46.98 and a SD = 14.19 compared to 48 h (+) IPTG, where 51% of the cells showed fluorescence with PkChl = 45.32 and SD = 13.17) (Fig. 4D). Of particular interest are the pSO1 transfected cells kept in the presence of IPTG for 24 h and then for 48 h in the absence of IPTG. A significant decrease was observed in the number of fluorescent cells as well as in the intensity of the fluorescence. Only 3% of the cells showed fluorescence (PkChl = 43.71,
FIG. 5. Surface plasmon resonance analysis as a RU versus time plot. The different DNA fragments were interacted with pSO1 transfected HeLa nuclear extracts in the presence (+) or absence (-) of IPTG and analysed. A: PCMV as template. B: PCMV-LacO as template. C: LacO as template. D: LacI as template (non-related internal control fragment).
TABLE II. SUMMARY OF THE BIAcore MEASURED DISSOCIATION RATE CONSTANTS

<table>
<thead>
<tr>
<th>DNA ligand</th>
<th>Protein analyte</th>
<th>(kd \times 10^{-4} \text{ s}^{-1})</th>
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<tr>
<td>PCMV</td>
<td>HeLa/(+)IPTG</td>
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</tr>
<tr>
<td>PCMV</td>
<td>HeLa/(−)IPTG</td>
<td>2.48</td>
</tr>
<tr>
<td>PCMV-LacO</td>
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<tr>
<td>Lacl</td>
<td>HeLa/(−)IPTG</td>
<td>2.20</td>
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PCMV: minimal cytomegalovirus immediate–early promoter (91 bp); LacO: minimal lac operator core sequence (21 bp); PCMV-LacO; 112 bp fusion of PCMV and LacO; Lacl; 110 bp internal sequence of Lacl in the presence (+) and in the absence (−) of IPTG.

SD = 17.96) as compared to 46% for the 72 h post-transfection (PChl = 52.33, SD = 14.44) (Fig. 4E).

3.5. Surface plasmon resonance

Double stranded DNA fragments representing PCMV, PCMV-LacO, Lacl and LacO, were immobilized via a biotin group at one 5’ end to a streptavidin-dextran surface and then reacted with HeLa cell extracts ((+)/(-) IPTG). The ability of the heterologous protein extract (containing the cellular transcription and translation factors) to interact and bind to the various ligands was investigated to support our analysis of a one step gene control system. Since we were working with cellular extracts, it was impossible to determine the concentration of the transcription or regulatory factors responsible for binding to the DNA control elements. For PCMV, no difference was observed between the RU versus time plots when the DNA was reacted with HeLa cell extracts in the presence or absence of IPTG (Fig. 5A) and the dissociation rate constants were identical, \(kd = 2.68 \times 10^{-4} \text{ s}^{-1}\) for PCMV/(+)IPTG and \(kd = 2.48 \times 10^{-4} \text{ s}^{-1}\) for PCMV/(−)IPTG (Table II). This was also the case for the negative control DNA and the internal Lacl sequence (no transcription elements are present in this DNA fragment) (Fig. 5D); the dissociation rate constants were \(kd = 2.35 \times 10^{-4} \text{ s}^{-1}\) for Lacl/(+)IPTG and \(kd = 2.2 \times 10^{-4} \text{ s}^{-1}\) for Lacl/(−)IPTG. However, for both the DNA fragments containing LacO, there was a significant difference between the sensorgrams and the relevant dissociation rate constants. For the PCMV-LacO control, the RU versus time sensorgram showed a different response when reacted with the (+)IPTG extract as opposed to the (−)IPTG extract (Fig. 5B). The dissociation rate constants were also different, \(kd = 5.6 \times 10^{-4} \text{ s}^{-1}\) for PCMV-
LacO/(+)IPTG and \(kd = 2.28 \times 10^{-4} \text{s}^{-1}\) for PCMV-LacO/(−)IPTG (Table II). The positive control LacO BIAcore sensorgrams also reflected this difference when reacted to the (+) and (−) IPTG HeLa cell extracts. The two sensorgrams differ (Fig. 5C), which is also reflected in their dissociation rate constants \(kd = 8.88 \times 10^{-4} \text{s}^{-1}\) for LacO/(+)IPTG and \(kd = 2.9 \times 10^{-4} \text{s}^{-1}\) for LacO/(−)IPTG.

4. DISCUSSION

The ideal vaccine might be characterized as safe, cheap, heat stable, containing protective immunogenic sequences from multiple pathogens and preferably administered as a single oral dose. Although no vaccine for human use meets all these criteria, current vaccines have been remarkably successful in reducing the morbidity and mortality associated with many common infectious diseases and, in the case of smallpox virus, have succeeded in globally eradicating a significant human pathogen [30, 31]. Current vaccines may be divided into two categories: firstly, live vaccines, which comprise traditional attenuated microbes, viral or bacterial, selected for reduced pathogenicity with maintained immunogenicity, and, secondly, recombinant vaccines, in which foreign antigens are expressed from a replicating viral or bacterial vector. Although recombinant vector vaccines are used in veterinary medicine, none has been approved for human use. Dead vaccines consist of killed whole pathogens or soluble pathogen proteins or protein subunits or the pathogen’s naked DNA.

We are interested in the precise control of gene expression, in particular, the switching on/off of specific genes located on naked DNA in the context of DNA mediated vaccines. Tightly regulated gene expression by an exogenous inducer has numerous applications. For example, inducible expression of the cre recombinase in transgenic mice [32] would allow for temporally specific inducible gene targeting of the adult or the developing embryo [33–35]. Inducible expression of toxins such as the diphtheria toxin would allow for tissue specific ablation [36]. Inducible vaccines, such as an African horsesickness VP2 (AHSV) specific DNA mediated vaccine, would allow specific expression of the correct AHSV VP2 serotype before the onset of a seasonal epidemic. Danko et al. [37] and Wolff et al. [3] have demonstrated that intracellular ‘naked DNA’ is stable for long periods of time and therefore ideal as a potential ‘controllable’ vaccine.

In search of regulatory systems that do not rely on endogenous control elements, several groups have demonstrated that the lac repressor/operator/inducer system of *E. coli* functions in mammalian cells. Three different approaches have been described: (i) prevention of transcription initiation by a properly placed LacO at promoter sites [38–40]; (ii) blockage of transcribing RNA polymerase II during elongation by a LacI/LacO complex [41]; and (iii) activation of a promoter responsive to a fusion between LacR and the activating domain of virion protein 16 (VP16) of
herpes simplex virus (HSV) [42, 43]. We report here the use of a repressible/inducible one step LacI naked DNA system as a potential inducer of gene activation in mammalian cells. This study involves an investigation of the controlling elements that regulate the expression of heterologous genes within the context of naked/episomal DNA and eukaryotic expression. It is very important to be able to control (the switching on/off of) the activity of the promoters responsible for the expression of the genes of interest once they are introduced into the target animal. This will enable the researcher to introduce several vaccines (targeted against a number of diseases) simultaneously (for example, at birth) into the target animal and selectively switch them on when required. This is particularly important in cases where delayed expression is important, for example where maternal antibodies play a role.

This study involved an investigation of the controlling elements that regulate the expression of heterologous genes within the context of naked/episomal DNA and eukaryotic expression. Our study involved the construction of a one step LacI repressible/inducible system. It is novel in that it contains on a single DNA plasmid construct all the cis and trans controlling elements necessary to manipulate (switch on-switch off) and control the expression of a reporter gene in a mammalian cell. The EGFP gene was cloned downstream of the PCMV IE, a chimeric intron composed of the 5' donor site from the first intron of the human β-globin gene and a lac operator (LacO) element in which expression of the reporter gene EGFP can be transcriptionally repressed by the expression of LacI under the control of a second PCMV IE (Fig. 1). This enabled us to study the activation of the PCMV IE promoter by removing the lac repressor from or, alternatively, preventing the formation of, the lac repressor/LacO complex via the addition of IPTG (for a summary, see Fig. 6).

After each cloning step, the construct was transcribed and translated in vitro to verify the PCR amplified genes and fragments (Fig. 2). The HNE/RRL experiment confirmed that the expected full length proteins were expressed and that premature termination due to the misincorporation of bases by the bio-X-act polymerase was absent. The observed sizes of the EGFP and the LacI proteins expressed were similar to the expected sizes of the proteins of the EGFP (28 kDa) and the LacI (38 kDa). The LacI protein is a homotetramer containing four polypeptide chains, each of molecular weight 38 kDa [44]. The EGFP protein has a molecular weight of 29.4 kDa [20, 21]. In these experiments we used truncated EGFP with a predicted size of 27.8 kDa. Since the HNE/RRL is an uncoupled transcription and translation system, it was expected to observe both the EGFP and the LacI protein bands (pSO1 as template) in the experiments (Fig. 2, lane 5). However, with the addition of 2 μL of the cold HNE/RRL (pSO1/(-) IPTG) solution to the transcription reaction of the pSO1 template followed by the normal RRL reaction, we were able to detect only the 38 kDa protein band (Fig. 2, lane 1). This demonstrated that in vitro the addition of LacI prevents the transcription of EGFP from the PCMV IE-LacO element. In the presence of 5 mM IPTG, two protein bands (Fig. 2, lane 7) were observed using the
same conditions.

Since the HeLa cell cultures could be maintained for only a limited period, all the experiments were conducted within 72 h of transfection. By investigating the transfected HeLa cell cultures directly under the microscope for the presence or absence of fluorescence, we were able to detect induction of EGFP expression in the presence of IPTG (Fig. 3). In the absence of IPTG, fewer cells expressed the EGFP protein and the fluorescence intensity was also lower. This confirmed the accuracy of the vector constructs and that the goal of a single ‘naked DNA’ repressible/inducible LacI vector within the context of DNA mediated vaccination was obtainable. IPTG has induced the expression of EGFP efficiently. In the pSOL transfected HeLa cell cultures (+) IPTG high levels of EGFP were detected by fluorescence microscopy (Fig. 3) compared to the pSOL transfected HeLa cell cultures (−) IPTG. The number of cells fluorescing was approximately the same for 24, 48 or 72 h post-transfection.

To quantify and demonstrate the tight control (i.e. the switching on/off of EGFP expression) of our repressible/inducible one step LacI system the transfected HeLa cells were subjected to a FACScan analysis (Fig. 4). In a population of 20 000 cells, a window of at least 4000 cells was selected and scanned for the presence of EGFP fluorescence. There was always a significant difference between the pSOL transfected cells in the presence (+) and absence (−) of IPTG in terms of the number of cells fluorescing (per cent of the population) and the intensity of the fluorescence. It seems that the highest level of fluorescence is achieved after 24 h post-transfection with a slight decrease at 48 and 72 h post-transfection. This might be
due to the HeLa cell's response to the continued treatment of the non-metabolizable galactoside IPTG. The deleterious effects of IPTG might be avoided by the use of lactose.

Furthermore, to demonstrate the ability of the system to be manipulated (i.e. to be switched on from a switched off position and, alternatively, to be the switched off from a switched on position), two experiments were conducted in parallel. Almost 40% of the pSO1 transfected HeLa cells, kept for 24 h in the absence of IPTG (i.e. repressed status) followed by 24 h in the presence of IPTG (i.e. induced status), responded to the induction and expressed actively EGFP (PkhCl = 45.32). A similar result was obtained for pSO1 transfected HeLa cells kept for 48 h in the absence of IPTG (repressed status) followed by 24 h in the presence of IPTG (induced status). 36% of the cells responded and expressed EGFP as determined by the presence of fluorescence (PkhCl = 48.70). This confirmed that it was possible to switch the expression of EGFP on from a switched off position. Alternatively, pSO1 transfected HeLa cells kept in the presence of IPTG for 24 h (induced status) and then for 24 h in the absence of IPTG (repressed status) showed a decrease in the number of fluorescent cells as compared to the 48 h (+) IPTG as reference (i.e. 24% at 24H+/24H- as compared to 51% at 48H+) with almost the same intensity of fluorescence (at 24H+/24H-: PkhCl = 46.98 compared to 48H+: PkhCl = 45.32). This was probably not due to the presence of the non-metabolizable IPTG (IPTG uptake and clearance from cell cultures is of the order of 2 h [45]) but rather to slow catabolism of the EGFP protein. Of particular interest are the pSO1 transfected cells kept in the presence of IPTG for 24 h (induced) and then for 48 h in the absence of IPTG (repressed), where a significant decrease was observed for the number of fluorescent cells (at 24H+/48H-: 3% as compared to 46% at 72H+). The intensity of fluorescence of the remaining 3% cells was again similar to that of the reference (PkhCl = 43.71 as compared to PkhCl = 52.33 at 72H+).

The plasmon surface resonance results confirmed the ability of the different extracts to prevent or reinstate transcription. The major advantages of the technique are that the protein-DNA interactions can be monitored in real time and that the BIAcore instrument performs calculations of kinetic parameters. In addition, the rate of ligand binding to DNA and the sequence specificity can be assayed easily and with high precision. The double stranded DNA fragments (PCMV, PCMV-LacO, LacI and LacO) representing the different controlling elements were immobilized via a biotin group at one 5' end to a streptavidin-dextran surface and then reacted with the relevant HeLa cell (+) or (-) IPTG extracts. Crude cellular transcription and translation extracts were used as a first step in the investigation of heterologous DNA binding proteins to study DNA control elements [26]. The main disadvantage when working with a heterologous analyte solution is that the association rate constants, which are concentration dependent, cannot be determined [24]. The dissociation rate constants, however, can be determined. The dissociation rate constants for the interaction of the
cellular transcription machinery with the minimal cytomegalovirus immediate–early promoter sequence (without the enhancer sequence) present in both the constructs PCMV and the PCMV-LacO (the latter in the presence of IPTG) \( k_{d_{\text{PCMV}}} = 2.68 \times 10^{-4} \text{s}^{-1} \) and \( k_{d_{\text{PCMVLacO}}} = 5.6 \times 10^{-4} \text{s}^{-1} \) (Fig. 5, Table II) were similar to that described by Bondeson et al. (1993), \( k_d = 3.4 \times 10^{-4} \text{s}^{-1} \) in their BIAcore studies, and by Phahl [46], \( k_d = 6.5 \times 10^{-4} \text{s}^{-1} \) in their classical enzyme kinetic studies.

The vector described in this report may have potential uses in addition to a regulatable DNA vaccine. Other uses could include the regulation of gene expression in cell culture systems using the simple inducer IPTG.

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**REFERENCES**


Epidemiology has evolved as a practical discipline, initially concerned with control of the infectious diseases. As many of the latter were conquered in the developed world, and animal enterprises were intensified, complex, frequently non-infectious, diseases increased in significance. A shift towards quantitative analytical procedures became necessary to identify the factors associated with these diseases and to assess the latter’s technical and economic impact. Recently, computer technology has facilitated data collection and analysis. In developed countries, there is a need for improved monitoring of disease and productivity, and for identification of indicators of health and productivity. Additionally, the results of such activities need to be transmitted to the producers. Qualitative aspects of food production are also increasing in importance, notably ‘clean’ food production and animal welfare. In companion animal practice, disease morbidity needs to be estimated more comprehensively, and risk factors identified through wider application of observational studies. Objective assessment of established and new therapeutic and prophylactic procedures, using well designed clinical trials, is also required. In developing countries — the focus of the paper — estimation of the impact of disease on health and production is a priority. This demands good survey design and rigorous evaluation of the diagnostic tests that are being used. Participatory Rural Appraisal is also a valuable data gathering technique. Epidemiological activity must be supported by adequate training. The subject has been taught as an identifiable discipline for over 30 years (usually with emphasis on qualitative components) and is expanding in undergraduate and post-graduate courses. Moreover, many governmental and non-governmental agencies are supporting epidemiological training. If this trend continues, the veterinary profession worldwide should be excellently placed to face the future challenges to animal health.
1. INTRODUCTION

1.1. Historical background

Epidemiology is a practical discipline which has evolved to assist medical and veterinary practitioners in their attempts to improve the health of human and animal populations. In early societies, the main constraints on the health of humans and their domesticated animals were infectious diseases. The main goal of early epidemiological investigations was therefore an increase in the understanding of the mode of transmission and maintenance of infections (their 'natural history'), so that effective control measures could be instigated. Such investigations were qualitative.

Infectious diseases continued to be the main concern of human and veterinary medicine globally until the first part of this century. However, the recognition of microorganisms as causes of disease in the 19th century, and the subsequent development of antimicrobial drugs and vaccines, brought dramatic improvements in the control of infectious diseases in the developed countries, and by the middle of this century non-infectious diseases (e.g. arthritis and cancer) had assumed importance in both humans and companion animals. Moreover, the increase in livestock numbers and intensification of farm enterprises in developed countries, facilitated by control of the major epidemic infectious diseases, resulted in an increase in the importance of non-infectious production diseases in farm livestock.

Many emerging diseases frequently had unknown causes, and when the causes were identified, they were often complex. Koch's postulates, which had proved to be so successful in identifying causes of the 'simple' infectious diseases (e.g. smallpox or rinderpest), became inadequate criteria for elucidating the causes of complex, non-infectious diseases. They were subsumed by a more comprehensive set of rules formulated by Alfred Evans [2], and, during the second quarter of this century, quantitative observational studies evolved to identify putative risk factors and their interactions [3].

The successes in disease control in the Western world were not, however, paralleled in the developing countries, where over half the world's livestock are found [4] and where infectious diseases continue to be major problems [5].

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1 The development of veterinary epidemiology in the context of the problems that have afflicted animal populations is discussed in detail elsewhere [1].
1.2. Contemporary epidemiology

Over the past twenty years, the discipline has become more quantitative, with the application of statistical techniques in collaboration with statisticians and mathematicians. The production of inexpensive computer hardware and writing of 'user friendly' software have facilitated an expansion of increasingly sophisticated and powerful analytical procedures (although not without dangers of overuse and misuse). An economic framework for the evaluation of animal disease and its control has also evolved, and the newer molecular techniques have increased the analytical sensitivity and refinement of diagnostic methods. The number of areas that have been included under the 'umbrella' of epidemiology has therefore increased; this is readily seen in the range of subdisciplines that are currently identified; for example, 'molecular epidemiology', 'statistical epidemiology', 'computational epidemiology' and 'genetic epidemiology'.

The current scope of veterinary epidemiology, including related topics, is presented in Fig. 1 (based on Ref. [1]). This symposium concerns many of the components of this figure, some of which are addressed fully elsewhere in these proceedings.

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2 It is therefore tempting to argue that quantitative epidemiology marks a temporal transition from what Gordon [6] described as 'old' (qualitative) epidemiology, but this reasoning is fallacious. The qualitative and quantitative components have evolved simultaneously, although emphasis may vary and may depend on current, albeit short term, crises and fashions, as exemplified by the recent flurry of mathematical models of bovine spongiform encephalopathy [7-9]. Such fashions may also be driven by the value that they appear to have to research grant awarding organizations (e.g. in terms of their topicality and relevance to the remits of the organizations [10]), rather than their pragmatic worth. The parallel growth of qualitative and quantitative epidemiology has been explored by various authors [11-13] and the merits of broadly based applied research (the core of epidemiology) have been posited by Erich Bloch, Director of the United States National Science Foundation [14].

3 The need for caution in using complex statistical procedures has been elegantly justified recently by Martin [15].

4 The creation of subdisciplines in epidemiology is, however, somewhat detrimental to a correct perception of the discipline. It implies distinct sets of techniques to solve problems whereas, in reality, a problem is usually solved by the integrated application of several techniques. A current example is elucidation of the natural history of bovine spongiform encephalopathy, which has involved molecular techniques, observational studies and mathematical modelling.
FIG. 1. Components of epidemiology. (Subjects relating directly to epidemiology, and that are discussed in other parts of these proceedings, are printed in bold upper case.)
This paper presents a broad view of epidemiology, with particular emphasis on its role in the developing countries and on subjects that require particular attention there, but avoiding those topics that are discussed in detail by other delegates. Predictions are only made for approximately the next 20 years. This is a realistic period because of the uncertainty surrounding the success of disease control campaigns\(^5\), in particular, and the rate of progress of medical and veterinary science, in general.

2. DEVELOPED COUNTRIES

2.1. The livestock sector

2.1.1. Monitoring herd health and performance

Over the past 30 years, a substantial literature on risk factors for diseases in intensively reared livestock has accrued \([1]\), providing the veterinary practitioner with essential information on determinants of disease that can be manipulated to reduce disease incidence. Beginning in the 1960s, herd health and productivity schemes evolved \([23]\). These combined the early preventive medical schemes with an assessment of productivity, their objectives \([24]\) being to:

1. identify disease problems on a farm;
2. rate the problems in order of importance, with reference to technical and economic criteria;
3. initiate suitable control techniques and measure their success, not only technically but also with regard to the economic efficiency of the utilization of resources at the national and individual farm level, thereby indicating which technique should be increased and which reduced.

These objectives therefore focused on providing a service to the individual producer. Systems were initially based on mainframe computers \([25]\) but many now run on microcomputers \([26]\).

\(^5\) Control campaigns are sensitive to various erratic events such as military and civil conflict. The beneficial effects of the African JP15 rinderpest campaign in the 1960s \([22]\), for example, were negated by civil strife, and wars have been responsible for spreading that disease over many centuries \([1]\).
A logical extension of these programmes designed for individual farmers is an integrated system to provide baseline data on health and productivity at the national level. The National Animal Health Monitoring System (NAHMS) has been developed in the United States of America to fulfil this requirement [27].

An imminent challenge is provision of an appropriate, efficient service to producers [28]. This involves:

1. definition of parameters to be monitored;
2. comprehensive data collection;
3. distribution of information.

Traditionally, attention was focused on monitoring clinical diseases, but this provides an incomplete picture which disregards the major subclinical diseases such as subclinical mastitis. The monitoring of such subclinical conditions (e.g. somatic cell monitoring for mastitis) therefore needs to be expanded. Moreover, production monitoring can be undertaken; this is the area most directly relevant to the producer. Techniques such as cusum charts [29] are available to detect trends beyond those due to random variation (e.g. reproductive performance in pig herds [30]). However, there still is debate over the most valuable parameters to monitor. For example, reproductive performance in dairy herds may be expressed as several summary parameters (e.g. calving to conception interval and calving index) or as more complex indices (e.g. the Fertex financial index [31]).

Data exist in a variety of isolated sources, for example, farms, abattoirs and diagnostic laboratories. Future progress to unify these is needed. Again, attempts are already being made: the Canadian Animal Productivity and Health Information Network (APHIN), established in the late 1980s, integrates data from farms, veterinary practices, government agricultural laboratories, the processing industry and the Atlantic Veterinary College [32, 33].

Finally, information needs to be distributed to producers. Early health and productivity schemes usually produced summary information as written reports, but graphical representation on microcomputers is now expanding [28]. The emerging field of informatics offers considerable scope for improved delivery of information.

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6 A distinction must be made between data and information. The former are essentially unprocessed numerical facts, whereas the latter comprises data that have been processed and organized so that meaning can be extracted from them.

7 Informatics is the supply of information through the medium of the computer.
2.1.2 Improving food quality

The intensification of animal production has raised fresh problems, notably in relation to food quality. Thus, bovine spongiform encephalopathy and *Escherichia coli* food contamination in the United Kingdom, and *Listeria* and *Salmonella* spp. infections more generally have raised public alarm over food quality [34]. Weak points in the food production chain, where contamination is a particular risk, are being identified by using techniques such as HACCP (Hazard Analysis Critical Control Points) [35].

Public concern over the welfare of animals in intensive production systems is increasing. Initially the remit of behavioural scientists, this subject is now being addressed by veterinary epidemiologists [36] and economists [37], and is a topic which will continue to attract attention in the near future.

2.2 The companion animal sector

Unbiased estimates of disease frequency in dogs, cats and other companion animals are largely unavailable because of the difficulty in obtaining data from primary care veterinary clinics. Hitherto, information has been collected either through questionnaires or telephone interviews, using breed society registers or lists of veterinary practices as sampling frames [38]. Data for observational studies and clinical trials have been collected in a similar way [39]. Some databases of clinical records generated at veterinary schools exist [40], but are clearly biased. A major goal for the next century therefore is the integration of primary care records from private clinics, using modern computer technology, so that epidemiological studies can be more readily undertaken. There have already been some attempts to achieve this goal [41].

2.3 Clinical trials

Until very recently, most treatments and preventive procedures were based on personal opinion and anecdotal evidence and were not scientifically assessed, in both human [42] and veterinary [43] medicine, using rigorous clinical trials. Although the latter have a long history [13], culminating in the modern randomized and controlled clinical trial [44], only recently have there been official requirements for formal testing of therapeutic and prophylactic procedures, before licensing, using clinical trials [45]. This is an expanding area of research in medical and veterinary science in the Western world, but is also directly relevant to the control of infectious and non-infectious diseases in developing countries where, for example, resistance against established drugs (e.g. antimalarials and trypanocides) is an emerging problem.
3. DEVELOPING COUNTRIES

3.1. Contemporary problems and prospects for their solution in developing countries

The major focus of veterinary attention in developing countries over the next two decades still will be control of the infectious diseases. Progress needs to be made both in the measurement of disease frequencies and in the implementation of control (notably eradication) campaigns.

3.1.1. Field surveys

The morbidity of many animal diseases is unknown, despite their widespread distribution. For example, the biologically transmitted (notably salivarian) and mechanically transmitted trypanosomes cause trypanosomosis in large areas of Africa and Asia, yet the precise incidence, and therefore the impact (both technical and economic), of these infections is poorly known because many earlier surveys were undertaken without proper regard to basic sampling principles (e.g. randomness and precision). The sampling theory is well established [1, 47, 48] and is now being applied. For example, a survey of Trypanosoma evansi infection of buffaloes has recently been undertaken in central Java [49].

Although accurate estimation of disease morbidity and mortality requires good ‘hard’ data, these may be difficult to obtain in developing countries, where illiteracy may be a problem [51], and overreliance on questionnaires can lead to disappointing results [52]. During the 1970s, the limitations of formal systems of data gathering were recognized, and Rapid Rural Appraisal (RRA) emerged [53]. This approach focuses on qualitative (rather than ‘hard’) data and farmers’ perceptions of major problems. The potential value of RRA (now renamed Participatory Rural Appraisal) to veterinarians in developing countries has been identified recently [54, 55], and it is likely that this technique will be used in conjunction with quantitative data gathering procedures to identify animal health problems and to assess the success of control measures.

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8 This should not imply, however, that many of the avenues currently being explored in developed countries are not applicable to the developing ones. Thus, there is a role for planned animal health and productivity schemes under extensive systems of management, and for observational studies of multifactorial conditions — infectious and non-infectious [46].

9 ‘Hard’ data are numerical, in contrast to qualitative, facts [50].
3.1.2 Disease eradication campaigns

The successful eradication\(^{10}\) campaigns so well documented in developed countries [57] have not been mirrored in most developing countries. (Indeed, the only infectious disease to be globally eradicated from the human population is smallpox [58].) However, eradication of the classical animal plagues is now being taken seriously. A current example is rinderpest [59].

Before eradication campaigns are undertaken, the following items are required:

1. all necessary technical resources (e.g. workforce);
2. the full support of the agricultural community to reduce the risk of illegal trade in infected animals;
3. adequate ‘policing’ of state borders;
4. adequate diagnostic tests, diagnostic facilities and other tools (e.g. effective vaccines).

These requirements pose considerable challenges to governments in developing countries, and a full consideration of them is beyond the scope of this paper. However, two important matters will be highlighted: (1) diagnostic tests and (2) adequate epidemiological training of personnel (an important component of workforce requirements).

3.2 Diagnostic tests

Diagnostic tests are applied in two main contexts: (1) in the individual patient (the classical clinical setting) and (2) in a population to determine either the presence or the extent of a condition (e.g. screening\(^{11}\)). These two contexts are different, and the same test may perform differently in the two circumstances. Only the application of diagnostic tests in populations is discussed here; this is common in both animal disease surveys and control campaigns (e.g. test–retest schemes to control tuberculosis).

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\(^{10}\) 'Eradication' has been used in various senses [56]. Notable uses are: (1) the complete, global extinction of an infectious agent; (2) reduction of infectious disease prevalence in a specified area to a level at which transmission does not occur; (3) reduction of prevalence to a level at which the disease ceases to be a major health problem, although some transmission may still take place; and (4) the regional extinction of an infectious agent. It is the last meaning which is usually assumed in veterinary medicine (e.g. the eradication of foot-and-mouth disease from the United Kingdom).

\(^{11}\) Screening is the presumptive identification of an unrecognized disease or defect using tests, examinations or other procedures that can be applied rapidly [60].
Despite a long history in veterinary medicine, many diagnostic (particularly serological) tests have not been assessed properly. For example, although antigen enzyme linked immunosorbent assays (Ag-ELISAs) to detect *Trypanosoma evansi* were developed several years ago [61], they have not been completely standardized or evaluated in the field. Most published investigations were undertaken in (comparatively few) camels [62, 63], although the test is also used in buffaloes. It is only recently that attempts have been made to standardize and evaluate the test in buffalo populations using sufficient numbers to attain acceptable precision [49, 64]. A poorly assessed test is of limited use. Thus, test assessment is a major goal during the next century.

3.2.1. *Spectrum of diagnostic tests*

A diagnostic test is any procedure for discriminating between sick and healthy individuals. The many procedures can be divided into three types according to the variables that are recorded:

1. discrete, dichotomous (e.g. meat inspection in an abattoir: affected or not);
2. ordinal (ranked) (e.g. single-serial-dilution antibody titres);
3. continuous (e.g. enzyme levels), or continuous approximations (e.g. blood cell counts).

Tests based on either ordinal or continuous variables require definition of a cut-off point below (or above) which an animal is defined as affected. The majority of serological tests fall into this category.

3.2.2. *Characteristics of tests*

The characteristics of diagnostic tests that can be identified are reliability and validity.

3.2.2.1. Reliability

The ability of a test to produce the same results when repeated under identical conditions.\(^{12}\) This can be affected by several factors including:

\(^{12}\) Some authorities draw a distinction between *repeatability*, defined as the degree of agreement between two or more sets of observations made on the same animals by the *same* observer, and *reproducibility*, defined as as the degree of agreement between two or more sets of observations made on the same animals by *different* observers.
(1) differences in interpretation between different observers (inter-observer variation);
(2) differences in interpretation between the same observer at different times (intra-observer variation);\textsuperscript{13}
(3) conditions within the laboratory or laboratories in which the test is conducted.

In developing countries the reliability of a test may be affected by variations in storage and other suboptimal conditions; the term 'robustness' is sometimes used to describe this component of the repeatability of a test affected by these conditions [65].

Reliability can only be effectively ascertained if the technical conditions under which a test is conducted are stable. This requires standardization of the test. There is now a clear awareness of the need to standardize tests — particularly those for widespread use (e.g. ELISAs [66]). This is complex, and needs to address: (1) random variation (e.g. reagent error in a single test) and (2) systematic variation (bias) (e.g. gradual loss of assay analytical sensitivity due to peroxidase lability in ELISAs). Quality control techniques such as cusum and Shewhart charts can address these issues [67].

Measures of reliability are dictated by the scale of measurement. Thus, dichotomous data can be assessed by computing the kappa coefficient of agreement [68], ordinal data by Cohen's weighted kappa [69] and continuous data by the intraclass correlation coefficient [70], with appropriate measures of precision such as the standard error or a confidence interval. However, the interpretation of these measures in relation to disease morbidity in the field is a neglected area; only recently, for example, has the impact of disease prevalence on the interpretation of kappa been realized [71, 72].

3.2.2.2. Validity

The extent to which, on average (i.e. in the long run), a test reflects the truth.\textsuperscript{14} It is expressed in terms of diagnostic sensitivity (the proportion of affected individuals that are test positive) and diagnostic specificity (the proportion of healthy individuals that are test negative). The need to estimate these is now widely appreciated, but point (sample) estimates are often quoted, without an indication of precision. Estimations based on only a few animals will yield almost useless values. Sample size determination, for estimation to a defined degree of precision, is elementary [1].

\textsuperscript{13} Using the convention of the preceding footnote, repeatability reflects intra-observer variability (e.g. when a test is conducted in the same laboratory by the same staff) whereas reproducibility reflects inter-observer variation (e.g. when a test is conducted in different laboratories).

\textsuperscript{14} It is helpful to distinguish between accuracy — the degree to which an individual result represents a true value — and validity.
Sensitivity and specificity are interpreted in the population in which they are applied in terms of the *predictive value* of a positive test result (the proportion of test positive animals that are true positives), and the predictive value of a negative result (the proportion of test negative animals that are true negatives). This depends on sensitivity, specificity *and* the true prevalence of the disease in the population.

Determination of sensitivity requires identification of an independent, trustworthy criterion on which to base true disease status (a 'gold standard'). Various methods have been used, including: (1) for positive status: experimental infections, and multiple testing strategies, and (2) for negative status: sampling of animals from disease free areas, and multiple testing strategies. However, these methods are not without problems. Thus, experimental studies tend to overestimate the value of a diagnostic test. Use of animals from disease free areas involves assessment of the test in a population *different* from that in which it is applied (this is, of course, also the case with experimentally infected animals). The results cannot necessarily be transferred to the candidate population and, coupled with imprecise estimates, can produce impossible results such as negative values for computed true disease prevalence. A further problem encountered in sequential testing is that test negative animals may not be followed long enough to determine their true status. 'Latent class' models have been proposed to handle this problem [73], but still are still likely to overestimate sensitivity and specificity.

Estimation of sensitivity and specificity has received much attention recently, but possibly at the expense of other methods for assessing the value of diagnostic tests using ordinal or continuous test variables. Thus, *likelihood ratios* [74] compare the proportions of animals with or without disease that record different levels of test result. These are usually more stable than sensitivity and specificity and can be used for different cut-off values. Similarly, a receiver operating characteristic curve (ROC) graphically depicts the effect on diagnosis of selecting a given cut-off point for a test [75].

### 3.2.3. *Meta-analysis*

Meta-analysis is the statistical analysis of data pooled from several studies to integrate findings.\(^{15}\) This technique, which is widely applied in clinical trials [78], can

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\(^{15}\) The term is derived from the Greek preposition μετα (meta) = 'alongside', 'among', 'in connection with'. A subsidiary meaning is 'after'. *Meta-analysis* is therefore either one that is done alongside/in conjunction with the normal analysis, or one that is done after the normal analysis, that is, at a later stage in the process. An alternative term — 'overviews' of research — has also been suggested [76]. The technique has its origins in educational research [77]. In contrast to the traditional, qualitative, review article, *meta-analysis* provides an objective, rigorous means of summarizing research data.
also be used to benefit in assessing diagnostic tests where several studies have generated results. For example, a meta-analysis of ELISA tests for the diagnosis of human and porcine trichinellosis has recently been undertaken [79].

Benefits of meta-analysis include:

1. increase in statistical precision;
2. resolution of uncertainty if there are conflicting results;
3. rigorous support for generalization of a diagnostic technique.

Methods for analysing continuous and categorical data are described in various publications on the technique [78]. However, it needs to be used with caution. In the assessment of diagnostic tests, for example, care would need to be taken to ensure that the populations from which the data were pooled were strictly comparable. Moreover, a meta-analysis should never be considered as a preferable alternative to a single study of suitable size.

3.3. Training in veterinary epidemiology

3.3.1. Early scope of training in epidemiology

Epidemiology has been an integral part of undergraduate veterinary education for many years. The 1971 edition of the World Directory of Veterinary Schools\textsuperscript{16} [80] listed 254 schools in 68 countries, of which 93 cited epidemiology in their curricula. This probably did not reflect the extent of tuition in the subject, but indicated the degree of acceptance of the subject (now nearly 30 years ago) by name. Inspection of the curricula showed that a variety of subjects of relevance to epidemiology were taught without specific reference to it; for example, statistics, animal ecology, biostatistics, veterinary public health, zoonoses, environmental science and agronomy. The focus was then on qualitative epidemiology, with particular reference to infectious diseases. Since then, there has been an expansion of teaching of epidemiology, although it is difficult to document in detail.\textsuperscript{17}

\textsuperscript{16} The only edition of the Directory published since then (1991) does not contain details of curricular content.

\textsuperscript{17} In the UK in 1976, for example, only one veterinary school taught epidemiology as an identifiable subject in the undergraduate degree course, whereas the number has now increased to three. Additionally, existing postgraduate courses are expanding their epidemiological component (e.g. the Masters course in Tropical Veterinary Medicine at Edinburgh University).
3.3.2. Curriculum development

Curriculum development in epidemiology in developed and developing countries has been discussed at meetings of the International Society for Veterinary Epidemiology and Economics [81–83], and veterinary educational reports have recommended an expansion of teaching of the subject [84, 85]. Moreover, several World Health Organization consultative documents have addressed the issue of training in veterinary epidemiology [86–89]. In all cases, the need for teaching of more quantitative techniques was identified, and most contemporary courses are attempting to fulfil this need.

Post-graduate training in veterinary epidemiology is now widely available in graduating and non-graduating courses in Australasia, Europe and North America. Additionally, agencies and organizations are supporting the training of professionals either on short courses or in the context of specific disease control campaigns. (The author is personally familiar, for example, with such courses supported by the European Union18, the Overseas Development Administration of the Foreign Office (UK), the British Council, the German Foundation for International Development (DSE), and the International Atomic Energy Agency.)

3.3.3. Short-course structure for developing countries

In a subject which is expanding rapidly, selection of an appropriate basic course becomes increasingly problematic and there is a danger of making it too advanced, fashionable and sophisticated for its users. A course should relate directly to the main veterinary activities in the country in which it is held. In developing countries, the main challenge over, say, the next 20 years has been identified, above, as the conquest of the major infectious diseases through structured control and eradication campaigns. A second goal is provision of baseline data on the morbidity of diseases not currently subject to national or regional control, on which disease control is initially dependent. Sampling theory (primarily relating to the ‘one sample’ case) should therefore be at the core of a course, supplemented by comparative techniques. Table I lists a suggested structure for such a short (one week or less) course. This should be capable of being conducted without recourse to microcomputers although, where available, the latter could usefully supplement calculator based computations. This structure therefore represents epidemiological ‘intermediate technology’, rather than developing expertise in the newer research areas.

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18 The EU finances training in veterinary epidemiology in two ways. First, it awards ERASMUS (now SOCRATES) and TEMPUS grants to support undergraduate and post-graduate courses, respectively. Secondly, EU funding of animal health projects in developing countries has included provision for epidemiological expertise.
TABLE I. STRUCTURE OF A SHORT COURSE IN EPIDEMIOLOGY APPLICABLE TO DEVELOPING COUNTRIES

1. Introduction
   Scope of epidemiology
   Requirements for developing countries

2. Relevant statistical theory
   Types of quantitative data
   Biological variation
   Sampling variation
   Distribution models
   Parameters of distributions

3. Expression of disease frequency
   Morbidity and mortality
   Crude, specific and adjusted measures
   Spatial representation (mapping)

4. Sampling
   Types of sampling
   Precision of sample estimates
   Sampling for field surveys
   Sampling for eradication campaigns

5. Diagnostic test evaluation
   Validation of tests
   Agreement between tests
   Multiple testing strategies

6. Data collection and management
   Questionnaires and interviews
   Participatory Rural Appraisal
   Databases

7. Comparative methods
   Significance tests
   Confidence interval estimation
   Observational studies
The prospects for tuition in veterinary epidemiology in the next century are therefore bright. Consolidation of training at the undergraduate level clearly offers the widest benefit and is less susceptible to the vagaries of funding than courses taught ad hoc in the context of specific projects.

REFERENCES


[40] WARBLE, A., Veterinary Medical Data Base (VMDB) Update, American Veterinary Computer Society Newsletter, September and October (1994) 8–10.


[79] GREINER, M., BÖHNING, D., DAHMS, S., “Meta-analytic review of ELISA tests for the diagnosis of human and porcine trichinellosis: Which factors are involved in


MONITORING/TRAINING

(Session 4)

Chairperson

M.H. JEGGO
FAO/IAEA
VALIDATION, STANDARDIZATION AND CONTROL OF ELISA TECHNIQUES FOR THE DIAGNOSIS OF INFECTIOUS DISEASES IN VETERINARY MEDICINE

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Abstract

VALIDATION, STANDARDIZATION AND CONTROL OF ELISA TECHNIQUES FOR THE DIAGNOSIS OF INFECTIOUS DISEASES IN VETERINARY MEDICINE.

The decision to use a new enzyme linked immunosorbent assay (ELISA) technique to diagnose infectious diseases in veterinary medicine must be based on careful validation and standardization of the test to ensure a high level of accuracy according to defined criteria. In particular, laboratories performing diagnostic tests related to control, surveillance or eradication programmes need to take care in producing accurate and precise test results in their daily routine. To ensure this, laboratories should have an acceptable level of organization and management that provides sufficient guarantee of producing reliable test results. Measures should include the performance of a well documented internal quality control (QC) programme in which internal control samples are calibrated against national or international reference standards where possible. The internal QC programme should be focused on test precision (random and systemic errors) and, if possible, also on test accuracy (correctness of test results). It is of importance that reference or specialized laboratories make primary or secondary reference standard samples available to diagnostic laboratories in order to enable careful calibration of internal control samples. An objective method of monitoring test accuracy is the participation in (inter)national external QC programmes organized by independent specialized and/or authorized reference laboratories. When possible, diagnostic laboratories should participate in such external QC programmes. Some aspects of the QC programme as used in veterinary immune diagnostic laboratories in the Netherlands are described.

1. INTRODUCTION

The diagnosis of infectious diseases in veterinary medicine must often take place through serological techniques in which antibodies directed against the
pathogen are detected. The use of enzyme linked immunosorbent assays (ELISAs) for
the detection of antibodies over classical techniques such as virus neutralization and
precipitation tests has the advantage of being easy and rapid to perform. Additionally,
guidelines for standardization, validation and control of ELISA techniques can be
given, which is of great importance when these tests are used in national or inter­
national programmes for the control, surveillance and/or eradication of infectious
diseases in veterinary medicine. To date, only limited international standardization
has been carried out. On the basis of a joint FAO (Food and Agriculture Organization
of the United Nations)/IAEA (International Atomic Energy Agency) consultants
meeting which took place in 1992, procedures have been described whose use as
guidelines is recommended for international standardization of ELISA techniques for
the detection of antibodies [1]. These procedures include methods of ELISA data
expression and of the preparation and use of primary reference standards and
recommendations for quality assurance and diagnostic validation.

Additional information on validation, standardization and control of ELISA
techniques, partially based on methods as performed in veterinary diagnostic
laboratories in the Netherlands, will be given in this paper.

2. RELIABILITY, VALIDATION AND STANDARDIZATION OF
A DIAGNOSTIC TEST

The reliability of a diagnostic test is defined by its sensitivity and its specificity.
The sensitivity of a test is its ability to detect animals with a specified disease (infec­
tion). The specificity of a test is its ability to detect non-diseased (non-infected)
animals (Table I).

TABLE I. DEFINITIONS OF THE RELIABILITY (SENSITIVITY AND SPECIF­
cITY) AND THE PREDICTIVE VALUE OF A DIAGNOSTIC TEST

<table>
<thead>
<tr>
<th>Condition of the animal</th>
<th>Infected</th>
<th>Non-infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>A</td>
<td>C</td>
</tr>
<tr>
<td>Negative</td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>A + B</td>
<td>C + D</td>
</tr>
</tbody>
</table>

Sensitivity = A / (A + B)
Specificity = D / (C + D)
Predictive value of a positive test (PV-positive) = A / (A + C)
Predictive value of a negative test (PV-negative) = D / (B + D)
Increasing cut-off value (arbitrary units)

FIG. 1. Test reliability (sensitivity and specificity) as a function of the choice of the cut-off value. Sensitivity and/or specificity are less than 1.0 when the test responses of positive and negative samples partially overlap. When a higher cut-off value is chosen, the number of false negative test results will increase, and the number of false positive test results will decrease.

It should be stressed that the selection of the positive/negative threshold (cut-off value) may strongly influence the reliability of a test because of overlap of test results from non-infected and infected populations (Fig. 1). In addition, as described by Wright et al. [1], the frequency distribution of samples from non-infected and infected animals may differ for a given test when used in different regions or in different countries. As a consequence, the diagnostic reliability of a given test may differ when used in different regions and should be determined by testing samples from well-defined negative (non-infected) and positive (infected) animals reflecting the target population. This demonstration of the diagnostic reliability of a test is called test validation. However, well defined negative and positive samples from the target animal population are not always available, and, as a consequence, the reliability of a test under practical circumstances often is not known and can, at best, only be estimated. When a gold standard test is available, a useful method of evaluating the diagnostic reliability of a test for the detection of antibodies is the examination of random positive and negative samples from the target population. Using the test results obtained, ‘receiver operating characteristics’ (ROC) curves can be created showing the relationship between sensitivity and specificity at different cut-off values. By this method, it is possible to make an objective choice of the optimal cut-off value and to determine the reliability of the test in the target population [2, 3].

Although sensitivity and specificity are important characteristics of a test, the clinician is especially interested in the predictive value of a test result. The predictive value is the probability with which a positive test result or a negative test result
predicts disease (infection) or non-disease (no infection), respectively (Table I). The predictive value of diagnostic test results depends on the reliability (sensitivity and specificity) of the test but, above all, on the disease prevalence in the target population [4]. For a given test system, the relationship between prevalence of a disease and the predictive value of a positive and negative test result is depicted in Fig. 2. By using a test with a specificity <1, the number of false-positive test results will increase when prevalence of disease (infection) decreases. In other words, when prevalence of disease decreases, the predictive value of a positive test result also decreases.

Results of investigations on serological tests for the detection of antibodies directed against bovine herpes virus 1 (BHV1), as summarized below, illustrate the need for standardization (harmonization) of tests. Because of control programmes and international trade activities in Europe, it is of great importance to detect all cattle (latently) infected with BHV1. Therefore, serological tests must be able to detect every BHV1 infected animal (i.e. sensitivity of 1.0) [5, 6]. To determine the ability to detect low levels of BHV1-specific antibodies, sixteen different commercial and non-commercial ELISAs employed in European countries were compared by using 41 bovine sera of well defined origin and containing different levels of specific antibodies. It was found that the ability to detect specific antibodies differed to a great extent between tests. Although all tests were able to identify most negative sera correctly (all tests detected at least 19 of 21 samples correctly), many antibody containing samples were incorrectly classified as negative by several tests. Of 20 positive samples, the percentage of incorrect test results ranged from 0 to 50%. In addition, the ability to detect low levels of specific antibodies as determined by
testing two fold serial dilutions of a positive serum differed greatly between tests: the highest dilution of the serum at which specific antibodies could be detected by the ELISA test kits varied between 1:64 and 1:16 384. Most assays were unable to detect specific IgM antibodies present in sera collected early after infection. The results of this comparative study clearly illustrated the need for standardization [7].

A European interlaboratory trial, organized in March 1995, also revealed large differences between ELISAs for the detection of BHV1 specific antibodies as used in diagnostic laboratories. The ability to detect positive serum samples varied to a great extent: the weak-positive European reference standard (EU2) [6] was correctly classified as positive in only 15 of 25 cases [8]. Moreover, serum samples obtained early after infection (about ten days post infection) were scored as positive by approximately half of the tests.

In summary, to ensure that various ELISA techniques being used by different laboratories in (inter)national control, surveillance and/or eradication programmes have a specified reliability, careful validation and standardization must be carried out beforehand.

On the basis of a given diagnostic application, test validation and standardization should be performed by using a carefully selected test panel comprising (experimentally obtained) samples containing antibodies with defined characteristics (e.g. specificity, avidity, isotype, and/or concentration), (inter)national reference standards and/or negative and positive samples taken from animals randomly selected from the target population.

3. INTERNAL QUALITY CONTROL

Once a test, based on careful validation and standardization, has been selected for diagnostic use, it is of major concern to ascertain that data produced in a laboratory by this test are accurate and precise at all times. Accuracy refers to the extent to which measurements agree with actual ‘true’ values, whereas precision refers to the magnitude of random (within-assay) and systemic (between-assay) errors. A given test may have an intrinsic random error which exceeds acceptable limits. In this situation, testing samples repeatedly and taking the average of the test results may lower this error satisfactorily.

Results which concern levels of specific antibodies cannot be referred to as ‘true’ values (exact concentrations) because of differences between test samples with respect to avidity, isotype and antigenic (or epitope) specificity of the polyclonal antibodies. As an alternative for referring to ‘true’ values (analytical meaning), one may define accuracy of an ELISA for the detection of antibodies as the correctness of test results, i.e. the proportion of samples in which the absence or presence of specific antibodies is correctly scored in the test:
Accuracy = \frac{TN + TP}{TN + FN + TP + FP}

where TN = true negative, FN = false negative, TP = true positive and FP = false positive.

For a given ELISA, accuracy may greatly be affected by variations and/or shifting in the detection limit. Monitoring the detection limit of an ELISA can be performed by incorporating, in each test run, one or more threshold positive standards and/or serial dilutions of positive standards (end point titration).

As agreed at the joint FAO/IAEA meeting in 1992, working standards, used in daily routine to monitor accuracy, should be compared and calibrated against primary reference standards which include a positive, a weak-positive and a negative sample. Data in indirect ELISAs are expressed as per cent positivity (PP), which is defined as the optical density of the sample relative to a positive reference standard. In competitive or blocking ELISAs, data are expressed as per cent inhibition (PI) [1].

As working standards in daily routine, it is our preference to use — in addition to a positive, a weak-positive and a negative sample — a negative sample spiked with a small volume of a positive sample. The spiked sample should contain specific antibodies just below the detection level of the test (see C2 in Fig. 3). By using these four

**FIG. 3. Hypothetical dose response curve of an ELISA (antibody concentration versus test signal).** Two negative (C1 and C2) and two positive (C3 and C4) control samples have been chosen of which C2 and C3 cover the linear portion of the curve. The controls should preferably be calibrated against reference standards. Shifting of the curve for any reason results in incorrect scoring of the control samples. In the figure, a shift of the curve to the left results in an incorrect positive score of control sample C2 (2'). In contrast, a shift to the right results in an incorrect negative score of control sample C3 (3'').
working standards in each ELISA test plate, random and systemic variation, affecting the accuracy of the test, can easily be detected. The results of an ELISA test plate are rejected when one or more of the two negative and the two positive standards are classified incorrectly (Fig. 4). In addition, the use of control charts to monitor the per cent positivity (PP, indirect ELISA) or the per cent inhibition (PI, competitive or blocking ELISA) of the working standards is easy to implement. By this procedure, control limits can be defined and data trends can be detected. Veterinary immune diagnostic laboratories in the Netherlands generally incorporate at least two independent internal control samples with antibody activity lying on the linear portion of the dose/response curve of the test. Control limits, usually the average PP or PI ± 2 standard deviations, are calculated from test results obtained on 20 different days. After that, control samples are incorporated in each of the ELISA plates that are run during routine testing, and the results of controls are monitored using control charts. Westgard rules have been used for acceptance or rejection of test results [9].

As an alternative, a simple and reliable internal quality control (QC) method for the detection of random and systemic variation is the use of a Shewhart cusum (cumulative sum) control chart method [10, 11]. This method uses three internal control samples, the responses of which fall within the linear portion of the dose response curve of the test. To include careful monitoring of accuracy, the internal controls should be calibrated against reference standards. A QC variable (\(\gamma_i\)) is calculated as being the mean of the test response of the three controls. Absolute upper and lower control limits are set using the standard deviation of \(\gamma_i\), calculated from data from at least 15 tests performed under routine diagnostic conditions. The procedure detects data trends by cusum calculation as well as out of control limits and allows a simple decision process to accept or reject a test run [10, 11].

In addition to the control methods mentioned above, the performance of a test within a laboratory can be checked by randomly inserting samples of known antibody level in between samples for routine testing.

It should be stressed that control samples forming part of a commercial test kit should never be used as samples for internal control. No useful information whatsoever can be obtained about the accuracy between kit batches when using control samples from the kit.

In order to provide a sufficient guarantee of producing reliable test results in daily routine, laboratories should have, in addition to a well documented internal control QC programme, an acceptable level of organization and management [12]. Both the Institute of Animal Science and Health (ID-DLO) and the Animal Health Service in the Netherlands have been formally accredited by the 'Council for Accreditation'. The criteria laid down by the Council are in compliance with ISO/IEC Guide 25 ("General Requirements for the Competence of Calibration and Testing Laboratories") and with EN 45001 ("General Criteria for the Operation of Testing Laboratories"). The criteria include aspects such as qualification and training of personnel;
descriptions of duties and responsibilities which go with the jobs; validation and documentation of diagnostic tests performed; strict application of approved standard operating procedures and work instructions; calibration and maintenance of equipment; approval and signing of reports of test results by authorized personnel; and periodic assessment of the effectiveness of the quality system. The aspects of a laboratory quality system as mentioned above clearly contribute to the improvement and maintenance of producing reliable and consistent routine diagnostic test results.

4. **EXTERNAL QC**

External QC primarily focuses on test accuracy (correct test result, see Fig. 3). ELISA techniques developed to detect specific antibodies, especially those used at several locations within the same control, surveillance and/or eradication programmes, should be able to classify defined positive, weak-positive and negative reference standards correctly. In other words, test results must be comparable between laboratories and fulfill defined reliability criteria. For this reason, reference standards or samples calibrated against these references should regularly be sent under code to the participating laboratories performing the test. When considering external QC programmes (i.e. interlaboratory investigation), the organization should be entrusted to a single laboratory, preferably a reference or specialized laboratory. In this way, the reliability of the diagnoses performed by each of the participating laboratories can then be evaluated without bias. All participants receiving the analysed results of their performance are enabled to identify inaccuracies and to correct the problems by evaluating methods, reagents, instruments and/or performance.

Besides participating in external QC programmes, the exchange between laboratories of coded positive and negative test samples provide an additional convenient means of assessing interlaboratory consistency.

As was mentioned earlier, random and systemic errors affect the accuracy of a test unfavourably. For this reason, it may be meaningful to incorporate objective checks for random and systemic errors in external QC programmes. Random errors can be checked objectively by distributing coded QC samples in duplicate to all participating laboratories. When tested in one test run, differences between duplicates are the result of random errors. When the same set of samples is distributed on a frequent (e.g. monthly) basis and coded differently at each occasion, analyses of the reported results will yield information about between-assay errors which may be the result of differences between analysts (performance), environmental conditions (temperature), reagents and/or instruments.

When different laboratories use the same ELISA technique from one producer or manufacturer, the possibility is offered to perform external QC on a more quantitative basis. For this purpose, serial dilutions of samples can be produced, covering
the linear region of the dose response curve of the test, and distributed under code, preferably as duplicates. The results produced by the testing laboratories should be expressed as per cent positivity (PP, indirect ELISA) or per cent inhibition (PI, blocking or competitive ELISA) relative to a reference standard [1]. By using these data, calculations can be performed to estimate the variation (precision) within assays, between assays and between laboratories. Alternatively, results can also be expressed as titres and titre variations.

Since 1992, an external QC programme has been running in the Netherlands, based on a method described in the International Standard ISO 5725-1986: ‘Precision of Test Methods — Determination of Repeatability and Reproducibility for a Standard Test Method by Interlaboratory Tests’. The six veterinary diagnostic laboratories that participate in the programme receive a coded set of six lyophilized serum samples every three months from the reference institute (ID-DLO). The six samples comprise three sera with different levels of specific antibodies. Each level was split beforehand into two slightly different sublevels, a and b, by adding negative serum (split level method; see ISO 5725-1986). From the data received from the participants, the reference institute calculates the geometric mean titres and repeatability and reproducibility values according to ISO 5725-1986 (see Table II). The repeatability (r) has been defined as the value below which the absolute difference between duplicate test results within one test run may be expected to lie ($|x_1 - x_2| < r$).

### Table II. Typical Example of Results of an Inter-Laboratory Investigation of an ELISA for the Detection of Specific Antibodies in Serum as Performed in the Netherlands

<table>
<thead>
<tr>
<th>Code of participating laboratory</th>
<th>Level 1 (log titre)</th>
<th>Level 2 (log titre)</th>
<th>Level 3 (log titre)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>a</td>
</tr>
<tr>
<td>1:</td>
<td>1.90</td>
<td>2.20</td>
<td>2.51</td>
</tr>
<tr>
<td>2:</td>
<td>2.20</td>
<td>2.51</td>
<td>2.81</td>
</tr>
<tr>
<td>3:</td>
<td>2.20</td>
<td>2.20</td>
<td>2.51</td>
</tr>
<tr>
<td>4:</td>
<td>1.90</td>
<td>2.20</td>
<td>2.51</td>
</tr>
<tr>
<td>5:</td>
<td>2.20</td>
<td>2.20</td>
<td>2.51</td>
</tr>
<tr>
<td>6:</td>
<td>2.51</td>
<td>2.51</td>
<td>2.81</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean (log titre):</th>
<th>Geometric mean (titre):</th>
<th>Repeatability (r):</th>
<th>Relative r:</th>
<th>Reproducibility (R):</th>
<th>Relative R:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.23</td>
<td>170</td>
<td>0.330</td>
<td>15%</td>
<td>0.551</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>2.73</td>
<td>538</td>
<td>0.246</td>
<td>9%</td>
<td>0.396</td>
<td>15%</td>
</tr>
<tr>
<td></td>
<td>3.17</td>
<td>1479</td>
<td>0.482</td>
<td>15%</td>
<td>0.748</td>
<td>24%</td>
</tr>
</tbody>
</table>
The reproducibility (R) has been defined as the value below which the absolute difference between the test results on the same sample, but obtained by two different laboratories, may be expected to lie (|x₁ - x₂| < R). It is easy to understand that repeatability is determined by the within-assay error (= random error), whereas reproducibility is determined by errors within the assay (random errors) as well as by errors between assays and between laboratories (= systemic errors). By using the same set of lyophilized samples that are differently coded on every occasion, each of the participating laboratories is able to carefully evaluate accuracy and precision longitudinally.

A disadvantage of the external QC method just described is the inaccuracy of the calculated repeatability and reproducibility when based on only a few investigations. The reason for this is the limited number of participants (n = 6). Additionally, calculations can only be performed and results between laboratories can only be compared when all participants use the same test for their routine diagnosis.

Recently, four of the laboratories that participated in the external QC programme in the Netherlands have been reorganized and merged. As a consequence, routine immune diagnostic tests are, at present, performed at only three or four locations. For this reason, external QC programmes will be organized in a different way. In the future, accuracy and precision will be monitored by using one and the same set of working standards by all the laboratories. Each participating laboratory is able to monitor within and between assay results by using these standards. In addition, at regularly time intervals the results of the standards as obtained by the participants will be compared. To monitor accuracy in a more objective manner, coded panels of positive, weak-positive and negative samples, will be sent regularly throughout the year by the reference institute.

REFERENCES


969–984.


Comparative study on sixteen enzyme-linked immunosorbent assays for the detection of

interlaboratory trial to evaluate the reliability of serological diagnosis of bovine


Shewart-cusum control chart for improved quality control in clinical chemistry, Clin.
Chem. 23 (1977) 1881–1887.

combined Shewart-cusum control charts in internal quality control of enzyme-linked
immunosorbent assays for the typing of foot and mouth disease virus antigen, Rev. Sci.

THE MONITORING OF THE PAN AFRICAN
RINDERPEST CAMPAIGN AND THE ESTABLISHMENT
OF DISEASE SURVEILLANCE SYSTEMS IN AFRICA

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Abstract
THE MONITORING OF THE PAN AFRICAN RINDERPEST CAMPAIGN AND THE
ESTABLISHMENT OF DISEASE SURVEILLANCE SYSTEMS IN AFRICA.

The background and the rationale for the monitoring of national and regional disease
control and eradication programmes in developing countries focusing on the Pan African
Rinderpest Campaign (PARC) are described. The technological and administrative aspects of
the establishment of a system to serologically monitor the success of vaccination against
rinderpest and the requirements to establish and maintain a diagnostic capability for the major
epizootics in laboratories of developing countries are discussed. The advantages of the enzyme
linked immunosorbent assay (ELISA), which evolved as the main diagnostic technology and is
now the most widely used serological technique in mass screening, are presented. The progress
towards the final eradication of rinderpest is regulated through a set of guidelines of the Office
international des épizooties (OIE) and culminates in the final (OIE) declaration of freedom
from infection. Countries are required to operate intensive disease surveillance programmes
based on statistically and epidemiologically defined standards. These programmes, which now
also include clinical surveillance, are described. They are based on active disease search as
opposed to passive disease reporting systems as relied upon in the past by veterinary services
in Africa.

1. BACKGROUND

1.1. Pan African Rinderpest Campaign (PARC)

After the reappearance of rinderpest in Africa in the 1980s the Pan African
Rinderpest Campaign (PARC) commenced as the second attempt to eradicate the dis-
ease from Africa. The rationale of PARC was to vaccinate national cattle populations
until immunity levels of around 80–90% were achieved. Several studies had clearly
shown that immunity levels of this order would stop the circulation of the rinderpest
virus. After a period of intensive vaccination with verified levels of immunity,
countries would cease vaccination and enter into a surveillance phase to identify any remaining pockets of virus activity.

Eradication is not achieved until the infectious agent is eliminated, and, in the case of rinderpest, since the causative virus cannot survive outside an animal, an intensive surveillance programme only needs to concentrate on identifying infected animals. This surveillance component was considered essential to prevent the failures of past programmes which were largely attributable to an inability to identify and report remaining foci of infection after the epidemic form of the disease had disappeared [1].

1.2. The OIE Pathway

The Office international des épizooties (OIE) has published guidelines defining a declaration of freedom from rinderpest. These guidelines demand active surveillance systems with statistically and epidemiologically sound structures [2]. A series of national and OIE declarations described as the ‘OIE Pathway’ culminate in a final OIE declaration of freedom from infection (Fig. 1) [3].

1.3. Monitoring and disease surveillance

Before the development of a suitable immunodiagnostic test for mass screening, disease surveillance was based on clinical surveillance for pathognomonic signs of a disease. Surveillance of the human disease smallpox, the only disease that has been eradicated globally, was based on such an approach and involved intensive clinical surveillance, supported through adequate legislation and a financial reward system [4]. In terms of animal diseases, the major epizootics in Europe were also controlled or eradicated through clinical surveillance and strict movement controls, despite the lack of a clinical test or even knowledge of the causative agent. Indeed, rinderpest was eradicated from the United Kingdom in 1877, long before the disease agent was identified as a virus in 1902. Similarly, contagious bovine pleuropneumonia (CBPP) was eradicated from the USA in 1892, before the etiological agent of this disease was known [5].

These successes were based on the existence of an effective government veterinary service with adequate financial, operational and legislative support to operate in all areas and to investigate all suspected cases. Equally important to the success, however, was the clinical severity of the disease being dealt with. Whilst this resulted in the disappearance of the major epizootics from developed countries many years ago, success against the less dramatic diseases has still been achieved through systematic surveillance, based on sound epidemiological sampling frames and laboratory based diagnosis and monitoring. Essential to the success of this approach is a cost–benefit analysis to demonstrate the economic importance of the disease in
FIG. 1. OIE Pathway.
question. Such programmes rely mainly on serological testing to monitor the progress of the programme and to verify the final eradication of the disease.

1.4. Diagnostic techniques

When PARC commenced in 1986, the majority of participating countries involved in rinderpest control had neither the field nor the laboratory capabilities necessary to diagnose and monitor rinderpest or indeed most of the other major epizootics [6]. Most of the national veterinary laboratories had very little in the way of operational funds and were limited in terms of the necessary technological capabilities to diagnose. At that time, to meet their diagnostic requirements, a multitude of diagnostic procedures were necessary, each one requiring specified equipment, reagents and training. In reality, few of these procedures were operational, and little diagnostic work was carried out. The situation was further compounded by a lack of laboratory vehicles and support resources to collect samples from the field, and few samples were being routinely submitted.

At that time, the ELISA was emerging as the most suitable technique for diagnostic screening; as it offered solutions to many of the problems being encountered by national veterinary laboratories in developing countries. Central to the adoption of the ELISA is its ability to diagnose a very wide range of diseases. Other advantages include simplicity of use, low cost, ease of automation, high sample throughput and ease of standardization [7].

2. ESTABLISHMENT OF A DIAGNOSTIC LABORATORY NETWORK

PARC from the outset required national veterinary services to monitor their vaccination campaigns. At precisely the same time, 1986, the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture began a new programme aimed at supporting the introduction and use of ELISA technology in developing countries for improving animal disease diagnosis and monitoring. The ELISA was quickly shown to be the ideal diagnostic tool for sero-monitoring the rinderpest vaccination campaign. It was highly appropriate therefore to link the sero-monitoring need of PARC with the this new support programme. But how best to transfer and use this technology? Following an evaluation of available laboratory services within PARC countries, it was apparent that a complete support package was needed, covering all aspects of the ELISA [6]. This included the equipment to operate the technology (ELISA reader, pipettes, plate shakers, etc.), the consumables and reagents (e.g. pipette tips, ELISA reagents) and the training and technical support (e.g. training courses, fellowships, expert visits). Since the laboratories would essentially be undertaking identical operations it was possible to develop a standardized approach to
almost all activities ranging from the ELISA kits themselves to the design of the sampling frames for sample collection.

Underlying the whole approach was the concept of a ‘network’ of national laboratories (Fig. 2), each monitoring its own national vaccination programme but using identical procedures in terms of sample collection, testing and reporting. This approach would not only ensure that results could be compared from country to country but would facilitate training, equipment and reagent supply and troubleshooting when problems arose.

Support for this network and the transfer of the technology was provided through both IAEA Technical Co-operation Projects (TCPs) — regional and national (Table I) — and an FAO/IAEA Co-ordinated Research Programme (CRP) partially funded by the Swedish International Development Agency (SIDA). In each country a research contract under the CRP was awarded to the national veterinary laboratory, which primarily paid for the ELISA kits and other consumables. The major items of equipment (ELISA reader, computer, etc.) were usually funded through national or regional IAEA TCPs. Each year a research co-ordination meeting was held at which the results from the previous year’s testing were discussed along with any problems that had arisen. The proceedings of these RCMs were published on an annual basis [8–11].

It should be noted that support focused on the transfer and application of the ELISA technology and not on the development of a national capability to produce ELISA kits. Financial resources and the availability of adequately trained
TABLE I. TECHNICAL CO-OPERATION PROJECTS

<table>
<thead>
<tr>
<th>Country</th>
<th>Related IAEA TCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burkina Faso</td>
<td>Non-IAEA Member State</td>
</tr>
<tr>
<td>Benin</td>
<td>Non-IAEA Member State</td>
</tr>
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<td>Cameroon</td>
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</tr>
<tr>
<td>Egypt</td>
<td>RAF/5/043</td>
</tr>
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<td>Gambia</td>
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<td>Ghana</td>
<td>RAF/5/043</td>
</tr>
<tr>
<td>Kenya</td>
<td>KEN/5/019</td>
</tr>
<tr>
<td>Mali</td>
<td>MLI/5/013</td>
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<td>Mauritania</td>
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<td>SUD/5/020</td>
</tr>
<tr>
<td>Uganda</td>
<td>RAF/5/043</td>
</tr>
<tr>
<td>United Republic of Tanzania</td>
<td>RAF/5/043</td>
</tr>
</tbody>
</table>

personnel are limited in developing countries, and it was felt that the focus should be on problem solving rather than on assay development and production. Added to this approach is the clear advantage of international standardization inherent in the use of a common validated and freely available assay protocol and reagents [12].

Beyond the task of sero-monitoring, rapid and accurate laboratory diagnosis to confirm or refute an initial clinical diagnosis is essential. National laboratories must also be capable of diagnosing rinderpest by using the agar gel immunodiffusion (AGID) and the ELISA for antigen detection [3]. Within the network, the national laboratories were also linked to the World Reference Laboratory (WRL), and systems for sample submission from the national laboratories to the regional reference laboratories and to the WRL were created.

Since the results of the diagnostic tests are the basis on which to make OIE declarations it is essential that such an ELISA is internationally validated and, once introduced into the national veterinary laboratories, its quality is controlled to give confidence about the validity of the results to national authorities and international
bodies. A system of external quality assurance (EQA) ensured the validity of the results produced by the national laboratories. A detailed account of the EQA system is given elsewhere [13].

3. THE ROLE OF FIELD SERVICES IN THE SURVEILLANCE

3.1. Sample and data collection

Sero-monitoring of the vaccination campaign requires a relatively straightforward approach to field activities. Whilst it is essential to explain to livestock owners why samples have to be collected and to maintain a dialogue with them to ensure that the process can be repeated on an annual basis, the logistics of organizing sampling teams is clear, and the greatest problem is to obtain the necessary resources [14].

Surveillance to detect any remaining disease or virus activity and to confirm eradication after the cessation of vaccination is considerably more complicated. Efficient surveillance systems must make use of all possible information sources. It is necessary that a strong public awareness of the objectives of the programme is created. Different target groups need to be identified and specifically addressed through extension programmes using radio broadcasts, posters, etc. It is essential that disease reporting is positively encouraged with, if appropriate, incentives (for example, in the small pox eradication campaign a reward was offered for the last report of a clinical case). Surveillance relies on the systematic collection of high-quality, accurate and valid information and samples.

The OIE Pathway demands that the post-vaccination surveillance procedure should be based on a random sampling frame which is sensitive enough to detect rinderpest with a probability of 95% if it is present in 1% of the herds [2]. These random surveys are supplemented through purposive surveys which are based on the experience of the surveyor and concentrate on areas and populations where the infection is likely to persist. Once again though, surveillance activities are standardized and structured to a high degree, and standardized questionnaires (Fig. 3) are used in the random sampling process and in the case of outbreak investigations to collect additional information.

In most countries the sero-monitoring component of the national PARC programme was primarily driven by the laboratory. Although sample collection was often done with the support of the veterinary field service, this was not a prerequisite. In terms of the surveillance phase which precedes the cessation of vaccination, whilst this has a laboratory component of serological testing and confirmation of a suspect field diagnosis, the emphasis is very much on the field collection of information and clinical surveillance. This phase of PARC, therefore, must be driven by the field service and not the veterinary laboratory. From the outset, many more people will be
FIG. 3. Rinderpest outbreak investigation report.

involved (Fig. 4), and the resources will be more complex. Veterinary field staff will need to be fully informed about all aspects of the programme and trained in the surveillance processes. Links to the laboratory will have to be effective and constant, with a two-way flow of information. Within PARC this process is only just under way.
To date, only Egypt and the Gambia have actually ceased vaccination and made an OIE Provisional Declaration of Freedom from Rinderpest. However, it is anticipated that many more countries in West Africa will make such a declaration in 1997, and all will require that a surveillance programme be in place.

3.2. Investigative work

Disease surveillance fails if rumours of disease are not adequately followed up and investigated. Rumours must be responded to without delay by the veterinary field services. The clinical inspection of suspected cases includes sample taking for laboratory confirmation (Fig. 5). Laboratory services should be involved from the outset and should feed back results as soon as these become available. If the suspicion is confirmed it must be followed up by tracing back the origin of the infection. All information should be documented, preferably in a standardized format.

4. REPORTING AND COMMUNICATION

The success of the surveillance phase will be based on effective information exchange between livestock owners, field veterinarians, animal health workers, laboratory personnel and those in government. A major barrier seen already in
rinderpest surveillance has been the lack of reporting from farmers upwards. To overcome these problems the surveillance of the rinderpest eradication campaign will use standardized structured reporting procedures. Generic performance indicators
have been developed which can be utilized by a country to determine if an effective system has been established and is working effectively and routinely. To help ensure that surveillance is an active and not a passive operation, the OIE Pathway guidelines stipulate a minimum number of herd investigations that must be carried out [2].

To assist data and information management, a number of computer software programs have been developed. At the field level, information should be entered in an FAO/IAEA computerized database (SID III), and all the diagnostic results produced at the laboratory are processed through an FAO/IAEA software program (EDI). The development of the surveillance network will be accompanied by annual RCMs and the publication of annual surveillance reports. The first of such meetings is planned to take place in Mali in mid-1997.

5. CONCLUSIONS

For the success of a large disease control programme such as PARC, it is critical that the various stages are monitored and that the eradication of the disease is verified. After vaccination has ceased, any decision to recommence vaccination should be taken only if rinderpest has been confirmed unanimously by laboratory diagnosis. These factors have created the need for more efficient disease surveillance in developing countries.

The diagnostic capability at the laboratory level to monitor large scale eradication programmes has been successfully established in the majority of national veterinary laboratories in Africa. The ELISA is no longer considered just a diagnostic test but an epidemiological tool in the monitoring process. It is essential that monitoring activities be integrated into any disease control programme and that an appropriate budget for these activities be available. Although the costs for the sample collection in the monitoring and surveillance process of a disease control programme seem substantial, they are minor to the overall cost of the programme and to the costs that arise if such a programme fails.

The laboratory network to support the surveillance of rinderpest and the diagnostic ELISA capability created in the other veterinary laboratories of Africa will benefit future disease control programmes once the disease is eradicated. The serum banks set up during the monitoring of the rinderpest vaccination campaign and in the subsequent clinical surveillance are now being used to investigate the distribution of other diseases such as peste des petits ruminants (PPR) and brucellosis.

In regionally based eradication programmes it is essential that procedures be harmonized. During the sero-monitoring phase of PARC, standardization in the data and sample collection and in the laboratory procedures has facilitated the implementation and the technical support of the programme enormously. Thus, the network approach and the operation of the external quality assurance programme ensured
that the data generated within the programme were reliable and transparent and could easily be compared. This created public confidence in the whole eradication programme.

Unfortunately, resources are frequently made available only during the early stages, when disease is rife, animals are dying and losses are great. Once the programme is effective in removing disease, such resources tend to become less, both in amount and in speed of availability. In any eradication programme it is vital to complete the task and verify success. PARC is entering this dangerous phase, and it is critical that resources continue to be made available to complete the elimination of the rinderpest virus from Africa. A critical delay in funding is now being experienced as PARC gradually changes from vaccination to surveillance. This could be fatal.

REFERENCES

[12] JEGGO, M.H., GEIGER, R., DARGIE, J.D., Economics of the development and application of biotechnology-based immunoassay tests for controlling animal diseases...
in developing countries. A viable proposition at the national level?, paper presented at the Symposium on Tropical Veterinary Medicine, Berlin, 1992.


THE FAO/IAEA EXTERNAL QUALITY ASSURANCE PROGRAMME FOR DISEASE DIAGNOSIS

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Abstract

THE FAO/IAEA EXTERNAL QUALITY ASSURANCE PROGRAMME FOR DISEASE DIAGNOSIS.

As part of the programme of support to scientists in developing countries, the Animal Production and Health Subprogramme of the Joint FAO/IAEA Programme has developed and distributed standardized and validated enzyme linked immunosorbent assay (ELISA) kits. These kits are used in epidemiological studies, in vaccination monitoring programmes and in control and eradication programmes. In order to assure that the test results from a laboratory using FAO/IAEA ELISA kits are valid and reliable an external quality assurance programme (EQAP) was implemented. The EQAP consists of three equally important elements: a questionnaire to provide evidence of routine use of internal quality control practices and satisfactory infrastructure services, the monitoring of the internal quality control (IQC) data as produced for each ELISA plate and an external quality control test panel of 'unknown' samples. At present, the EQAP is operational for the following FAO/IAEA ELISA kits: the rinderpest competitive ELISA, the brucella indirect ELISA, the trypanosomosis direct sandwich ELISA, the foot-and-mouth disease virus (FMDV) indirect sandwich ELISA and the FMDV liquid phase blocking ELISA. The results clearly indicate that the EQAP is a valuable tool in the assessment of both the results derived from and use of the ELISA kits provided through the Joint FAO/IAEA Programme. Furthermore, the EQAP can assist counterpart scientists to establish and implement quality control/quality assurance (QC/QA) procedures for conducting the FAO/IAEA ELISA and to advise on the implementation of similar QC/QA procedures in other laboratory activities. In the long term, it is envisaged that the FAO/IAEA EQAP would provide a model for an internationally accepted veterinary laboratory accreditation system. This type of system will be essential to veterinary authorities in developing countries to establish credibility in their animal disease reporting schemes and, thus, in their ability to engage in the international trade of food animal commodities under the conditions established by the World Trade Organization (WTO).

1. INTRODUCTION

For any testing laboratory, it is essential that assurance can be given that test results are accurate and reliable. Equally important is an ability to compare results
between different laboratories involved in similar assessments. Many diagnostic tests contain an element of subjectivity in their interpretation of results, which renders both internal and external assurance difficult to operate. One of the distinct advantages of an enzyme linked immunosorbent assay (ELISA) based system is the objectivity of the results and the ease with which data can be processed. Furthermore, it is possible to incorporate a high level of internal quality control for every ELISA test plate.

In 1992, an FAO/IAEA consultants meeting was convened to define and establish standards for internal quality control and data expression for ELISA. Internal quality control comprises not only the monitoring of variables which might affect assay performance but also the use of internal quality control (IQC) samples on every ELISA plate [1]. The recommendations of that meeting have now been incorporated into all FAO/IAEA ELISA kits and adopted by the Office international des épizooties (OIE).

The primary purpose of conducting internal quality control is to ensure that the assay is performing within defined limits [1, 2]. Equally important is the assurance, not only to the test operator but to all outside interested bodies such as (national) veterinary authorities, international organizations, donor organizations and trading partners, that the results derived by the testing laboratory are valid and reliable. In order to confirm this assurance a routinely conducted external quality assurance programme (EQAP) becomes essential [3, 4].

In 1990, an external quality assurance exercise was carried out for the FAO/IAEA rinderpest indirect ELISA kit. These results have been published in detail [5]. In 1991, laboratories involved in the Pan African rinderpest campaign (PARC) changed over to a competitive ELISA rinderpest kit. In determining an external quality assurance for this competitive ELISA, a test panel of 40 unknown sera was distributed among the PARC laboratories. In 1992 to 1994, such a panel was sent out to, respectively, 20, 21 and 17 participating laboratories [6–8].

In September 1994, an FAO/IAEA consultants meeting was convened with the aim of extending and further improving this EQAP for veterinary laboratories in developing countries utilizing FAO/IAEA ELISA kits. The meeting focused on establishing procedures that would ‘recognize’ veterinary laboratories as competent in utilizing FAO/IAEA ELISA kits for specific diseases. The improved EQAP as formulated by the consultants is based on the proof of the presence and use of quality assurance/quality control (QA/QC) systems, the continued satisfactory performance of processes and output, and the use of external quality control procedures applied in conjunction with FAO/IAEA ELISA kits [9].

To obtain such proof, the EQAP consists of three critical elements:

A. **Survey questionnaire**

A questionnaire based survey, conducted twice a year, of individual laboratories to provide a regular system of monitoring the presence and use of a number of
key quality elements such as the presence of trained staff, maintenance and calibration procedures of ELISA equipment, and available laboratory facilities. The information collected through the questionnaire should be updated at least once in a year by the officer in charge\(^1\) and should be supplemented by information from the appropriate staff of the Animal Health and Production Subprogramme.

**B. The evaluation of IQC data**

The routine performance of the ELISA will be determined by using the IQC data as they are produced for each ELISA plate. In the initial stages of the EQAP, it is acceptable for laboratories to provide relevant IQC data to the co-ordinator for evaluation. However, laboratories should implement the regular use of these control charts as a component of the routine internal monitoring of test performance. In the future the laboratories will be asked to generate suitable control charts for assessment by the EQAP co-ordinator.

**C. Proficiency testing by means of an external quality control (EQC) test panel**

For interlaboratory proficiency testing, each laboratory conducts the designated test method on a defined panel of five test samples (the EQC test panel). Identical panels of test samples are dispatched simultaneously to participating laboratories for concurrent testing. This exercise is conducted twice a year.

2. **EQAP PROCEDURES**

The EQAP itself is a continual process. However, the questionnaire and the EQC test panel are sent out twice a year, and at the same time the participants are asked to provide the EQAP co-ordinator with the necessary IQC data to assess the test performance. Twice a year the EQAP co-ordinator evaluates the test performance within a laboratory on the basis of the information collected through the questionnaire, the assessment of the IQC data and the analysis of the EQC test panel results. The EQAP co-ordinator and the other staff members of the Animal Production and Health Subprogramme are in a constant dialogue in order to assure prompt action if the EQAP identifies problems within a participating laboratory.

The EQAP results and evaluations are presented in a report prepared by the EQAP co-ordinator and distributed among the participants, the staff of the Animal Production and Health Subprogramme and other outside interested bodies. For EQAP

\(^1\) The officer in charge is the officer responsible for diagnosis and monitoring disease in an EQAP participating laboratory.
reporting purposes, laboratories are identified only on the basis of unique code numbers.

The EQAP in its current format focuses on FAO/IAEA ELISA kits for disease diagnosis; this means that per EQAP programme the participating laboratories are all using the same standardized FAO/IAEA ELISA kit. This use of a standardized assay by the participants is critical to the approach that was adopted for the FAO/IAEA EQAP.

2.1. Questionnaire

The Questionnaire consists of eight categories, A to H:

A: Administrative information
B: General information on other diagnostic activities performed in the laboratory
C: Laboratory facilities
D: Maintenance and calibration of equipment
E: Handling of test results
F: Monitoring of IQC data
G: Laboratory staff
H: Other QA procedures within the laboratory

The majority of the questions are of a multiple choice type. During each EQAP round, the officer in charge is asked to review the last completed questionnaire and update it whenever appropriate. The collected information is categorized and presented in each EQAP report.

2.2. IQC data

The obligatory routine use of a defined set of IQC samples on every ELISA plate as part of FAO/IAEA ELISAs provides the basis for the internal quality control assessment. Included on every ELISA plate, the IQC data not only indicate whether an assay was operated within acceptable limits but also contain information on the variability within and between assays. Hence, these data are very valuable in assessing the test performance in an individual laboratory. They also enable the test operator to foresee problems, even before they arise, through continual use of control charts; the data are essential for effective trouble shooting in the assay [2, 4, 10, 11]. The routine and continual evaluation of the IQC data should therefore be considered a very critical component of the EQAP.

The IQC samples for the FAO/IAEA trypanosomosis and the brucella indirect ELISA consist of four replicates of the high positive control (C++), of the medium positive control (C+), of the negative control (C−) and of the conjugate control (Cc). The IQC samples for the FAO/IAEA rinderpest competitive ELISA consist of four
replicates of the C+ and C++ controls, four replicates of the monoclonal control (Cm), and two replicates for the C− and Cc controls. The FAO/IAEA FMDV ELISA for antibody detection consists of four replicates for the C++, C+, C− and the antigen (Ca) controls. No fixed upper and lower limits for IQC samples of the FMDV indirect sandwich ELISA for antigen detection are given (the replicates for the C++, C+ and the buffer control (Cb)). However, if the mean of the optical density (OD) replicates for the Cb is below 0.1 for all FMDV serotypes and the corrected mean OD values for the moderate and high controls (C+, C++) are greater than 0.1 and 1.0, respectively, the test results can be accepted unconditionally [12–16].

The IQC samples have been tested extensively, 200 to 350 times, under routine diagnostic conditions, either by the FAO/IAEA Centre for ELISA and Molecular Techniques in Animal Disease Diagnosis, an OIE ELISA collaborating centre (the IQC samples for the brucella indirect ELISA, trypanosomosis direct ELISA) or in a world reference laboratory2 (the IQC samples for the rinderpest competitive ELISA and FMDV ELISA, both antibody and antigen detection). Given that the variation in OD values and percentage positivity/percentage inhibition (PP/PI) values should be normally distributed, the ±2 or 3 standard deviation (SD) ranges have been calculated. These values are used to set the upper control limit (+2 or +3 SD) and the lower control limit (−2 or −3 SD) of each IQC serum sample and are provided with each ELISA kit [12–16].

Laboratories using an FAO/IAEA ELISA kit are provided with the FAO/IAEA computer software program ELISA Data Interchange (EDI). This program automates the reading and calculation of the test results when using FAO/IAEA ELISA kits and works with most Multiskan or equivalent ELISA plate readers. The EDI program produces a standard format per ELISA plate with all plate details, such as date, test operator identification, plate identification, IQC data and their limits, and the OD values and PP/PI values of the test samples. The EDI program has been written specifically for use with the FAO/IAEA ELISA kits. The EDI program automatically creates an ‘instat.qc’ file when the first ELISA plate is read. Each time an ELISA plate is read the EDI program automatically writes the IQC data and plate identification number to this ‘instat.qc’ file.

As part of the EQAP, the participating laboratories received a floppy disk containing a batch file to copy the ‘instat.qc’ file from the computer linked with the ELISA reader on to the supplied floppy disk.

Where a laboratory was not using an EDI version to read and calculate the ELISA plates, a request was made to send the IQC data of the last 30 to 40 plates in a table format.

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2 Institute for Animal Health, Pirbright Laboratory, United Kingdom, FAO World Reference Laboratory for Rinderpest and Foot-and-Mouth Disease.
FIG. 1(a). Mean OD values of the C++ per ELISA plate for T. brucei (error bars indicating a ±2 SD range). Laboratory 5.

FIG. 1(b). Mean PP values of the C+, C- and Cc per ELISA plate for T. brucei (error bars indicating a ±2 SD). Laboratory 5.
The following types of analysis are used to evaluate the IQC results [9]:

1. **Control charts**

   Control charts provide a criterion for detecting lack of statistical control. Lack of statistical control in data indicates that the observed variation in the measurements is greater than it should be on the basis of chance. For the IQC evaluation, as part of the EQAP, the mean ±2 times the SD of the replicate values for each IQC per ELISA plate are plotted together with the UCL and LCL for that IQC sample (Fig. 1).

2. **Frequency distributions**

   These histograms provide a visual reference for the distribution of the measurements of each IQC sample (Fig. 2).

3. **Basic statistical parameters**

   The following statistical parameters are presented for each IQC sample: number (N) of ELISA plates, minimum and maximum, range, mean, median, variance, SD and the coefficient of variation.

2.3. **EQC test panel**

   Since the participating laboratories are using standardized FAO/IAEA ELISA kits with identical protocols and reagents, an EQC test panel consisting of five test samples can provide acceptable levels of test reliability [9].

   The EQC test panel comprises:
   - one sample which will produce an unequivocal negative result;
   - one sample which will produce an unequivocal weak positive result;
   - one sample which will produce a medium to strong positive result.

   The remaining two test samples are selected from any combination of the above three categories. The final decision on the inclusion of suitable samples is based on individual test requirements for additional evaluation of assay performance. The EQC test panels for the trypanosomosis ELISA and for FMDV antibody detection ELISA consist of test panels of five samples composed as above per species or serotype. The EQC test panel for the FMDV antigen detection consists of two negative samples in total and three positive samples per serotype.

   For an individual test sample to be acceptable for inclusion in the EQC panel, it has, as far as is practicable, to meet the following criteria. It should
FIG. 2. Frequency distributions of the values for C++, C+, C− and Cc for T. brucei. Laboratory 5.
— be derived from a single animal;
— be undiluted (however, if the above is not possible, then a positive test sample should be prepared by diluting a stronger positive sample in the sample that is used to prepare the negative sample);
— not be lipaemic;
— not be haemolysed (for sera);
— not contain secondary clots (for sera);
— not be contaminated
— have not been repeatedly frozen and thawed;
— be supplied to participating laboratories in a volume sufficient to perform at least one repetition of the test in question;
— be such that the initial assignment of weak positive should ensure that the sample does not give equivocal results;
— be such that all aliquots of a given sample are derived from a single production batch, if possible lyophilized.

All test samples are allocated unique identity codes, and the individual test samples sent to different laboratories have different identities to reduce the potential for collusion between laboratories. For the purpose of selection of a sample for inclusion into the panel, the initial assignment of a sample status will be based on the reactivity determined by the co-ordinator. The final determination of the sample status will be based on the results of testing by the participating laboratories. This status will be determined only when over 80% of the laboratories obtain the same result.

The EQC test samples are prepared either by the FAO/IAEA ELISA or in the World Reference Laboratory (for the rinderpest competitive ELISA and FMDV ELISA both antibody and antigen detection).

The laboratories were requested to provide the EQC results not only in terms of positive or negative for each sera but also to submit the computer printout as produced by the ELISA software EDI program, thus including the OD values and PP/PI values of the IQC data and the PP/PI values of each EQC test sample.

The following types of analysis are conducted on the EQC test panel results [9, 17, 18]:

(1) Frequency analysis demonstrating the distribution of laboratory results;
(2) Measurement of intra- and interlaboratory variance by identifying repeatability and reproducibility extreme results using Dixon’s test;
(3) Determination of systematic and random error through simplified Youden plot analysis.

2.4. Logistics

Central to the whole process is the timely dispatch of the EQAP items. Whenever possible, the dispatch time of the EQAP items is co-ordinated with the
activities of the participating laboratories. The EQAP items are sent by courier whenever possible. Only the EQC test panels for the EQAP for trypanosomosis ELISA were hand carried on ice.

A set time limit of five weeks is allowed for the return of the EQAP results to the EQAP co-ordinator. Such a period commences from the date of confirmation of EQC test panel receipt by the participating laboratory. To facilitate the operation of this system, the EQAP co-ordinator notified the participating laboratory by fax or telex of the date of dispatch of the EQAP items. The participating laboratories were asked to notify the EQAP co-ordinator of the receipt of the EQAP items within three weeks after the date of dispatch. In the event that such notification was not received by the co-ordinator, a follow-up investigation by the EQAP co-ordinator was undertaken to determine the reason for the delay. Whenever participants expected problems in keeping to this time limit, they were asked to contact the EQAP co-ordinator as soon as possible. In the event a laboratory was not able to fulfil all EQAP requirements, they were asked to submit the remaining EQAP results within the given time schedule.

It was not possible to ascertain the travel time and conditions of each individual EQC test panel. However, from experience it was not expected that the shipment time would add any significant variable to the EQC test results.

3. RESULTS AND DISCUSSION

The first round of the EQAP for the FAO/IAEA rinderpest competitive ELISA started in October 1995. The results are published in the EQAP interim report for the FAO/IAEA rinderpest competitive ELISA [19]. The first round of the EQAP for the FAO/IAEA brucella indirect ELISA started in December 1995. The results are published in the EQAP interim report for the FAO/IAEA brucella indirect ELISA [20]. The first EQAP round for the FAO/IAEA trypanosomosis antigen detection ELISA and the FAO/IAEA FMDV ELISAs both for antibody and antigen detection, started in August 1996 [21]. The second EQAP round for the FAO/IAEA rinderpest competitive ELISA and the FAO/IAEA brucella indirect ELISA started at the end of 1996 [22, 23].

Table I shows the distribution of participants for each EQAP.

The percentage of results returned in the different EQAP is acceptable (Table II). However, the objective is to have a 100% return of results within the time schedule. The establishment and maintenance of good communication lines is essential and needs improvement in several cases. However, it is anticipated that once the EQAP has become more routine for all involved, it will be easier to adhere to the set time schedule.
TABLE I. OVERVIEW OF PARTICIPATING LABORATORIES PER EQAP

<table>
<thead>
<tr>
<th>EQAP for</th>
<th>Participating laboratories 1995/1996</th>
<th>Africa</th>
<th>Latin America</th>
<th>Asia</th>
<th>Total</th>
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<tr>
<td>Rinderpest competitive ELISA</td>
<td></td>
<td>21</td>
<td>—</td>
<td>8</td>
<td>29</td>
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<tr>
<td>Brucella indirect ELISA</td>
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<td>16</td>
<td>16</td>
<td>2</td>
<td>34</td>
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<tr>
<td>FMDVa ELISA</td>
<td></td>
<td>—</td>
<td>b</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Trypanosomosis direct ELISA</td>
<td></td>
<td>16</td>
<td>—</td>
<td>—</td>
<td>16</td>
</tr>
</tbody>
</table>

*a Both liquid phase blocking ELISA and the indirect sandwich ELISA.
*b Next EQAP round, laboratories in Latin America will join.

TABLE II. OVERVIEW OF SUBMITTED RESULTS PER EQAP ROUND

<table>
<thead>
<tr>
<th>EQAP for</th>
<th>EQAP Round 1995A</th>
<th>EQAP Round 1996A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Q&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rinderpest competitive ELISA</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>Brucella indirect ELISA</td>
<td>31</td>
<td>23</td>
</tr>
<tr>
<td>FMDVa ELISA</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Trypanosomosis direct ELISA</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*a Total: total number of participating laboratories.
*b Q: questionnaire.
*c IQC: internal quality control data.
*d EQC: external quality control test panel.

There has been extensive correspondence between the participants and the EQAP co-ordinator. In almost all cases, there was a valid reason why a laboratory could not fulfil all the EQAP requirements within the given time schedule.

It must be emphasized for laboratories currently using FAO/IAEA designated diagnostic kits that participation in the EQAP is essential and the supply of kits in the longer term will be contingent upon the participation of individual laboratories.

3.1. Questionnaire

The information collected through the questionnaires is categorized and presented in detail in the different EQAP reports. The following areas were identified as needing improvement in almost all laboratories:
— Checking for calibration of the pipettes and ELISA reader.

— The routine monitoring of IQC data: almost all laboratories are using the IQC data to evaluate whether the results of an individual ELISA plate should be accepted or rejected on the basis of whether the IQC data are within the upper and lower limits. Only few laboratories indicated monitoring of IQC data by using control charts.

As part of the EQAP, 'reference' plates are being made for use with ELISA readers and will be distributed among the participants.

The implementation of routine monitoring of the IQC data is essential. A manual is being prepared which will enable test operators to plot control charts themselves. Two kinds of charts will be recommended for QC of the FAO/IAEA ELISA kits:

(a) the detailed daily data chart (similar to the control charts as presented in this paper), which keeps the actual data for various parameters for each plate used;

(b) the summary data chart which summarizes data for all tests performed in a given period of time.

3.2. IQC data

In general, the participating laboratories are producing reliable results as the majority of the IQC results are within the upper and lower limits with acceptable repeatability and precision [19–23]. However, several laboratories showed high 'within plate' and 'between plate' variation in their IQC results. The most common causes for variation in the IQC data, such as water quality, test operator or pipetting techniques, are discussed in the EQAP reports.

It is important to emphasize that the IQC data expressed in OD values and PP/PI values are used to determine whether or not the test has been performed within acceptable limits of variability and therefore whether or not the test data may be accepted for any given ELISA plate. While if the value of a control falls just outside the upper and lower control limit, the assay is likely to still give a correct positive or negative value to the test sera, the results as such are questionable. The assay must be examined in this situation and the cause for the failure to obtain controls within limits determined; it is not acceptable to carry on testing sera with controls consistently falling outside the limits.

In order to evaluate the IQC data of an individual laboratory correctly, basic information such as ELISA kit batch number, test date, test operator and any other additional information on a specific ELISA plate is needed. For example, if an empty ELISA plate is run several times to test the system, these plates should be identified on the control charts. This kind of information was not available for the EQAP coordinator and therefore not incorporated in the control charts and in the IQC
evaluations as presented in the different EQAP reports. As soon as routine monitoring of IQC data has been implemented in a laboratory, the IQC assessment as part of the EQAP will be based on control charts of the last 40 plates prepared by the test operators on which the necessary information will be written.

3.3. EQC test panel

The majority of the laboratories in the different EQAPs identified the EQC samples correctly as positive or negative [19–23].

Only the qualitative EQC results of the first EQAP round for the brucella indirect ELISA showed a discrepancy between the different laboratories (Table III) [20]. This was caused by the fact that the laboratories are using different cut-off values to determine whether a sample is positive or negative (Table IV). For the EQC test panel

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>high positive</td>
<td>weak positive</td>
<td>weak positive</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>Brucella indirect ELISA</td>
<td>95</td>
<td>35</td>
<td>30</td>
<td>100</td>
</tr>
</tbody>
</table>

**TABLE IV. CUT-OFF VALUE FOR BRUCELLA INDIRECT ELISA PER LABORATORY**

<table>
<thead>
<tr>
<th>Laboratory code</th>
<th>Cut-off value (%)</th>
<th>Laboratory code</th>
<th>Cut-off value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19</td>
<td>16</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>18</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>22</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>46</td>
<td>24</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td>35</td>
<td>27</td>
<td>?</td>
</tr>
<tr>
<td>11</td>
<td>?</td>
<td>28</td>
<td>32</td>
</tr>
<tr>
<td>14</td>
<td>35</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>32</td>
<td>15</td>
</tr>
</tbody>
</table>
preparation, a cut-off value of 15% was used. Table V shows the qualitative data per laboratory based on this 15% cut-off value; all laboratories would identify sample 1 as positive and samples 4 and 5 as negative (100% agreement). Two laboratories would identify sample 2 as negative, one laboratory would identify sample 3 as negative, using a cut-off of 15% (90% agreement for sample 2, 95% agreement for sample 3).

![Simplified Youden plots for samples 1, 2 and 3, EQAP for the Brucella indirect ELISA (BRA/1995a).](image)

**FIG. 3(a).** Simplified Youden plots for samples 1, 2 and 3, EQAP for the Brucella indirect ELISA (BRA/1995a).
As it is the cut-off value that determines whether a test sample is positive or negative, it is of utmost importance that a laboratory should use a correctly chosen cut-off value. It is stressed that each laboratory should be able to demonstrate on what grounds the cut-off value was chosen.

This also shows that one should be careful to interpret only the qualitative results of an external quality control test panel as participating laboratories can use different cut-off values.

For each EQAP round, the positive EQC samples are also plotted on a simplified Youden diagram (Fig. 3(a–c)). Such a diagram consists of a rectangular plot, on which each laboratory’s results are presented by a point. The abscissa (horizontal component) of each point is the laboratory’s results for sample X, while the ordinate (vertical component) contains results for sample Y. The grey coloured rectangle in the Youden diagram represents the mean value ±1 SD range for both samples.

The Youden diagram helps to identify systematic versus random differences between laboratories. Laboratories with systematic error components are either on the upper right hand quadrant (as formed by the mean lines for both samples) or the lower left hand quadrant (Fig. 3(a)). A laboratory positioned in the upper right hand quad-

![Simplified Youden plots for samples 1, 2 and 3, EQAP for the Brucella indirect ELISA (BRA/1995a).](image-url)
FIG. 3(c). Simplified Youden plots for samples 1, 2 and 3, EQAP for the Brucella indirect ELISA (BRA/1995a).

rant, outside the +1 SD range, could indicate that the laboratory values for both positive samples are too high, possibly because of increased sensitivity of the test in that laboratory. A laboratory positioned in the lower left quadrant of the diagram, outside the –1 SD range, could indicate that the laboratory reports results too low for both positive samples, which can result in a decrease in test sensitivity. Laboratories reporting results with random error will be placed either in the upper left hand or lower right hand quadrant, outside the ±1 SD range.

Figure 3(a–c) shows the simplified Youden plots for the EQC test samples of the first EQAP round for brucella indirect ELISA [20]. The plotted pairs of serum samples of the EQC test panels mostly lie at 45° to the main axes, indicating that the majority of the variation was systematic rather than random. For laboratories 7 and 22, this resulted in a decrease in sensitivity of the test since they would identify sample 2 as negative if a cut-off value of 15% was used. Laboratory 22 would identify sample 3 as also negative when a cut-off of 15% was used. These laboratories also showed lower PI values for the other EQC samples.

The statistical summary (Table VI) indicates some variation for samples 2 and 3. However, no extreme results in repeatability and reproducibility could be identified by using Dixon’s test. Unfortunately, there was no IQC evaluation available for
TABLE VI. SUMMARY STATISTICS OF EQC TEST SAMPLES OF EQAP FOR BRUCELLA INDIRECT ELISA

<table>
<thead>
<tr>
<th></th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>95</td>
<td>35</td>
<td>30</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Minimum</td>
<td>32</td>
<td>10</td>
<td>9</td>
<td>-2</td>
<td>-2</td>
</tr>
<tr>
<td>Maximum</td>
<td>110</td>
<td>57</td>
<td>52</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Range</td>
<td>78</td>
<td>47</td>
<td>45</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Mean</td>
<td>79</td>
<td>30</td>
<td>27</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Variance</td>
<td>419</td>
<td>140</td>
<td>112</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>20</td>
<td>12</td>
<td>10</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Coefficient of</td>
<td>25</td>
<td>41</td>
<td>39</td>
<td>&gt;&gt;</td>
<td>&gt;&gt;</td>
</tr>
<tr>
<td>variation (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard error</td>
<td>4.5</td>
<td>2.6</td>
<td>2.4</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Median</td>
<td>84</td>
<td>27</td>
<td>26</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

Repeatability: extreme results (for sample pairs): none
Reproducibility: extreme results: sample 1 — none; sample 2 — none; sample 3 — none; sample 4 — none; sample 5 — none.

laboratory 22. The IQC data of laboratory 7 indicated no major problems in conducting the assay.

4. CONCLUSIONS AND RECOMMENDATIONS

QC/QA procedures are essential to testing laboratories as they provide confidence in test results. The assurance that the produced test results are reliable is not only of importance to the test operator, scientist or the animal owner but for all interested outside parties. To achieve this end, the Animal Health and Production Subprogramme initiated an external quality assurance programme (EQAP).

It can be concluded that especially monitoring and evaluation of the IQC data need more attention in most laboratories. In general, the laboratories are producing reliable results as the majority of the IQC results are within the upper and lower limits with an acceptable level of repeatability and precision. Most laboratories have identified the EQC samples correctly. However, several laboratories should be alerted by the high 'within plate' and 'between plate' variation in their IQC results and hence attempt to reduce it. These laboratories were informed of the most common causes for increase of variation in the IQC data.

The implementation of routine and continual monitoring and evaluation of the IQC data is an important objective of the EQAP. The assessment of the test
performance based on the IQC evaluation should be considered the most critical element of the EQAP.

The EQAP has proven to be a valuable tool in the assessment of both the results derived from and use of the ELISA kits provided through the Joint FAO/IAEA Programme. Furthermore, in the short term, the EQAP can assist counterpart laboratories to establish and implement QC/QA procedures for conducting the FAO/IAEA ELISA and to advise on the implementation of similar QC/QA procedures in other laboratory activities.

The EQAP as currently conducted is based on a consultants meeting, “The Establishment of External Quality Assurance Procedures for Use with FAO/IAEA ELISA kits”, Vienna 1994. There will be an external review at the end of 1997 on the operation of the EQAP in its current format, to determine its effectiveness, to improve the programme where necessary, to assess the feasibility to extend its scope to other diseases and diagnostic systems and to develop into an internationally accepted veterinary laboratory accreditation scheme, thereby providing veterinary authorities in developing countries with an essential tool to establish credibility in their animal disease reporting schemes and, thus, in their ability to engage in international trade of food animal commodities under the conditions established by the World Trade Organization (WTO).

ACKNOWLEDGEMENTS

The author would like to thank all participants in the EQAP for their contributions and kind support. He would also like to express his gratitude to the staff of the Animal Production and Health Subprogramme, especially M. Jeggo, M. Robinson and W. Richards.

REFERENCES


[14] JOINT FAO/IAEA DIVISION, ANIMAL PRODUCTION AND HEALTH SECTION AND ANIMAL PRODUCTION UNIT, FAO/IAEA foot and mouth indirect ELISA kit, indirect sandwich enzyme immunoassay for detection of antigen to FMDV.

[15] JOINT FAO/IAEA DIVISION, ANIMAL PRODUCTION AND HEALTH SECTION AND ANIMAL PRODUCTION UNIT, FAO/IAEA foot and mouth liquid phase blocking ELISA kit, liquid phase blocking enzyme immunoassay for detection of antibodies to FMDV.


APPRIOPRIATE POST-GRADUATE TRAINING TO SUPPORT SURVEILLANCE AND CONTROL OF ANIMAL DISEASES IN SOUTHERN AFRICA

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Control Programme for Southern Africa,
Avondale, Harare,
Zimbabwe

Abstract

APPROPRIATE POST-GRADUATE TRAINING TO SUPPORT SURVEILLANCE AND CONTROL OF ANIMAL DISEASES IN SOUTHERN AFRICA.

Appropriate training at the MSc and PhD degree levels as well as short, skills oriented, certificated modules to support surveillance and control of infectious and parasitic diseases of livestock in southern Africa are discussed. The paper is divided into three parts. The first part reviews the context within which training is given. This is followed by a discussion of a new post-graduate training strategy to accommodate students with different backgrounds and experience and, lastly, the post-graduate programmes that are already in place or which are envisaged in the region are briefly reviewed. There are currently six veterinary faculties and numerous technical colleges that offer training in veterinary diagnostics in the countries of the Southern African Development Community. At these institutions, training is focused mostly on formal, full time post-graduate courses for those who seek academic advancement and excellence. The paper highlights the results of consultative workshops on training in veterinary diagnostics in southern Africa. Current approaches are also critically reviewed, and the basis for a new approach to supporting sustainable disease surveillance and control in the subcontinent is outlined.

1. INTRODUCTION

This paper focuses on appropriate post-graduate training at the MSc and PhD degree levels as well as on short, skills oriented, certificated courses to support surveillance (including diagnosis) and control of infectious and parasitic diseases of livestock (cattle, sheep, goats, pigs, horses) in southern Africa. We hope that our
thoughts stimulate productive debate in the Southern African Development Community (SADC), which will require a harmonized approach to training in its various sectors.

The University of Pretoria’s Faculty of Veterinary Science and, in particular, the Department of Veterinary Tropical Diseases (DVTD) have in recent years been inundated with requests from potential students to enrol in post-graduate studies. The applicants are from different environments and backgrounds in the SADC region and other parts of Africa as well as from further afield. This has stimulated us to review the existing post-graduate programmes critically so that students with different backgrounds, experience and academic goals can be accommodated in a new post-graduate training programme. A concern frequently voiced by faculty staff members is that this change might mean a lowering of standards.

The question that should be asked is: Why do students want to do post-graduate studies? Is it to obtain education, training, knowledge, skills, analytical capabilities, a combination of these, or is it just to achieve their own academic goals? These terms are often used loosely but, according to the Oxford English Dictionary, they have quite distinct and different meanings, as indicated below:

**Education** is defined as

- the process of bringing up young persons;
- the manner in which a person has been brought up;
- the systematic instruction, schooling or training given to the young (and, by extension, to adults) in preparation for the work of life; the whole course of scholastic instruction which a person has received; hence, culture or development of powers, formation of character; often qualified as intellectual and/or moral.

**Training** is defined as serving

- to instruct and discipline in some particular art, profession, occupation or practice, to exercise, practise, or drill;
- to prepare a racehorse for its work;
- to bring, by diet and exercise, to the required state of physical efficiency for a race.

**Knowledge** is defined as

- acquaintance with facts or a range of information;
- the sum of what is known.

**Skills** are defined as

- to have discrimination or knowledge, especially in a specified matter;
— to have practical knowledge in combination with ability, cleverness or expertness.

Analytical capabilities are defined as

— the resolution of anything complex into its simple elements;
— the tracing of things to their source.

Clearly, a strategy is required to match the demands of the workplace with the wishes and academic goals of prospective post-graduate students. Students require specific training, certain skills and analytical capabilities in order to improve the effectiveness of the laboratories or institutions in the subcontinent which are involved in disease surveillance and control. It is important to recognize that learning is a lifelong process!

This paper comprises three broad parts. The first part reviews the context within which training is given. This is followed by a discussion of a new strategy for post-graduate training to accommodate students with different backgrounds and experience and, lastly, we briefly review post-graduate programmes that are already in place or which are envisaged in the region to support animal disease surveillance and control of infectious and parasitic diseases of livestock in southern Africa.

2. TRAINING IN CONTEXT

Tropical and subtropical Africa, while renowned for its wealth of flora and fauna, is equally notorious for the large variety of infectious and parasitic diseases, which are periodically responsible for severe losses in domestic and wild animals. Many wild animals also act as carriers or reservoir hosts of infectious and parasitic agents of domestic stock [1, 2]. Scientists in the region have therefore had the opportunity of studying a wide spectrum of diseases under natural and experimental conditions, to an extent that is probably unequalled in most parts of the world.

The countries in southern Africa comprise communities at different socio-economic stages of development, and animals are consequently kept under markedly different conditions which influence the occurrence of diseases. The human population of southern Africa is increasing rapidly, and a large proportion of it is dependent upon small scale farming where livestock is important not only as a source of food, investment and transport, but also for its cultural value [3]. In the context of the environment in which we operate, the importance of livestock production in the national economy of most southern African countries, and in the well-being of its people, cannot be overemphasized. To illustrate the value of the livestock sector, the exports of animals and animal products from the SADC countries to the countries of the European Union totalled some ECU 377 665 000 (or US $490 million) in 1995
An outbreak of foot-and-mouth disease in Zimbabwe in 1989 was estimated to have cost Zimbabwe US $17 million in lost earnings. Consequently, animal diseases are an important non-tariff barrier to trade, and the countries of southern Africa need to improve and sustain the control of, or eradicate, those diseases that reduce productivity and threaten trade.

The region invests heavily in producing its veterinarians: it costs about US $17 000 to educate a veterinary undergraduate each year. The annual salaries of graduates working in government service in the region range from US $1 500 to US $36 000; the earnings of private veterinary practitioners are considerably higher. There is a need to ensure that these expensive veterinary graduates have adequate resources and sufficient incentives to practise their profession and apply their knowledge effectively to safeguard the region’s livestock industry.

The Faculty of Veterinary Science of the University of Pretoria, the Onderstepoort Veterinary Institute and the Onderstepoort Institute for Exotic Diseases are situated within a two kilometre radius of each other. The latter institute is one of very few high security laboratories in Africa where highly contagious diseases, such as foot-and-mouth disease, can be studied. Southern Africa also has other training and research institutions and diagnostic laboratories. The existence of these facilities and this expertise in the region presents unique opportunities for co-operation in studying tropical and subtropical infectious and parasitic diseases of livestock. These opportunities are unequalled elsewhere in Africa and, possibly, in the world. However, overall, the region is poor and its economic hardships continue to reduce the resources made available for disease surveillance and control. It is, therefore, essential that staff in the region, and beyond, collaborate closely to ensure that the region’s expertise and facilities are used efficiently and effectively, and that unnecessary duplication is avoided. To enhance the impact of such collaboration on the region’s diverse production systems people should be prepared to work in multidisciplinary teams.

The Faculty of Veterinary Science at Onderstepoort has a firm historical basis of training (more than three quarters of a century of ‘tradition’). While it has had many decades to test and establish its undergraduate and post-graduate programmes, most other faculties in the region (e.g. in Zambia, Zimbabwe, Mozambique and South Africa’s MEDUNSA veterinary faculty) were established in the last decade or two and are still developing their post-graduate programmes. The adoption of curricula developed by and for faculties in the ‘developed world’ may have short term benefits in rapidly establishing training programmes. However, faculties in the region should not model their curricula on European or American concepts in terms of production systems, perceptions, needs and service provision. Although we can learn a great deal from their experience, it is essential that faculties in Africa develop an approach that will make training more appropriate to the environment in which we function; this will require a different emphasis from that given in other parts of the world. Our training programmes should utilize the region’s resources (such as infrastructure and
workforce) more efficiently and effectively to address our unique situation; we are faced with a wide spectrum of infectious and parasitic diseases that occur in diverse ecosystems. An appropriate response to the region’s needs will ensure international recognition of our post-graduate training programmes, which will appeal to students not only from Africa but from all over the world, as we have already experienced.

3. POST-GRADUATE TRAINING STRATEGY

It is imperative that the undergraduate curricula and the post-graduate programmes be complementary. Currently the undergraduate curricula of the veterinary faculties in southern Africa vary greatly; they urgently need to be revised and, as far as possible, standardized. It should be accepted that, broadly, undergraduates primarily receive an ‘education’ with limited ‘training’. Many post-graduates require specific training to acquire skills needed for their work; fewer are likely to benefit from further ‘education’. However, difficult decisions have to be made to avoid ‘information overload’, and curricula and course contents should be designed with care, taking into account the context within which veterinary skills are needed.

Various post-graduate degrees and diplomas are offered by the region’s veterinary faculties and other institutions related to disease surveillance and control [4]. To meet demands, it is important that these post-graduate training programmes be flexible and respond to the different entry and exit requirements that people have for personal, financial or other reasons. Courses should be designed in line with a strategy of accommodating students with different backgrounds and work experience. To maintain and improve relevance, courses should contribute to specific custom made portfolios to meet trainees’ individual needs at the workplace; this may not be the same as career needs.

The delivery of training should be decentralized to enable a more field oriented and practical (‘hands-on’) approach to be adopted. It should be refocused to address more effectively the broader needs of the livestock producers (the base of the pyramid) and not continue to focus mainly on the peak of academic excellence (the ‘ivory tower’). To support disease surveillance and control, training is needed at all levels (e.g. livestock owners and producers, animal health officers, field veterinarians as well as scientists, technical and other staff members in provincial, national and regional laboratories). The training of trainers and the evaluation of the impact of training are integral parts of the strategy to deliver appropriate training. To evaluate and modify training to meet changing needs more effectively, training institutions should actively participate in policy analysis and formulation.

The Office international des épizooties (OIE) sponsored a workshop that was held at Onderstepoort from 12 to 14 July 1995. Its participants recognized that training of personnel at all levels is a high priority to ensure a sustainable and high
quality diagnostic service for southern Africa [4]. At a meeting held during October 1996, the Technical Committee of SADC's Livestock Production and Disease Control Sector (which comprises the chief veterinary officers of the region) agreed to develop a new regional programme to support livestock disease control. It suggested that the programme should focus on the surveillance and control of priority diseases, including tsetse and trypanosomosis, tick and tick borne diseases, foot-and-mouth disease, contagious bovine pleuropneumonia and rabies.

There are good arguments for adopting a regional approach to post-graduate training. By creating a training network, not only will human, financial and material resources be more efficiently utilized, but a network of trainers and trainees will be established. This would build up the region's capacity and promote the interchange of ideas; it would also promote the critical evaluation of training programmes. Furthermore, trained people would be encouraged to train others, which would replicate the development of appropriate skills and improve disease surveillance and control.

It is important to be able to evaluate the success of all training programmes but, first, objective evaluation criteria have to be devised to determine their impact. A programme's success cannot be determined by the number of candidates that apply for or attend its courses. Similarly, examination results reflect student performance and the standard of tuition, not the relevance of a course. While student and staff feedback (course evaluation) is valuable, perhaps the feedback from the client group, including animal owners or employers (impact evaluation) is of greater significance. It is not enough to report that the sponsor's money has been spent and that the students have passed their examinations!

4. ESTABLISHED MODULAR OR OTHER COURSES IN THE REGION TO SUPPORT DISEASE SURVEILLANCE AND CONTROL

4.1. Faculty of Veterinary Science, Zimbabwe

4.1.2. Master of science/diploma in tsetse and trypanosomosis control

This MSc degree/diploma course is co-ordinated by the Department of Paraclinical Studies of the Faculty of Veterinary Science, University of Zimbabwe, and the Training Section of the Regional Tsetse and Trypanosomosis Control Programme for Southern Africa (RTTCP).

The RTTCP started in 1986 and aimed to eliminate the tsetse fly from the common fly belt affecting four countries of southern Africa through a regional effort. To fulfil this task, skilled manpower is required. Professionals and middle level personnel involved in the fight against the disease and its vector need to update their knowledge and skills in control, management and research. Although
the focus of the RTTCP has shifted recently to disease control rather than eradication, specific training is still needed to build up the region's capacity to control this important vector borne disease.

The objectives of the course are to offer specialized training based on modern concepts to improve the control of vector borne diseases, in particular, tsetse transmitted trypanosomosis. The course aims at providing veterinarians and biologists with the appropriate technical, planning and management skills required for co-operation with development programmes, in teaching and research and in public and private animal health agencies.

The course is organized on a part time basis and has a modular structure (Table I). There are three core modules in the first year and three specialization modules in the second year; studies can be completed by research done in an additional third year. The part time nature of the training encourages students to use their newly acquired knowledge as soon as they return to their workplaces. There is also the opportunity for some participants to attend only those individual modules that are of greatest relevance to their needs.

On successful completion of Parts I and II, participants will be awarded the post-graduate diploma in tsetse and trypanosomosis control. The MSc will be awarded after successful completion of module 10 in a third and final year and acceptance of a thesis.

TABLE I. STRUCTURE OF THE POST-GRADUATE TRAINING PROGRAMME AT THE UNIVERSITY OF ZIMBABWE

<table>
<thead>
<tr>
<th>Part I: Core Modules (M 1–3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three modules of four weeks each</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Part II: Specialization modules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three modules of six weeks each</td>
</tr>
<tr>
<td>Option A:</td>
</tr>
<tr>
<td>Tsetse unit (M 4–6)</td>
</tr>
<tr>
<td>Option B:</td>
</tr>
<tr>
<td>Trypanosome unit (M 7–9)</td>
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<tr>
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4.2. Faculty of Veterinary Science, Onderstepoort

4.2.1. The approach to post-graduate training

A modular approach is central to the post-graduate training strategy of the Department of Veterinary Tropical Diseases (Fig. 1). This ensures that students with different backgrounds and work experience can enter the post-graduate training programme. An integrated approach has been adopted particularly for practitioners, field veterinarians and research workers, and modules have been devised to cater for the more laboratory oriented candidates. Apart from the modules, selected information days, congresses, symposia and workshops that are attended locally or abroad may be credited and may count towards obtaining a BVSc(hons).

The MSc and PhD degrees require candidates to successfully complete a research project and, in addition, an examination based on the topic of the research project may be required. Depending on the student’s background and experience, registration for the MSc degree may depend on prior completion of certain course work and skills oriented modules. The DVSc degree is awarded on the basis of the candidate’s post-doctoral publications.

Continuing education is necessary for professional and personal development. While few people can afford the time and money to attend full time post-graduate courses, they may need to acquire new knowledge and skills to work more effectively. A modular approach is followed in the post-graduate continuing education

* Selected information days, congresses, symposia, workshops may also be credited.

FIG. 1. Post-graduate programme of the Faculty of Veterinary Science, University of Pretoria.
certificated courses to accommodate the particular needs and interests of the student. These skills oriented modules are of one to five weeks in duration and are given annually, depending on demand. Students are taught in small groups (5–15 candidates) to ensure that they achieve practical competence. Each candidate who satisfactorily completes a module is awarded a certificate. Credits are also given for each completed module and these may subsequently contribute towards obtaining a post-graduate degree (Fig. 1), or towards further career development.

Through the department's increasingly close collaboration with the universities of the region, it is envisaged that the credits obtained for the modules will eventually be recognized by all member countries of SADC. To achieve this aim, modules are offered in collaboration with veterinary faculties and other institutions within the SADC.

4.2.2. African epizootic diseases module

Epidemic diseases, including foot-and-mouth disease, African horsesickness, bovine contagious bovine pleuropneumonia, Rift Valley fever, lumpy skin disease, African swine fever, anthrax, theileriosis and Newcastle disease, are widely prevalent in sub-Saharan Africa. Although they cause widespread epizootics only periodically, they have severe socioeconomic effects. Other epidemic diseases, such as rabies, pose a direct threat to human health.

Because of their irregular occurrence and, in most African countries, the lack of biologically secure facilities in which to study the diseases and causative agents safely, few veterinarians are sufficiently knowledgeable about the diagnosis and control of these diseases to avert disasters early in the course of an epizootic.

To address this problem, two of the institutions at Onderstepoort, namely the Onderstepoort Institute for Exotic Diseases (OIED) and the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, provide an annual course devoted to these epidemic diseases. Cases of some of the above mentioned diseases are reproduced in the high security facilities of the OIED during the course. For both ethical and economic reasons, such use of animals for teaching purposes will largely be replaced as soon as practically possible by high quality audio-visual teaching material.

Experts from all over the world are engaged as tutors on this course, thus ensuring that participants receive the most relevant information.

4.2.3. Modules in the laboratory diagnostic series

At the OIE sponsored workshop on the Requirements of a Sustainable Veterinary Diagnostic Service in Southern and Eastern Africa, in July 1995, it was resolved that the maintenance of a functional veterinary diagnostic service is
dependent on the provision of appropriate training at all levels involved in veterinary diagnostics. A follow-up meeting was organized to discuss the harmonization of training in the region. This meeting was sponsored by the OIE and the RTTCP and took place at Matobo Hills, Zimbabwe, from 4 to 8 March 1996. The outcome of the workshop was summarized in a report [4].

The Faculty of Veterinary Science, University of Pretoria, has recognized the need to provide short, formal, structured skills oriented courses in diagnostics and disease control. The Departments of Veterinary Tropical Diseases, Pathology and Pharmacology and Toxicology have introduced continuing education certificated modules in the laboratory diagnostic series that can meet the requirements of veterinarians, other scientists and laboratory personnel who are engaged in veterinary diagnostic work. This series comprises modules in bacteriology and mycology, ectoparasitology and protozoology, helminthology, immunology and serology, virology parts I and II, toxicology, histopathology laboratory practice and diagnostic pathology.

Although candidates are encouraged to find their own funding for these courses, it was found necessary to provide scholarships to some candidates, particularly from African countries, to offer them the opportunity to attend a course.

One of the aims of the modules in the Veterinary Laboratory Diagnostic Series is to collaborate with candidates upon their return to their respective countries or regions, in applied diagnostics or research projects that can benefit those countries. In this way, students can apply the skills acquired during their training, and their capacity, and that of their laboratories, is enhanced. In addition, it is envisaged that participants, particularly from faculties and institutions in the region that are active in veterinary diagnostics, will be involved as tutors in subsequent modules. The establishment of a network of tutors and trainees will contribute towards harmonization of veterinary diagnostics in the region and facilitate the evaluation of the impact of the training they received.

4.3. Faculty of Veterinary Medicine, Zambia

4.3.1. Master of veterinary medicine (MVetMed) in veterinary diagnostic medicine

The School of Veterinary Medicine, University of Zambia, has launched a course in diagnostic veterinary medicine, for the degree of Master of Veterinary Medicine.

The Master's degree programme comprises two parts: Part I consists of advanced courses (diagnostic pathology, clinical microbiology, clinical parasitology and scientific methodology) equivalent to an academic year of study. Part II consists of supervised research on an approved subject carried out during the subsequent 12 months, which leads to the submission of a dissertation. Normally, no candidate may proceed to Part II unless he or she has passed all the courses in Part I.
5. **NEEDS STILL TO BE ADDRESSED**

Specialized training is needed in other subjects, and these have still to be defined. To supplement the modules that are already in place in the region, other modules, each of a few weeks' duration, should also be developed after the needs have been prioritized. These should cover such subjects as good laboratory practice, research methodology, information management, practical epidemiology, socio-economics, draught animal management and state veterinary medicine.

6. **CONCLUSIONS**

Post-graduate training in southern Africa requires revision. The new approach should focus on ensuring that training courses have tangible impact on disease surveillance and control. This in turn will support improved animal production, which will benefit the people of southern Africa. A strategy should be implemented that pools the region's resources and establishes a network. The resultant improved collaboration would promote capacity building and improve disease control. Several initiatives are in progress which should be refined and co-ordinated to ensure that the increasingly scarce resources are used with greater efficiency and effectiveness. Since training is centred on people, training programmes should respond more readily to the needs of individuals to improve their skills; this will require greater flexibility on the part of the institutions charged with delivering education and training.

The provision of appropriate post-graduate veterinary training can justifiably be regarded as an exciting challenge of high priority for the region's institutions and trainers. A start has been made to increase the relevance of the region's training, and critical evaluation of its impact and further refinement are essential.

**REFERENCES**


INFORMATION TECHNOLOGY/MODELLING

(Session 5)

Chairperson

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FAO/IAEA
MANAGING KNOWLEDGE: PERSPECTIVES FOR INFORMATION TECHNOLOGIES IN ANIMAL HEALTH IN THE 21st CENTURY

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Abstract

MANAGING KNOWLEDGE: PERSPECTIVES FOR INFORMATION TECHNOLOGIES IN ANIMAL HEALTH IN THE 21st CENTURY.

The paper considers the potential of information technologies in the prevention and management of animal diseases.

1. INTRODUCTION

What can information technologies offer those responsible for the prevention and management of animal diseases at the dawn of the 21st century? What enabling infrastructure of communications should be put in place to facilitate the delivery of key information to the decision maker rapidly at the point of decision? What changes in veterinary working culture and in the processes of sustaining animal health will be needed to make animal health a reality in the Global Village? How far will the rapid advance of disease management for human medicine be replicable in the world of animal health? How far can we improve the way we manage and use knowledge?

A recent report by Coopers and Lybrand [1] on human disease management could hardly place a higher emphasis on the role of information systems as the fundamental enabler of change:

"The key to these developments [European consensus on disease management] is information management. European style managed care demands information exchange and sharing, not just in relation to particular patients, but for whole communities."

The proposition behind this paper is that animal health, like human health, will show an increasing emphasis on effective management of health, which in turn demands a strategic approach to the management of information about health and about disease. The principles of exchange and sharing identified by Coopers and Lybrand apply with emphasis to information in the domain of animal health because of the intense socio-economic significance of the animal population in so many of the world's societies and economies. The means? Wide area networking. The Internet.
Predictions about the dawn of the age of the Global Village have been common since Marshall McLuhan first propounded the idea in the 1950s; but now the dawn seems finally to have arrived. Strikingly, the communications instrument that has forced the issue is not, as McLuhan predicted, the television, but rather the computer as gateway to the Internet — the name given the worldwide network of computers which constitute the Global Village communications channel. (Some technology providers are, however, hedging their bets and providing Internet access through the television monitor.) This channel, now increasingly capable of supporting multimedia (text, image, animation, sound, even video), is opening for veterinary uses, and not just the ‘surfing’ of the college student.

The global spread of the Internet is rapid, incoherent and cross-cultural. The speed with which it is developing, however, is not just a function of the remarkable developments of computing technology (which in some ways is still not very efficient) but of the steadily decreasing cost of access. The opportunity now exists, almost irrespective of where in the world you are, to access information of the highest quality and immediacy at the cost of a local telephone call — or even by mobile telephone operating from batteries in the field. Putting this opportunity to work in the service of disease control — or perhaps, better, disease management — through such initiatives as GREP and EMPRES, is a challenge now worth facing seriously.

At the heart of the mission of global eradication of disease is the circulation of information and data of the highest quality and credibility. Imagine that a veterinary officer could stand in a field next to an animal suspected of being sick, capture visual and diagnostic data from that animal by camera or by, for example, ELISA, transmit the images and data for further analysis to a national laboratory, or one in Paris, Rome, Vienna or Pirbright, and receive the analysis back again into the field setting. Imagine every village had such a capability, reinforced by support materials suited to the local culture and conditions, but at a price that each village could afford without subsidy; imagine the impact this could have on the capacity to prevent and control diseases.

It is time to plan for that scenario since the technologies that enable it are now within reach, not just technologically, but, perhaps more critically, economically. Economists recognize certain magic thresholds in the adoption and spread of new technologies; what we are experiencing is our global passage through such a threshold.

If the premise is accepted that we now all share in the concerns of a Global Village, then diseases such as rinderpest, which have hitherto been seen as in the ownership of the countries which have them, are now problems which affect the village as a whole. We are all stockholders (stakeholders) now. And the issue is not simply
one of mobilizing a global strategy for controlling and eradicating a key disease; the world's veterinary services are in a process of evolution and realignment to bring livestock production and national veterinary activity closer into liaison. This in turn reflects a new interest in achieving common scientific standards, for example in diagnostics and disease control, as a basis for a common trade order. Such standards form the basis of mutuality of interest and trust.

3. IMPROVING ACCESS: THE INTERNET AS VIRTUAL UNIVERSITY OR TRAINING CENTRE

While nothing adequately substitutes for face to face tuition, there is a great deal the Internet can do to support training, either as part of a structured programme for acquiring university or college level qualifications, or as part of an in-service continuing veterinary education (CVE) package. The value of such support lies in the immediacy and cost of access to high grade knowledge which in principle the Internet can facilitate.

For a developing country, the economics of training delivered through the Internet may be extremely attractive. The most significant example is perhaps at the level of advanced professional qualification, such as a doctorate. Estimates of current cost here vary, but are in the range of US $500 000 over three years. This figure is calculated as follows: fees and subsistence over three years, family travel (and upheaval), replacement of key worker during study absence (in effect, the country sponsoring the student pays two salaries); but also there is a risk that the person concerned will not wish to return home with the additional skills but rather exercise them in the country where they were acquired. The Internet offers itself as a means of providing various modalities of training without removing the target population from their posts or their social and economic circumstances. The savings can obviously be considerable, even if for supervision reasons the student is still required to make a number of short study visits to the host institution.

There is a further aspect to the economic perspective: there is a degree of concern in developing countries that the centres of scientific excellence are still predominantly in Europe and the USA, which renders their knowledge and expertise expensive and hard to get at; even a hint of old style colonialism appears to cloud some relationships. The opportunity of the Internet is to make available state of the art, operationally vital knowledge to any user to whom it may be of value, on equal terms. Of course, that raises the question as to whether or not such centres will be willing to share freely what they know. At a time of increased funding constraint many may be reluctant to do so.
4. ORGANIZATIONAL IMPACT

As the culture of the Global Village increasingly impacts international bodies, public or private, the management of change in response to the new features of village life becomes of decisive significance to any organization's future. There are many ways to characterize change, and also substantial risks inherent in mistaking one form of change for another, however closely they may resemble each other. Two modalities of change most concern this paper: first, change led by a need for strategic repositioning, and, secondly, change driven by technological innovation, especially in the field of communications.

What is not in doubt as such, though still far from clear in its practical implications, is that the global culture will bring with it profound structural changes. Large centralized organizations are giving way to small, empowered units, virtual groupings, business units, etc., which is all very well, but the risk to coherence is great. The potential for small groups of well motivated terrorists for holding communities to ransom is not simply indicative of a problem in the political arena. Small groups can also exercise very strong influence over the world of information, and if this influence is wielded malevolently, or even partially so, the risk to the integrity of information can be very real. For example, in a trade dispute, one country could deliberately falsify data on a key process or industrial sector to discredit another.

What is unlikely to survive the process of organizational change is a structure based on a large and complex hierarchy. The typical organizational pyramid, captured in the conventional organogram, is under pressure from a culture of peers, working in teams to address project based or problem based tasks.

4.1. Strategic change

The debate prior to the appointment of the new Secretary-General of the United Nations highlighted the dilemmas of managing world organizations in a period of change. Institutions created in the wake of World War II need restructuring in the light of changed circumstance and expectation. But what goals are now appropriate? What means of achieving those goals present themselves? Whatever the means to the solution, the requirement for greater transparency and accountability is in the ascendant. Value has to be demonstrated and not assumed. And to demonstrate value is what the United Nations is now tasked to do; but it is not alone in having to face a profound process of reflection on how best to achieve its goals. Only recently the World Bank has also adopted a policy of change into its plan for the coming years.

The drivers behind the strategic forces at work in the United Nations are also affecting the goals defined for many veterinary services. The underpinning rationale of public health services is now complemented, or even replaced, by goals defined by the need to support animal production and essential economic activities associated
with animals as sources of food, power, transportation and productive value (e.g. leather). The World Trade Organization and the freer world trade order demand a new emphasis on common standards for defining states of health and disease amongst live and dead animal stock. Standards for disease management and standard operating procedures (SOPs) for key processes are becoming the order of the day. How these standards are defined and implemented impacts directly on the working cultures of animal health professionals, who will need new tools to meet their new challenges.

In such a world order, the capacity of communication systems to make previously centralized and inaccessible information and expertise accessible much more widely and at greatly reduced cost opens the possibility of the genuinely distributed or ‘virtual’ organization. In other words, large bodies such as the United Nations, and component parts of it, can be visible and present on people’s desks all over the world. This invokes the need for a conscious policy of pursuing strategic change in which the true value of the expertise and support that a global body can give can be made visible to the end user community in a way that makes such a value more tangible and even measurable.

4.2. Communications

Throughout the history of communications the intrinsic link between medium and message has been well understood: McLuhan argued that the one was the other in his famous dictum: “The medium is the message.” As the Cold War ended, the statement gathered political weight as it became clear that the television and the computer network (e-mail) were main carriers of dissident communications. It was even rumoured that when Moscow was briefly under siege the rebels forgot to cut e-mail connections to the outside world and so were under the illusion of controlling the situation when in fact they did not. In essence, what has happened is a radical democratization of the channels of communication.

But such a process is not without its complexities and complications. For democratization also brings responsibilities, of which quality control and quality assurance are perhaps top of the list. For while one defence of a centralized and centrally driven information culture was the ability to control quality, in a free-for-all such as the Internet it is by no means certain whether information to which a user has access is accurate, up to date or validated. Achieving quality will require a great deal of attention to consensus formation and to communications, and it is not yet clear that either the enabling infrastructure or the culture of open communications is everywhere in place to meet the new challenge.

The cultural issue also speaks to the historical shift now in process from paper to digital forms of communication. The communications process has hitherto been dominated by the dominant medium of communications — paper. This medium will not be replaced; but it will be complemented and probably superseded in importance
by digital communications, notably the laptop PC (or smaller communication devices, such as pagers), as communications point, linked directly or indirectly to the mobile phone. In both cases, the agent of change is not just the medium of storage and transmission but also the mobility of the users. Both laptop and mobile phone technologies (and they are now being combined in handheld devices) share the characteristic that the user, theoretically, can access the communications infrastructure from anywhere, and even the dependence on electricity is removed with long life batteries. Hence the AVIS (Advanced Veterinary Information System) vision of a veterinarian standing in a field with a sick animal, shipping and receiving data in the real time management of individuals and groups of animals.

This theoretically opens a wide range of possibilities for the animal health professional:

— direct access to centralized information and decision support resources
— real time feedback
— on-line or asynchronous disease management support.

The potential benefits in terms of the speed and quality of information flow are remarkable, given that both have been problematic in the past.

4.3. Knowledge

The combined impact of strategic change and communications change is delivered in the domain knowledge of any professional, particularly in life science based organizations and industries, where a further pressure for change is exerting a radical influence. Nowhere is the evolution of our knowledge about the world occurring more rapidly than in the life sciences. The WHO estimates that the half-life of medical knowledge is seven years, and reducing. The same holds true for veterinary knowledge. This phenomenon is changing the basis on which power structures are based.

The tradition of acquiring, owning and protecting knowledge in the life sciences has been heavily influenced historically by the guild structures and cultures of medieval Europe. In guilds small groups of masters surrounded by apprentices and journeymen dominated a given area of knowledge and expertise and protected their livelihoods at both a personal and a group level by retaining to themselves the ‘mystery’ of their domain — knowledge that was transmitted from person to person but never written down. When the printing press arrived in the 15th century, masters began to break ranks and commit some knowledge to paper; medicine and pharmacology to an extent followed in this path, but still the medical professions and their sister professionals, the veterinarians, retained essential mysteries to themselves. As the digital revolution continues, the residual guild knowledge is further under
challenge since the opportunity exists in multimedia to capture more and more of the
mystery in a form that even quite unskilled people can use effectively.

But paradoxically, the digital revolution has highlighted a domain of knowledge
which seems to grow in value and significance the more the computer is used, namely
what Michael Polanyi characterizes as “tacit knowledge”, the knowledge of experi­
ence and intuition — which is still a fundamental attribute of the skilled life science
professional. Polanyi does not dispute that this knowledge can eventually be codified,
but what is at stake is the apparent paradox that the more one knows the less one is
able to explain one’s knowledge to a third party: “One just knows.”

Given the need for acute attention to quality issues already identified, one of
the most substantial challenges ahead for the computerized delivery of knowledge
is to find an appropriate methodology to elicit the tacit knowledge from the experts
in a way that does not de-skill them and degrade their knowledge, for the computer
is not a good instrument with which to make finely balanced judgements. It does
not, and will not, think as a human will in a given situation. This may sometimes
be helpful, sometimes it may not. A computer can well be programmed to make
rational judgements about the world; but humans also make reasonable judgements,
where ‘reasonable’ accommodates emotional (softer) influences on decisions than
purely rational thought. If I think rationally about a slaughter policy for BSE I may
well agree to it; if I own the cattle to be slaughtered it is entirely reasonable that I
disagree.

But there is also a pragmatic challenge at stake, that simply of keeping people
up to date with the state of knowledge in their domain. If the half-life of their knowl­
edge is only seven years, the old guild structure (the higher you go the more valuable
the knowledge) is turned on its head; the more recently you have graduated the more
likely you are to be right. The obvious need, which again the computer well can meet,
is for life long learning. But who is to build the curriculum; how are jobs to be struc­
tured to permit time for such learning to take place?

4.4. Managing the knowledge

In working across the life sciences for the past ten years a list of general
requirements has become commonplace, each of which expresses one of the needs,
and therefore values, of the life science professional. In no particular order of impor­
tance these themes are part of daily life:

— harmonizing work practices and SOPs
— regulatory compliance
— international standardization
— quality control and certification
— rapid knowledge update
To meet these requirements, certain knowledge acquisition and management tools need to be created which achieve the following goals:

- automated event description
- intelligent event charting
- rapid response to divergence or the unforeseen
- proactive use of decision support tools
- data filters for effective leverage
- trend recognition.

But the tools are not in themselves enough; the relationship between the user and the computer also needs to be restructured to make more effective use of the capabilities of both; in essence, this means creating increasingly automated ways of capturing data, but deploying users, especially expert users, to leveraging value from data, which means in turn creating a classic value chain:

\[
\text{DATA} \rightarrow \text{INFORMATION} \rightarrow \text{KNOWLEDGE}
\]

As chaos theory has taught us, the value mechanism within this chain is not an easy one to understand, for the intrinsic quality of the knowledge depends critically on the intrinsic accuracy of the data; and fractal-like, one wrong point can cause a cascade not of value but of damage.

5. DISEASE MANAGEMENT

In an internal position paper for the FAO, April 1996, B. Geering identified three key questions to which enhanced information systems will need to enable the provision of answers:

- what is going on?
- what does it mean?
- what must be done about it?

These questions are at the heart of the practice of disease management. They are also the fundamental questions of knowledge management: how do I organize my knowledge (epistemology) and how do I interpret it (hermeneutics)? What the computer brings to both questions, which is new is the simple capability of cross-referencing. While, for example, Denis Diderot’s Encyclopédie well knew that knowledge needs to
be cross-referenced to be really useful to a professional, cross-referencing large paper knowledge bases (tomes of his Encyclopédie) is physically impossible. This is what the computer does well (keyword searching, etc.) and this is the key characteristic of the Internet language HTML, for the HT (Hypertext) is no more than an elaborate, but efficient, means of cross-referral. The point is that the cross-referencing tool now exists, which fundamentally means that Geering’s needs can, at least in theory, be met.

Interpreting Geering’s questions in terms of systems requirements, there are three superordinate goals;

— reporting
— analysis
— risk management.

To these three I would add a fourth, fundamental goal: knowledge management. Taken together, and not by explicit design, they constitute a classic quadrant model (Fig. 1).

6. EFFECTIVE MANAGEMENT OF DISEASE:
AVIS AND OTHER ENABLING TECHNOLOGIES

In preparation for the exploitation of the opportunities the new global communication technologies offer, a consortium was formed in 1993 to develop the AVIS. The founding consortium members were the FAO, Rome; the Institute for Animal Health, Compton and Pirbright; the Office international des épizooties, Paris; and the Telos Group, London. Membership on an associate basis is open to public and private sector bodies with an interest in promoting standards in the field of veterinary services, especially in the prevention, management and eradication of diseases.

AVIS is a state of the art system for the management of a range of key diseases — List A, and selected List B. Diseases currently addressed include: rinderpest; foot-and-mouth disease; SVD; poultry diseases (including Newcastle disease and avian influenza); poultry hygiene; CBPP; PPR; lumpy skin; rabies; BSE; swine fever.

In association with FAO, there is also a module on EMPRES.

Each module is submitted to peer review (usually at least four different readers), including one reader from each partner in the consortium. Where appropriate the full text, cross-referenced, of any guideline or regulation is included in full. SOPs and standard documentation also are added as required.
AVIS was intended to capitalize on the potential of powerful but increasingly affordable computing technology (computers have roughly doubled in power and halved in price every year for the past forty years) to build a custom made, easy to use system for veterinary medicine, integrating three key functions: training and information; data capture and management; and decision support.

Decision support in particular is growing in significance, given the increasing volume and complexity of regulations and guidelines to which decision makers have
to respond. Tools for risk management and for disease prevention and emergency response are in development.

6.1. Deployment

The logic of deployment is as follows:

(1) Education and training improve the basic efficiency of primary care, enabling greater awareness of accuracy in the diagnosis of diseases, but also a more managed approach to treatment and control.

(2) Data capture, management and analysis enable correct identification of diseases, efficient case management and overview of case mix, audit tracking and outcome analysis.

(3) Decision support tools enable overall sector management, epidemiology, cost–benefit analysis and overall performance and quality control.

Programmes may be delivered in any appropriate format — diskette, CD-ROM, Internet or Intranet — or combinations thereof. They may run in stand-alone, LAN or WAN mode.

6.2. Mission

This combination of purposes led to the following mission statement:

"To develop, make and distribute information technology products and services for animal healthcare, aimed at consciousness raising, human resource development, local capacity building and cost effective delivery, to prevent, alleviate or cure suffering."

6.3. AVIS: The friendly colleague

AVIS recognizes the needs of general veterinarians and other animal healthcare workers, by allowing for the fact that "memory is treacherous". Even the most thoroughly informed professionals need reminders "to make them master of the situation and enable them to prescribe exactly what their judgement tells them is needed for the occasion" (Merck Manual). AVIS aspires to be a friendly colleague to the animal health professional.

6.4. AVIS users

The principal users of AVIS are animal health workers, with particular attention to need in the primary sector. But systems are also envisaged in the educational setting, such as in schools, colleges or universities, and in the workplace.
AVIS was born from a long and well conceived process of consultation and testing with users. AVIS was confirmed as an internationally accepted project at the OIE General Session 1995, where it was formally presented to the Delegate Assembly.

At the OIE Regional meeting in Rabat in January 1995, the following priorities were established:

1. Rapid access to information of the highest quality, based on agreed standards
2. Quality control of data going into the system
3. Capacity to access data from paper sources
4. Timeliness of reporting
5. Observation of national standards
6. Cost effective training and SOP type support for field work.

This list has subsequently been reconfirmed.


The AVIS development process is as follows: Phase 1: Rinderpest: the pilot disease; Phase 2: foot-and-mouth disease; ELISA: A manual; Phase 3: modular roll-out of full AVIS system.

AVIS has kept very much to schedule with diseases addressed including rinderpest, foot-and-mouth disease, Newcastle disease, poultry hygiene, CBPP and BSE. Other EMPRES diseases, such as Rift Valley fever and lumpy skin, and priority list B diseases, such as rabies, tuberculosis and salmonella, are in advanced script development.

6.6. Rinderpest: The pilot disease

Rinderpest was chosen to initiate the AVIS project for a number of reasons, of which one in particular stands out. It is now a common, though poorly explained, experience in the application of the computer to standards (such as SOPs, diagnostic standards, etc.) that computerization greatly facilitates and reinforces consensus formation, and through consensus standards emerge by practice and consent rather than by fiat. Capturing this process is intrinsic to the widespread recognition of what standardization could bring in the way of benefits to the global process of disease eradication and/or control.

Rinderpest also acts quickly, which challenges AVIS to produce a system capable of rapid response. So the longer term vision of AVIS is real time support for the field worker. To achieve this takes time and financial resources but, most importantly, close attention to the user at work and the requirements that user might have as the computer plays an increasingly large part in his or her life.

In the pilot phase of rinderpest a number of more precise goals were defined:
(1) Introduce target users to the potential of multimedia and test assumptions about generic aspects of the system itself.

(2) Deliver a modular information and training package on rinderpest, containing information on:
- the disease, its agent and clinical symptoms
- pathology
- control, vaccines
- the ELISA system
- sero-monitoring
- legislation and regulations.

(3) Develop synergy with related EC and WHO activities, such as PARC, WAREC and SAREC.

The pilot phase realized these goals.

The AVIS project is now rolling forward into its next three year cycle. In the coming period the key objectives are:

- integration of disease reporting and risk management programmes with AVIS training and reference modules
- broadening of the consortium base to include other languages and cultures
- deployment of the integrated system to senior decision makers
- evolution of a consensus based standard for managing key diseases.

6.7. AVIS goals: The main phase, 1997–

The goals set in 1993 for AVIS have been reviewed at the end of the pilot stage and substantially confirmed. They will be kept under review as the main phase moves forward:

(1) To build a modular, multimedia information system providing easy, rapid, low cost access to standardized disease management information on a global basis through a standard multimedia PC platform.

(2) To create, by national consultation meetings, user seminars and working groups at local level, a network of key users capable of steering the AVIS project from a user perspective.

(3) To define a methodology for integration of AVIS into existing animal health information systems.

(4) To create a communications network enabling AVIS users to be in direct communication with each other, and with other on-line sources of information.
(5) To create and provide an AVIS Box, a personal computer either portable or desktop in nature, which contains all the essential tools and techniques required to support the practice and management of animal primary care.

(6) To derive management information from and for AVIS users sufficient to enable authorities at district and national levels to enhance disease prevention, management and control.

6.8. The AVIS System: Template and content

From the outset, AVIS has focused on creating a template for presenting disease information common to all diseases. Users thus learn the system once and it then works in the same way for each disease. Overall, the information is structured to make it mostly possible to access any information within three mouse clicks. Cross-referencing on key terms is standard as are facilities such as a glossary, keyword searching and printing.

A standardized template not only assists users; authors work to a predetermined structure which makes the organization of information (and the mechanics of reviewing and updating) significantly easier. This does not mean, however, that the template is inflexible. Each disease has different requirements and where the author(s) cannot accommodate these within the generalized AVIS template, it is varied.

Users have a variety of navigational aids such as a map from which point to point navigation is possible within and across any module; and a ‘preferred pathway’ is offered to new users for training purposes.

The information itself is structured into two main modalities: ‘encyclopedia’ and ‘tools’.

6.8.1. Encyclopedia

Each disease is treated in encyclopaedic detail, resulting in a template with six major modules per disease, each capable of running as a stand-alone program or being linked in one single disease information system. The content of each module is written by one or more acknowledged experts and editorial sign-off is based on a peer reviewed assessment by at least four reviewers.

There are four main information sections for each disease:

— What is [the disease]: overview
— The disease
— Diagnosis
— Management and control.
Within the encyclopaedic view, five user types are envisaged:

— senior decision makers
— disease specialists/veterinary officers
— laboratory workers
— field workers
— livestock owners/villagers.

Each user type has a module targeted at their needs but is able to access other modules through the same cross-referencing technique as works within modules.

6. 8. 2. Tools

Extensive analyses of user behaviour and requirements have shown that in the long term the ability rapidly to access specific parts of any program is of greatest value to experienced users: for example, an epidemiological technique, a diagnostic checklist, a standard report form or a laboratory technique. These are integrated into the ‘tools’ within each program.

An advantage of the ‘tools’ modality is that target groups, such as laboratory workers, can install the modules for all diseases and relevant techniques. The same facility enables field veterinarians to access support tools for primary response.

7. LEVERAGING THE PRICE: PERFORMANCE RATIO OF MODERN COMPUTING

AVIS was intended to capitalize on the potential of powerful but increasingly affordable computing technology to build a custom-made, easy to use system for veterinary medicine. In particular, the spread of cost effective portable systems — laptop computers and mobile phones with data transmission and reception capabilities — opens up new opportunities in supporting the work of veterinarians and others concerned with animal production and health.

As a system, AVIS is designed as a client–server environment, with the capacity to run disease training and reference programs in stand-alone mode on client multimedia PC machines. The architecture conforms to open systems integration standards, and the design approach uses object oriented programming with an object broker on the server.

7.1. Internet /World Wide Web/Intranet

In October 1996, agreement was reached with FAO to deploy some AVIS products via the Internet. A web site was built by Telos on the FAO server in Rome for
the World Food Summit (November 1996), from which programs can be downloaded. A home page has been built for EMPRES, and a pilot site has now been built for rinderpest, offering a synoptic summary of the rinderpest modules. Once testing has finished this will also be installed on the FAO server. The design of the Internet solution permits also an Intranet deployment. In the domain of disease reporting, this is likely to be a preferable solution to the Internet for reasons of data security.

8. WIDENING THE SCOPE: MUTUALITY AND TRUST

In the same report by Coopers and Lybrand that identified systems integration as the essential enabling condition of an improvement in disease management, another key condition for progress is identified — trust. Only if stakeholders trust each other enough to share data, information and knowledge will a real impact be noticed, with measurable beneficial outcomes. How feasible is this?

Among the many lessons to be learned from the BSE crisis is that animal health offers a natural and important opportunity to link all stakeholders in the community, probably more rapidly than in human medicine. But equally, the same crisis underlines what happens when trust fails. The outlook is not promising and will take considerable political will, and patience, to resolve in a positive way. This type (and level) of difficulty is what in the end will settle the fate of information technologies in the coming century; what the technologies can do, and what people will allow them to do are not necessarily the same thing. Or, put in a more germane way, you can take a horse to water but you cannot make it drink.

ACKNOWLEDGEMENTS

The AVIS project has received remarkable levels of support from a wide variety of colleagues; in a sense, naming is invidious, but the following individuals deserve nevertheless full recognition for their outstanding contribution to date: J. Anderson, J. Blancou, J. Bourne, Y. Cheneau, A. Donaldson, P.C. Lefevre, M. Moussaid, E. Moynihan, M. Reece and M. Rweyemamu. The author is grateful to P.C. Lefevre for insights in connection with Section 6.8.1.

REFERENCE

INTEGRATION OF MATHEMATICAL AND INFORMATION MODELLING APPROACHES FOR DECISION SUPPORT IN AFRICAN ANIMAL PRODUCTION AND HEALTH SYSTEMS

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Abstract
INTEGRATION OF MATHEMATICAL AND INFORMATION MODELLING APPROACHES FOR DECISION SUPPORT IN AFRICAN ANIMAL PRODUCTION AND HEALTH SYSTEMS.

The use of tools such as mathematical models, databases and expert systems has an important role to play in the provision of relevant expertise and technologies to farmers in developing countries. However, these tools are often deficient when used in isolation; their full potentials can only be realized through integration. The integration of tools is not, in itself, sufficient to ensure the effective and efficient use of information. The specific information needs of different decision makers will vary, and one cannot afford to create distinct systems for each type of user. These differences may be more apparent than real, representing different routes through the same information structure. The concept of information integration describes an attempt to organize data and knowledge in a single cohesive unit. The resultant complexity requires the provision of tools which provide appropriate access to, and navigation within, the information structure. Such an integrated information system is currently under development for the domain of African livestock production. It is anticipated that a system will enhance the diagnosis, treatment and management of cattle disease, increase understanding of animal nutrition and allow health and productivity records to be more effectively maintained. The paper illustrates the types of integration described above through the development of an integrated information system.

1. INTRODUCTION

African agriculture is faced with a huge challenge in meeting the current and predicted demand for foodstuffs within the continent. The problem stems from the high rates of population increase in sub-Saharan Africa. The human population of this
region is growing at a rate of 3.1% per year, which is the highest rate of increase in any area of the world. If such growth rates continue, the total population of the region will have doubled in around 25 years. Humanitarian and economic interests require food production to rise at a steeper pace than population size. Such a development would provide scope for the improvement of the nutrition level of African communities and for the reduction of food imports, with a resulting saving in foreign exchange. The World Bank has specified a target of a 4% annual increase in sub-Saharan food production. The increase in population which has occurred over the past thirty years has already wrought dramatic changes on the African environment. As demographic change accelerates, it becomes even more important that any technological developments that are introduced to increase food production should be sustainable in the long term, both economically and environmentally. It is vital to conserve the natural resource base for future generations. The past record of agricultural development in Africa would suggest that there is little room for complacency. During the period of 1962 to 1987, meat production increased at an average rate of 2.6% per year and dairy production at an average rate of 3.2% per year [1]. Past levels of productivity growth must be improved upon in the future if essential agricultural goals are to be achieved. Dairy farming is one particular example of an area with a large potential to contribute high value, high protein food products to an increasingly urban population. The bulk of east African milk production takes place in the smallholder sector, and this structure probably has the greatest potential for growth in the near future.

2. INFORMATION INTEGRATION

The diversity of information relating to agricultural production has often led information providers to the conclusion that each user need and information type pairing must be served by a separate system. While it would be foolish to ignore the fact that each user's needs must be taken into account during design and implementation, the extension of this client focused view to the creation of a large number of individual systems brings with it a number of problems. One of the most obvious problems is that of sharing information between systems which were conceived in isolation and use widely varying tools and approaches. This is, of course, not a problem unique to the agricultural sector but, in a recent paper, Parsons suggests that this type of incompatibility has been a factor in the generally slower than expected uptake of information technology in the agriculture sector [2]. In the system described here, for example, it was found that the expert system cannot provide useful advice without access to a detailed on-farm database, while such a database is itself of little value in evaluating the impact of technology changes unless it is, in turn, integrated with predictive mathematical models for farm management. Details of some approaches for dealing with this type of integration problem will be given in Section 4.
While difficulties in sharing information between systems is a real issue, a much more fundamental problem raised by the 'individual systems' approach are the artificial boundaries which are created between subject matters which should be considered in a unified manner. One of the major themes of sustainability is the need to view systems in a holistic rather than a segmented fashion. In the same way, information provision to aid decision making in a sustainable environment must ensure that appropriate linkages exist and that the 'whole' is not obscured by an overemphasis on individual parts of the picture. Apart from any theoretical arguments as to the completeness or elegance of this approach, this type of information integration has important economic implications. Rather than assuming that each group of users has a unique information need requiring a separate system, assume that the needs of different users can be met from different positions or dimensions in one space. If this is the case then the maintenance of information in a unified but suitably flexible manner will enable differing user perspectives to be captured within a common information structure. This approach is illustrated in Fig. 1, where the conceptual model of an 'information cube' is introduced. Note that the dimensions used in this figure are not the only possibilities, and in reality there will certainly be more than three important dimensions. While incorporating these would cause a problem in our geometric analogy, it would not prove particularly problematic within a faceted information system [3].

![Diagram](image-url)

**FIG. 1.** Differing perspectives on information for agricultural decision support.
Using a structure such as the ‘cube’ presented in Fig. 1, it should be possible to map the information needs of any given user to an area of ‘information space’. Thus, for example, a farmer’s information needs will tend to be satisfied by information of ‘local’ scope, ‘short’ time horizons and be of both a ‘structured’ and ‘unstructured’ nature. The dairy yields of cattle on the farm in the recent past (‘structured’) or word of mouth advice from extension agents and fellow farmers on current best practices (‘unstructured’) are examples of such classes of information. Some of a farmer’s informal information sources may fall into the ‘long’ term time dimension. An example for this might be the valuable resources of oral tradition, passed down the generations. The farmer’s characteristic needs are very different from those associated with the planner or policy maker, who typically requires highly processed information spanning lengthy time frames and larger geographical areas. Moreover, different segments of the information cube not only represent the variety of possible user needs but also imply the need for different types of tools to process and present the information. Thus, while database and spreadsheet packages will be appropriate to the ‘structured’ levels of information, hypertext and other information retrieval tools will be required for the ‘unstructured’ levels.

A simple type of conceptual integration was pioneered in the area of librarianship with the development of classification systems such as Dewey and UDC to give conceptual content tags to books. This work was continued and expanded into the discipline of information science, where tools such as thesauri and faceted classification systems were introduced to supplement the earlier hierarchical or enumerative approaches. Because of its origins, most of the work in the area of information retrieval has been focused on dealing with unstructured, textual information. However, recently it has been noted that classifications for a given domain can be used to index information and knowledge of other types. The most notable attempt to move classification tools into this wider context have been the UMLS and the GALEN projects within the medical domain [4, 5], though developments are still at an early stage. It is hoped that insights gained from these areas will aid the development of a fuller theoretical framework, which will in turn underpin the work carried out within decision support projects. The ways in which such a framework might be utilized are illustrated in the next two sections, which consider the implications of providing information to a wide range of differing users through the use of a diverse range of tools and techniques.

3. INTEGRATED DECISION SUPPORT: THE USER DIMENSION

It is important to stress that the type of system being constructed within such projects is a decision support system, not a decision making system. Some of the
components which are being incorporated into the system, such as mathematical models and expert systems, have at times been misinterpreted as decision making devices. However, the idea of a computer system which tells the user what to do is a discredited one. Decision support systems do not supplant the decision maker. They should provide information in an appropriate format, enabling users to make use of as much (or as little) of the available resources as they wish, allowing them to make better decisions. As discussed earlier, different users will have different information needs, as well as having different capabilities in utilizing information which is presented. These differences can be illustrated by considering how a decision support system might handle information on the subject of animal disease and how different users would interact with the system. A description of the proposed system which places more emphasis on the animal health aspects of software development is available in Ref. [6].

To illustrate the integration of information from a user perspective, the manner in which such a system deals with an important protozoan disease is considered. East coast fever (ECF) is a disease of cattle caused by infection with a protozoan parasite, *Theileria parva*. The presence of this disease is a major constraint on dairy production in east Africa, owing both to the mortality and morbidity losses which it causes and to the indirect losses which it imposes on the farmer. The most important of these is foregone production. If ECF were not present, farmers would be able to keep non-native, more highly productive cattle. Reliable drugs are now available to treat infected animals, but prompt intervention is required if treatment is to be effective. This situation has led to the introduction of a type of immunization in which cattle are inoculated with living *T. parva* parasites. Simultaneously, the animals are injected with a long acting dose of the curative drug. Typically, the animal develops an infection, which is, however, inhibited and eventually eliminated by the drug without further intervention by the veterinarian. This infection is sufficient to provoke an immune response within the animal, which will henceforth be immune to the strains of parasite against which it has been challenged. A new technology is being introduced to Africa. The potential benefits of this 'infect and treat' methodology are vast, but incorrect usage could introduce huge risks to the farmer. Let us consider how information relating to this technique is presented to, and used by, a variety of groups supported by the decision support system. Farmers are the owners of the cattle. It is they who will make the decision to use 'infect and treat' on their animals; it is they who will either benefit from, or suffer the consequences of, this decision. They need to be provided with guidance on the suitability of their animals for this procedure and with estimates of the likely costs and benefits that will ensue. This information is being incorporated in the form of an expert system, linked to mathematical models for disease dynamics and productivity. Farmers should not, however, be expected to assimilate technical veterinary information nor do they require a description of the details of the 'infect and treat' procedure. The
technique requires specialist materials and skills and is, therefore, regarded as outside the expertise of a farmer; this is reflected in the scope of information which is made available. It is, however, important to watch treated cattle with care in order to identify a small minority of animals which are likely to develop a serious clinical form of ECF in response to the immunization. Hypermedia resources which detail the clinical signs of ECF can be used to prepare farmers for this task. The materials needed to achieve this are likely to be present already in the system because they are of even greater importance to the next group of users we shall consider.

Veterinary surgeons and veterinary health assistants are the people who will provide additional advice to farmers and carry out the ‘infect and treat’ procedure. They also need to be provided with guidance on the suitability of different breeds and different situations for immunization, but this can be based on their provision of more technical specifications than would be possible for a farmer. The logical inferences which the expert system uses, however, remain similar to those used to process the input of a farmer. There is also scope to provide veterinary assistants with a detailed description of the correct procedure to be followed during the ‘infect and treat’ procedure. This consists of a small expert system module which is integrated with a hypermedia database consisting of text, archetypal datasets, diagrams and photographs illustrating the technique.

The information and resources used in this detailed description of the mechanics of ‘infect and treat’ would also be of use in the training of student veterinarians and veterinary health assistants. The information could be presented to these users in a form which emphasizes the educational aspect of the material. Different situations could be presented to students, who would subsequently have to select the most appropriate action, which could then be compared and contrasted with the best practice as recommended by the expert system [7].

It is envisaged that one further group might benefit from the information structures which are contained within the ECF related module of the decision support system. Mathematical models [8] will be used within the system to process data and to provide estimates of the future incidence of disease subject to different control strategies. These models will be of considerable interest to research scientists, such as biologists and epidemiologists, who can use them to examine the effect that different biological hypotheses might have on the dynamics of the disease. Hypotheses which give rise to unrealistic disease systems can then be dismissed. Research scientists would be given scope to change the properties of these mathematical models, whereas farmers and veterinary users would not wish to alter these specialist information structures. In fact, it is anticipated that the models which are used to give advice to the farmers and veterinary surgeons could be parametrized by exploring the disease complex with veterinary epidemiologists, using the tools which are provided for them in the system.
4. INTEGRATED DECISION SUPPORT: THE SYSTEMS PERSPECTIVE

Numerous attempts have been made to develop computer systems to aid decision making. These include the development of database management systems, mathematical model based systems, expert systems and multimedia systems. A database management system is a software package which stores data sets in a logical manner and provides tools which allow users to easily manipulate and analyse data. A mathematical model utilizes mathematical structures to obtain insight into dynamically evolving systems. Such models are usually based on systems of equations which incorporate parameters that can be estimated from available data sets [9]. An expert system is a program which attempts to interpret information automatically in a manner equivalent to that used by a human expert. Conventional expert systems are usually based on heuristic rules which can be obtained by interviewing domain experts [10]. Recent advances in the development of expert systems use more sophisticated techniques such as belief networks to incorporate the uncertainties which are involved in the decision making process of an expert [11]. A multimedia system is a framework which will allow the user to make more effective use of stored but unstructured information such as literature, pictures, sound and video clips.

The above techniques have been applied to the solution of problems in a wide variety of domains. However, these applications are usually limited to a small and specific domain, comprise tools designed for a particular type of user and restrict the access of these users to a limited range of information. Another factor which has reduced the utility of existing information tools is the tendency to use them in isolation. The users of a database management system may sometimes find it hard to understand the outputs from the system when these are presented as uncontextualized, uninterpreted data elements. A mathematical model can be used to produce estimates of otherwise unobservable pieces of information, but users may find it hard to accept and understand such predictions without suitable guidance. An expert system, in mimicking the decision making process of human experts, may not be able to complete its inferential process without access to the information contained in a detailed database. Integration of these techniques can combine the advantages of each method while overcoming their individual shortcomings. An additional benefit of this commitment to the integration of tools is the flexibility in system design which it provides. It is likely that for each subject of interest within the decision support system, one type of tool will be found to be more suitable for manipulating information than the others. This optimal tool can then be applied for this purpose. This policy will ensure that the system as a whole makes the best possible use of the available information. It is obvious that a database system should be used in the management of large quantities of data. A mathematical model might profitably be used in the analysis of numerical information. A conventional rule based expert system is effective in the presentation of information which has been extracted from human
experts in the form of rules of thumb, while belief network based expert systems are invaluable in the analysis of situations which involve a degree of uncertainty. The benefits of a multimedia system will only be fully appreciated when it is acting as a resource for other information tools, providing background information and supporting the interpretation of the output from these other tools. An example of the use of an integrated hypertext/expert system is given in Ref. [12].

This variability in suitability is closely related to the variability in the types of information which these tools can process. Rule based expert systems provide a means of structuring qualitative knowledge which has been gathered from experts. The output of such a system will typically be a single 'conclusion' based on the factual information provided by the user. Belief networks update quantitative, probabilistic information in order to express knowledge about the relative likelihood of different scenarios. Mathematical models utilize qualitative information in the formulation of the mathematical rules which describe how the model will evolve, while using quantitative data to estimate the numerical parameters which are also needed. The output from these models is quantitative, but other properties of the information will depend on the choice of model.

The smooth transfer of information between different tools is essential if information integration is to be achieved. In some situations, the process is simple. Output from a stochastic mathematical model can be presented as a set of probabilities, which are easy to reinterpret as a set of beliefs for use as input to a belief network. The results of a deterministic mathematical model can be used as input to a rule based expert system since both tools operate within a paradigm that ignores randomness. Factual information which is used in a rule based expert system can easily be recalled from a database. Deterministic mathematical models can use datasets stored in a database to estimate average rates at which events take place. It is also operationally simple to pass deterministic information to a tool which operates within a stochastic paradigm. If output from a rule based system or deterministic model is passed to a belief network, this particular input can be interpreted as having a probability of one. Concern may be expressed about the validity of stochastic inferences based on such a procedure. However, if the incorporation of deterministic information creates severe inaccuracies in the belief network, this is a sign that the information which is being processed within the deterministic tool is inherently stochastic, a fact which should have been addressed in the choice of information tool.

It is more complex to pass information from databases to mathematical models. Firstly, qualitative information might be required to choose the correct rules to describe the evolution of the disease complex. It is not possible to pass on this form of information directly, but it would be easy to write a rule based expert system to interpret information in the database and specify the rules which are most appropriate to a given situation. Once these rules have been specified, it is necessary to estimate numerical values in a stochastic model which identify when each rule will be invoked.
Where a data set related to these random variables is available, automated statistical procedures can be used to produce a series of pseudorandom variables with the correct distributional properties. Techniques such as the parametric and non-parametric bootstrap [13] can produce meaningful statistical analyses on an automatic basis.

The scope and utility of information integration from a systems perspective can be illustrated by considering some of the information exchanges which might be devised for use within a decision support system for dairy production:

— A database can be used to store past weather conditions.
— The data can be used to produce empirically derived statistical distributions which are then used to simulate typical pseudorandom weather patterns. (Care must be taken to maintain a flexible design so that weather pattern data can be taken from a geographical information system module should one exist for the area being considered.)
— The simulated (and real) weather predictions can be passed to a mathematical model which uses this information to model the population dynamics of the vector which transmits a particular disease. This stochastic model is then used to simulate the disease dynamics which will be observed in cattle that have been exposed to certain conditions.
— These probabilistic predictions can be used to train a belief network based expert system which summarizes the influences of different conditions on the probability of disease outbreak. When this belief network is provided with values from another database which stores information about the conditions which are currently being seen in the field, such as rain patterns or animal health status, it is possible to estimate whether there is a high risk of a particular disease breaking out in the immediate future.
— If the current disease risk appears likely to be very high, a rule based model dealing with the prevention of disease will be invoked, giving advice on the immunization and animal management practices which might be used.
— Should animals become ill, in spite of the advice which has been given on the prevention of disease, a belief network expert system can be used to provide a diagnosis of the disease agent, based on the clinical signs observed in the sick animals.
— A rule based advisory model can then be called to give advice on the treatment of whatever disease has been diagnosed.
— During this advisory session, the system can extract data from specific databases whenever they are required. For example, information on drug treatment can be stored in a drug information database which incorporates detailed information on the use of various drugs in the treatment of different diseases.
— A multimedia facility is available which can be called from within any of these modules whenever the user requires further information on a particular topic.
This type of integration is very powerful and helps address many of the limitations inherent in using any one of the tools in isolation. It can be seen that the flow of information between different tools will enhance the value of the individual components of the system.

5. CONCLUDING REMARKS

The experience of the authors in developing decision support systems for African livestock production has convinced them of the necessity of integrating different information tools and data structures into a single cohesive framework. We have described how this might be attempted through flexible design and a focus on information structures rather than client needs. The presence of diverse groups of potential users therefore presents both a challenge and an opportunity. There is a great deal of useful material which might be integrated in this way and then delivered to agricultural communities in Africa, and the need for such an information transfer is urgent. A wide range of users might be able to make use of this large mass of information, but this will only be possible if suitable tools are made available to the different users to achieve their individual ends.

The information tools which have been discussed in this paper can be combined to form a hybrid information system which allows users to interact with the information base in a flexible manner. Most of the individual subsystems within such a configuration are already being used, though often in isolation, to assist decision makers in agriculture. By bringing these subsystems together within a cohesive information matrix, each component subsystem will enable the user to make better use of the others, ensuring that the value of the whole system is greater than the sum of its parts.

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REFERENCES


SATELLITES/CLIMATE
(Session 6)

Chairperson

D.J. ROGERS
United Kingdom
AN INTRODUCTION TO THE GEOGRAPHIC INFORMATION SYSTEM AND ITS USE IN LIVESTOCK DISEASE CONTROL PROGRAMMES

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Abstract

An introduction to the Geographic Information System and Its Use in Livestock Disease Control Programmes. The concepts and applications of the geographic information system (GIS) and remote sensing (RS) are explained and details of computer software and hardware requirements are given. A review of applications of GIS and RS in livestock disease control programmes and land use planning is presented. The database development process and data analysis procedures and requirements are briefly outlined. The integration of the GIS and decision support systems is discussed in order to provide a cost–benefit analysis for GIS implementation.

1. GEOGRAPHIC INFORMATION SYSTEM (GIS)

The GIS is a collection of computer hardware and software, organized to store, view, update, analyse and manipulate geographically referenced data for the production of reports and maps [1]. The GIS can handle large amounts of data for analysis and perform spatial overlay operations when a new spatial area has to be derived from an existing map. For this operation a cartographer who uses traditional methods would need a long period of time. With the use of a computer, the GIS can rapidly compare, combine and, subsequently, evaluate maps and thus illustrate relationships among variables in a novel way (Figs 1 and 2).

1.1. History of the GIS

Different geographical aspects of the earth’s surface are not independent of each other and can be evaluated in an integrated, multidisciplinary way. The conventional method was to overlay transparencies of resource maps on light tables and identify places where the various attributes on the maps coincided.
During the 1960s, computer technology was utilized to produce simple maps of statistical values on a grid of plain paper and to overlay the grid values by using line printer characters to produce various intensities of grey colour representing the statistical values. This was known as a grid cell or raster system. The results were not sufficiently refined to find general acceptance among cartographers.

By the late 1970s, the technology of computer map production had progressed while, at the same time, advances had been made in a number of related fields, including soil science, surveying, photogrammetry and remote sensing. The result was a linkage of different sets of spatial data into truly general purpose geographic information systems.

By the early 1980s, the GIS developed as a computer technology that was sophisticated, relatively inexpensive and widely accepted. At present, GIS systems are being used by public agencies, research laboratories, academic institutions, private industry, public utilities and the military.
Previous versions of the GIS required either workstation hardware or powerful personal computers. A recent development in software has been the emergence of the desktop GIS, which is targeted at individual users and small firms who need data integration, simple statistical data analysis and presentations in the form of charts and maps. This now represents some 30% of the GIS users; their hardware and software requirements for desktop GIS are modest (Fig. 3 and Table I).

1.2. Remote sensing

Remote sensing (RS) is the science and technology of obtaining information on an object, area or phenomenon through the analysis of data acquired by a device that

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\text{TABLE I. SOFTWARE AND HARDWARE SPECIFICATIONS FOR A DESKTOP COMPUTER GIS ENVIRONMENT.}
\]

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIS software</td>
<td>ARC/INFO version 3.5 and ArcView 3.0 [12]</td>
</tr>
<tr>
<td>Operating system</td>
<td>Windows NT or Windows 95 or MS-DOS</td>
</tr>
<tr>
<td>PC minimum configuration</td>
<td>Pentium (1 GB hard disk, 16 MB RAM, etc.)</td>
</tr>
<tr>
<td>Monitor</td>
<td>Super VGA, size 14 or 17 in.</td>
</tr>
<tr>
<td>Peripherals</td>
<td>Digitizer (size), plotter (design/ink-jet), printer (laser-jet), scanner (1200 dots per inch)</td>
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is not in contact with the object, area or phenomenon under investigation [2]. Orbiting satellites are fitted with electromagnetic energy sensors to acquire data depending on the way various features of the earth's surface emit and reflect electromagnetic energy, usually infrared (Fig. 4).

The RS data are analysed by using an image processing application (IDRISI) to analyse measurement, classification and estimation of a specific parameter or to compare them through other parameters, i.e. soil or vegetation [3].

The applications of RS for arthropod vector surveillance and control have been reviewed recently [4]; for example, RS has been used as a landscape epidemiologic tool to identify villages at high risk from malarial transmission in Chiapas, Mexico [5]. Similarly, mortality rates and population densities of tsetse flies were found to correlate with data from meteorological satellites. Satellite imagery could thus be used to predict the abundance of tsetse over large areas of the African continent and to produce maps of high risk areas for the disease [6].

2. DATABASE DEVELOPMENT AND MANAGEMENT

2.1. Database development

Database development involves converting existing data into a digital format and entering the data into the GIS (Fig. 5). The development requires experienced

FIG. 6. Example of data analysis procedure for modelling land suitability for crop production.
personnel and is labour intensive. The database specifications and analysis procedures must always be defined. Written procedures are needed for source data collection, interpretation, accuracy, verification and the preparation of data for input. User groups must be co-ordinated, and the types of output requirement must be defined. A high standard for data preparation and quality control should be maintained at all times.

2.2. Data analysis

A unique feature of the GIS is spatial analysis, which can be used not only to answer questions about what existed at some point in the past, or exists now, but also to predict the consequences of proposed activities. However, as an initial step, the geographic information needs to be organized so as to optimize the convenience and efficiency with which the data can be used. Some of the operational procedures available within a GIS to perform a spatial analysis are listed in Table II. An example of a data analysis procedure for modelling land suitability for crop production is shown in Fig. 6. Thus, GIS provides the tools for combining relevant sequences and developing models. Moreover, the model may reveal new or previously unidentified relationships within and between data sets, thus increasing our understanding of the data. Results of GIS analysis and modelling can be presented as maps and/or reports.

3. APPLICATION OF GIS AND RS IN LIVESTOCK DISEASE CONTROL PROGRAMMES

3.1. Background

Cattle, goats, sheep and camels are important sources of nutritious food — milk and meat — for smallholder families in Africa and at the same time can generate cash income through the sale of animal products. In addition, livestock provide tractive power for the transport of goods or for the cultivation of land. Furthermore, the animals produce manure, which can be used as fuel or fertilizer. Efforts to increase livestock production in sub-Saharan Africa have been hampered by seasonal shortages of fodder, a multitude of livestock diseases as well as the lack of a land tenure system. In order to increase animal production in Africa, the Animal Production and Health Section of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture has concentrated part of its efforts on the improvement of disease diagnosis by promoting the use of techniques based on nuclear and related technologies. Following the development of the enzyme linked immunosorbent assay (ELISA) in 1971 [7], the technique was soon introduced as a standard test to diagnose a wide range of diseases [8]. For example, an ELISA was developed for the detection of
### Table II. Listing of Some of the GIS Operational Procedures to Perform Spatial Analysis

<table>
<thead>
<tr>
<th>Analysis procedure</th>
<th>Type of information generated from the GIS database</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overlay operations</strong></td>
<td>Arithmetic and logical overlay includes such operations as addition, subtraction, division or multiplication of each value in a data layer by the value in the corresponding location in a second data layer.</td>
</tr>
<tr>
<td><strong>Neighbourhood operations</strong></td>
<td>Neighbourhood operations evaluate the characteristics of the area surrounding a specified location, i.e. counting the number of livestock within a 5 km radius of a village.</td>
</tr>
<tr>
<td><strong>Line-in-polygon and point-in-polygon operations</strong></td>
<td>To identify points and lines contained within a polygon area, i.e. the roads and their respective lengths passing through a district.</td>
</tr>
<tr>
<td><strong>Topographic functions</strong></td>
<td>Surface characteristics, i.e. the relief of an area.</td>
</tr>
<tr>
<td><strong>Interpolation</strong></td>
<td>Interpolation is the procedure of predicting unknown values by using the known values at neighbouring locations.</td>
</tr>
<tr>
<td><strong>Contour generation</strong></td>
<td>Contour lines are used to portray surface relief as a set of lines connecting points of the same value, i.e. to connect points with the same altitude (in metres above sea level).</td>
</tr>
<tr>
<td><strong>Proximity</strong></td>
<td>Proximity is a measure for the distance between features, i.e. a buffer zone, being an area of a specified width drawn around one or more map elements.</td>
</tr>
<tr>
<td><strong>Network functions</strong></td>
<td>Network functions are a net of interconnected linear features that form a pattern or framework, i.e. routing or resource allocation.</td>
</tr>
</tbody>
</table>

*a Adapted from Ref. [1].

trypanosomal antigens in cattle [9] and was applied to the diagnosis of trypanosomosis [10–12], one of the most important diseases affecting livestock in Africa.
This particular ELISA was transferred by the Joint FAO/IAEA Division to a number of African research institutes [13] to monitor tsetse and trypanosomosis control and eradication programmes [14].

As additional tools for assessing disease distribution and vector population dynamics, monitoring the effectiveness of disease control and predicting the results of interventions, other technologies such as GIS and RS are being introduced [15, 16].

3.2. Theileriosis GIS

The GIS has been used to represent geographically the known distribution of diseases caused by *Theileria parva* and their vectors in Africa, to identify, categorize and map the data in relation to selected variables relevant to the epidemiology of the diseases and to define the zones that are environmentally suitable for the survival and development of the vector [17]. Variables studied included major hosts (cattle and buffalo), the vector ticks (*Rhipicephalus appendiculatus* and related species) and the reported presence of East Coast fever, corridor disease and January disease. In addition, the distribution of climatic suitability for *R. appendiculatus* was assessed. The considerable value of GIS is its ability to provide a systematic geographical representation of the available data relevant to the epidemiology of theileriosis and to improve the definition of the environmental parameters that influence the distribution and prevalence of tick borne disease in Africa. Additional studies attempted to quantify and evaluate the variables affecting the distribution of *R. appendiculatus* in various parts of Africa [18]. Recently developed interpolated climate databases for the region were used to drive the climate matching model CLIMEX to show that much of the highlands of Ethiopia are suitable for the survival of *R. appendiculatus* [19]. In Zimbabwe the seasonal vegetation differences in both communal and commercial grazing areas were evaluated by using the advanced very high resolution radiometer (AVHRR) normalized difference vegetation index (NDVI), which is a good measure of green leaf biomass and surface area [20]. This information has been very useful in defining and quantifying vegetation greenness differences between various grazing areas during the year and can provide significant information for decisions on tick and tick borne disease control strategies, now and in the future.

3.3. Trypanosomosis GIS

The application of RS to studies on African trypanosomoses showed the NDVI to be of great value in monitoring tsetse mortality rates and the seasonal incidence of sleeping sickness in the human population [21]. A theoretical framework was developed and examples were given on how an intensive study in one or two places might be used to make extensive predictions about vector and disease distribution. In addition, the GIS was used to introduce spatial and temporal elements in the
epidemiological studies on human and animal trypanosomosis in Africa [22]. It was argued that the GIS provides the techniques for simple analysis of data and the tools to manipulate the data within a geographical framework and, thus, increases our understanding of the variations in space and time of tsetse and trypanosomosis distribution and abundance [23].

A field study in the Gambia reported the spatial and temporal distribution of herds of cattle in relation to tsetse and calculated an index of challenge based on the ratio of vectors to hosts over the livestock ranging area [24].

Previously, the lack of quantitative multidisciplinary data constrained ex ante evaluations of trypanosomosis control at a continental level [25]. However, the advent of computerized data storage, analysis and display systems in the form of a GIS now makes it possible to assess the economic, environmental, demographic and sociocultural factors influencing land use decisions in Africa and the impact of land use change associated with trypanosomosis control on these factors [25]. For example, tsetse flies were shown to be a better discriminator (73% correct) of human population density than rainfall (67% correct) or elevation (65% correct). This suggested that tsetse may be an important constraint to human land use. Furthermore, a positive relationship was detected between land use intensity and human population density in Burkina Faso, Zambia and Mali. However, the relationship between tsetse presence and the intensity of agricultural land use varied in each of the three countries, being a negative one in Zambia, variable in Mali and a positive one in Burkina Faso [25].

3.4. Applications of GIS and RS in land use planning

Land use planning is the systematic assessment of land and water potential, alternatives for land use, and economic and social conditions in order to select and adopt the best land use options [26]. Various options of rural land use are assessed for agriculture, pastoralism, forestry, wildlife conservation and tourism [27].

The Government of Canada was one of the first to use the GIS on a nation-wide scale. The Canada Geographic Information System (CGIS) has been operational since the late 1960s. It evolved from a project to develop a national land capability classification and to compile an inventory of potential productive land in Canada. Since then, various other land information systems have been developed (Table III) [28–33].

A study commissioned by the FAO explored the possibilities of using techniques inherent in the GIS to produce a database containing information on global livestock distributions, human demographic data and agro-ecological information [34]. The results showed that GIS techniques can be useful in deriving livestock populations from the distribution of people, and that even the simplified analyses used could produce useful insights into livestock systems defined by agro-ecological and human demographic criteria.
TABLE III. LISTING OF SOME OF THE LAND INFORMATION SYSTEMS (LIS) DEVELOPED SINCE 1960

<table>
<thead>
<tr>
<th>Area</th>
<th>LIS</th>
<th>Year</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada</td>
<td>CGIS-CLDS</td>
<td>1960</td>
<td>Started as a project to make an inventory of potential productive land in Canada; includes forestry, recreation and wildlife information; provides services to governmental agencies and specialized agencies of the United Nations.</td>
</tr>
<tr>
<td>USA</td>
<td>GIS for LRP</td>
<td>1982</td>
<td>GIS techniques for rural land use planning in Dane county, a major agricultural area in Wisconsin.</td>
</tr>
<tr>
<td>USA</td>
<td>GIS for SCP</td>
<td>1985</td>
<td>Developed by Oklahoma State University and used to predict soil erosion and assist with erosion control programmes.</td>
</tr>
<tr>
<td>Europe</td>
<td>CORINE system</td>
<td>1990</td>
<td>Developed by the European Union for comparative analysis of agricultural and other land use issues of member states.</td>
</tr>
<tr>
<td>Kenya</td>
<td>Agro-ecological assessment</td>
<td>1993</td>
<td>Computerized land resources database of Kenya and the crop, livestock and fuelwood productivity models derived from land suitability assessments together provide a basis for estimating potential productivity of land resources [28].</td>
</tr>
<tr>
<td>IGADD</td>
<td>Crop production system zones (CPSZs)</td>
<td>1995</td>
<td>Numerous variables (502) describing the physical and biological environment were used to define 44 crop production system zones, which describe actual farming in the subregion. The CPSZ database viewer software provides more direct access to the actual data [29].</td>
</tr>
<tr>
<td>Togo</td>
<td>Tsetse fly and its control</td>
<td>1994</td>
<td>Grid based sampling surveys of Togo provided valuable data sets on tsetse, cattle and trypanosomosis throughout the country. A combination of ground based meteorological and remotely sensed satellite data, within linear discriminant analytical models, enabled description of the observed distribution of the five tsetse species occurring in Togo [30].</td>
</tr>
<tr>
<td>Africa</td>
<td>Tsetse fly and its effects on agriculture in sub-Saharan Africa</td>
<td>1995</td>
<td>Computer simulation models were designed to forecast the patterns of human and cattle population densities and those of arable land use in the moist subhumid zone of Nigeria. The data were transferred to GIS for mapping purposes. The model predicted the results of interactions between such variables as human and cattle populations, their growth rates, the intensity of cultivation and the influence of tsetse/trypanosomosis [31].</td>
</tr>
<tr>
<td>Kenya</td>
<td>GIS for land use planning</td>
<td>1995</td>
<td>GIS was used to analyse and perform spatial overlays to develop models on agricultural expansion, land suitability, land use conversions, wildlife distribution and human–wildlife conflict areas [32, 33].</td>
</tr>
</tbody>
</table>

CGIS = Canada Geographic Information System; CLDS = Canada Land Data Systems; LRP = Land Records Projects; SCP = Soil Conservation Planning; IGADD = Intergovernmental Authority on Drought and Development; FAO = Food and Agriculture Organization of the United Nations; OAU = Organization of African Unity.
4. DECISION SUPPORT SYSTEMS

An overview of models available to predict the occurrence of diseases and their effects on animal health was presented at the Africa GIS 1995 meeting [35]. It was observed that the use of the GIS by the epidemiologist has evolved from a simple display of mapped data to fairly sophisticated modelling and decision support systems. Case studies in Uganda, Namibia and Zimbabwe were presented, showing that the GIS can be integrated with modelling, expert systems and statistical analysis to provide information to decision makers in a form that is timely and easy to use. GIS tools have evolved such that simple analysis can be performed by the decision makers, rather than by the GIS specialists [35].

In the next decade, it is expected that the use of the GIS for epidemiological applications will evolve further, so that the massive amount of information available can be profitably used not only to improve animal disease control but also to assist the integration of animal disease control efforts with other attempts to improve agricultural production [35].

A sero-monitoring database system for rinderpest and brucellosis has been developed at the IAEA's Animal Production and Health Section, which can be linked to a GIS system as an initial step in developing a decision support system (Fig. 7).

FIG. 7. Flow chart for integration of database systems with GIS.
REFERENCES


SATELLITE IMAGERY AND THE PREDICTION OF TSETSE DISTRIBUTIONS IN EAST AFRICA

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Abstract

SATELLITE IMAGERY AND THE PREDICTION OF TSETSE DISTRIBUTIONS IN EAST AFRICA.

The paper briefly reviews the debate over whether the aim of tsetse suppression is eradication or control, concluding that control is the only viable solution for many areas of Africa. This approach requires a fuller understanding of the natural dynamics of tsetse populations and of the environmental limits to their distribution than does the alternative approach aimed at eradication. Various types of remotely sensed satellite data are correlated with the meteorological records that have in the past been used to interpret tsetse distribution and abundance patterns, and have the advantage over such records of complete spatial coverage at acceptable resolution for country, regional or continental studies. The paper describes both the temporal Fourier processing of such data from the NOAA and Meteosat series of meteorological satellites and the application of these processed data to describing the distribution of five species of tsetse in East Africa, Glossina morsitans (s.l.), G. pallidipes, G. austeni, G. longipennis and G. fuscipes fuscipes. Observed distributions are described with accuracies of between 85 and 97% (G. morsitans and G. austeni, respectively) when, within non-linear discriminant analysis, variables are selected on the basis of maximizing the minimum multivariate distance between alternative categories of presence and absence training set data. Inaccuracies in the predicted maps arise for several reasons: the distribution maps used to define tsetse and non-tsetse areas are now rather out of date; some of the areas for which predictions are made are very different from any of the training set areas, although the analysis may be forced to assign such areas to one or other category; and, finally, non-tsetse areas may indeed be climatically suitable for flies, but not inhabited by them for a variety of reasons. Examples are given of each for the case of G. longipennis, a species unrecorded from relatively large areas of Kenya which appear to be suitable for it. The conclusion highlights the need to test many of the ideas presented in this paper using contemporary tsetse and satellite data, to establish a real time monitoring system for the changes that lie ahead.
1. INTRODUCTION

The role of remotely sensed satellite data in defining the natural habitats of tsetse flies in East Africa is becoming increasingly important as it is now generally accepted that tsetse eradication over vast areas is prohibitively expensive, even if it were ecologically possible, and that some form of long term tsetse control is the only viable solution. This realization requires a reassessment of the aims and objectives of the applied research that is required before any intervention. Eradication campaigns involve a high level of investment over a short period of time. It is important that eradication is achieved quickly so that the benefits may be reaped as rapidly as possible, thus ensuring the economic viability of the eradication exercise. The rapid execution of an eradication programme can often only be achieved with a high degree of involvement of government or external agencies and expertise, and programmes must be planned and executed with almost military precision. Almost by definition, such involvement cannot be long term since government agencies have many other activities to carry out, whilst external agencies need to justify their activities to international donors. The great danger of eradication programmes that fail is that there is often little to show for them several years after they have ended. Neither the peasant farmers, faced with the same degree of the problem as before, nor government services, trained in techniques which have demonstrably failed, benefit from the exercise. Eradication exercises require little beyond a minimal knowledge of the pest’s biology: they are an expensive way to remain ignorant.

Vector control, rather than eradication, is now seen as the only viable solution to the tsetse problem in Africa. Such control involves low cost, locally producible and maintainable traps or targets, or the use of pour-on insecticides or trypanocidal drugs in domestic livestock populations. Several conditions need to be met before such control can be contemplated. The stake holders — generally the livestock owners — must acknowledge that trypanosomiasis is a constraint on production worth addressing by the expenditure of capital or the investment of time (e.g. to make or service traps or targets). This is the same as saying that the benefits obtained by removing the constraint are obvious and translatable, at least in theory, into cash returns that are equal to or greater than the cost of the control in the first place. The second condition is that the individual indulging in control should experience directly the benefits of it. Individual behaviour, the returns of which are only felt at the level of society, will almost certainly not persist beyond the initial flush of enthusiasm that all new projects generate. The role of government and external agencies in this approach to trypanosomiasis control is to provide the environment in which individual stakeholder involvement is sustainable in the long term. Governments might be expected to work at the community or higher level to support the activities of the individual farmers by, for example, the provision of access roads to existing, or the building of new, markets; the provision of education or other materials explaining
the local options for trypanosomiasis control; training in the construction and siting of traps or screens and, finally, monitoring the success of local activities. Most importantly, government services need to identify the areas of each country in which all these activities may be profitably encouraged. In particular cases it may be necessary for governments to maintain barriers preventing fly movement from one area to another, thus protecting the activities of many individual farmers.

Recent work suggests that only in a few areas are the right conditions met for government level trypanosomiasis control to be justified. In southern Africa, for example, the emphasis is upon those areas infested with tsetse that are adjacent to other areas where land pressures are already considerable; the areas to be controlled must be of a sufficiently large size to relieve land pressure for a sufficient period of time for the exercise to be economically worthwhile [1]. Whether or not the same considerations apply to situations in which individuals carry out all, or nearly all, of their own trypanosomiasis control activities independently of government is still uncertain. Given the past, rather anarchic, spread of the illicit use of trypanocidal drugs, a practice that is likely to extend to insecticidal pour-on applications, it seems unlikely that governments will have much influence on trypanosomiasis control methods that they do not directly fund. This should only be of concern if the individual farmer’s behaviour has long term, adverse consequences that society as a whole eventually pays for; increasing land degradation through over-stocking is one consequence, environmental pollution with pesticides (rare in tsetse control) is another. Such consequences are internal to each country, and are important matters for societal debate. Trypanocidal or insecticidal resistance are other consequences with ramifications beyond a country’s borders; the worldwide spread of malaria resistance to chloroquine is a cause of international concern and research action. In a sense, factors such as these, with external consequences, may be less severe from an individual country’s point of view; the bigger the problem, the more likely it is that somebody else will deal with it (the commercial sector may also have an interest in maintaining their market share of drug or pesticide sales by developing new products, although the track record for this in Africa is disappointing).

The role of research in either government or individually led trypanosomiasis control is to define the local conditions of disease transmission in ways that lead to sensible and efficient transmission reduction. Given that one of the crucial factors in determining transmission rates is the vector mortality rate (which determines equilibrium disease prevalences non-linearly), research could sensibly concentrate on this key parameter. Vector mortality rates are probably the most variable of the various demographic rates (i.e. birth, death, immigration and emigration) affecting tsetse and so will almost certainly also determine whether or not tsetse occur in any area. In the past, these rates have been correlated with environmental temperature and/or saturation deficit, measured at standard meteorological stations [2]. The recent increased availability of remotely sensed satellite data, many of which are strongly correlated
with ground based measures of climate or weather [3], now allows us to replace the rather sparse coverage of meteorological station records in Africa with the comprehensive data from these satellites, and so to begin to make predictive maps of tsetse and disease distributions as inputs to GIS decision taking exercises. This paper describes how to make predictive maps of the distribution of key species of tsetse in East Africa.

2. MATERIALS AND METHODS

2.1. Satellite data

Many of the satellite data come from the National Oceanographic and Atmospheric Administration (NOAA) series of meteorological satellites, each in polar orbit and providing two images per day (a daytime and a nighttime image) of every area on earth. The advanced very high resolution radiometer (AVHRR) on board each satellite images the earth in five wavebands, one visible and four infrared, and both individual wavebands and combinations of wavebands have been used in the past to monitor surface phenomena. Cloud contamination, a frequent problem in remotely sensed imagery, is reduced by compositing the daily data over intervals of time (10-day or monthly periods) and selecting the least contaminated value in the interval (this usually involves selecting the maximum value for any particular picture element, or pixel, since contamination usually reduces the signal). Even after such maximum value compositing, however, individual wavebands tend still to be attenuated by atmospheric dusts and aerosols, and various waveband combinations have been suggested as indices of greater reliability than the individual channels. Foremost among these is the normalized difference vegetation index (NDVI), the ratio of the difference between channels 2 (near infrared) and 1 (visible) and their sum. As its name implies, the NDVI is a measure of the photosynthetically active radiation at the earth’s surface, but it has also been correlated with biomass and drought conditions in Africa [4].

AVHRR channels 3 to 5 contain information about thermal conditions at the earth’s surface; in general channel 4 appears to show the best correlation of the individual channels with surface air temperature but, once again, a combination of channels 4 and 5 (using ‘split-window’ techniques that remove some of the aerosol effects [5]) provides the best overall correlation with this variable over large areas in Africa [6].

Whilst clouds are an unwelcome contaminant of AVHRR imagery, they form the signal of interest in imagery from the geostationary Meteosat series, placed at the equator above the Greenwich meridian. By selecting iteratively chosen thresholds for cloud-top temperatures, the TAMSAT programme in West Africa deduced a cold
cloud duration (CCD) index with the highest correlation with local rainfall (a quantity notoriously difficult to interpolate between weather stations, because of its great spatial variability) [7]. CCD images are now routinely produced by the ARTEMIS programme at FAO and made available to the research community.

For present purposes the 8 km spatial resolution NDVI series for 1982 to 1990 was chosen (recently made available on CD-ROM by FAO, Rome), together with a six year series of AVHRR channel 4 data for the period 1987 to 1992, from the archives of NASA's Global Inventory Monitoring and Modelling Systems (GIMMS) group at the Goddard Space Flight Centre, Maryland, USA. Finally, the five year average monthly CCD data from the Meteosat series for the period 1988 to 1992 were kindly made available by the FAO ARTEMIS programme.

In addition to these satellite derived data, a set of digital elevation model (DEM) data for Africa were acquired from the Global Land Information System (GLIS) of the US Geological Survey, Earth Resources Observation Systems (USGS, EROS) data centre. These original 0.083 degree resolution data were resampled to the same pixel size as the remaining data (i.e. about 8 km on a side) before further analysis.

2.2. Tsetse distribution data

The Ford and Katondo [8] maps of the distribution of tsetse in Africa were digitized and transformed to longitude/latitude co-ordinates, for registration to the satellite data layers. These maps are now acknowledged to be somewhat out of date, especially in West and West-Central Africa, which has suffered from the effects of the extended dry period culminating in the drought year of 1984. Nevertheless, the maps represent the best 'historical' picture of tsetse distribution, with which recent changes may be contrasted.

2.3. Image processing before analysis

The AVHRR and Meteosat signals tend to be repeated on an annual basis, reflecting climate and habitat seasonality. These monthly time series of satellite data \{x_t\} were described by their temporal Fourier series representations:

\[
x_t = a_0 + \sum_{p=1}^{N/2-1} \left[ a_p \cos\frac{2\pi pt}{N} + b_p \sin\frac{2\pi pt}{N} \right] + a_{N/2} \cos\frac{\pi t}{N}
\]

\[(t = 1, 2, \ldots N)\]
with coefficients \( \{ a_p, b_p \} \) defined as follows:

\[
\begin{align*}
    a_0 &= \bar{x} \\
    a_{N/2} &= \sum (-1)^i x_i / N \\
    a_p &= 2 \left\{ \sum x_i \cos(2\pi pt / N) \right\} / N \quad p = 1, \ldots, (N/2) - 1 \\
    b_p &= 2 \left\{ \sum x_i \sin(2\pi pt / N) \right\} / N
\end{align*}
\]

The component at a frequency \( \omega_p = 2\pi p / N \) is called the \( p \)-th harmonic, and for all \( p \neq N/2 \) these harmonics may be written in the equivalent form

\[
    a_p \cos \omega_p t + b_p \sin \omega_p t = R_p \cos(\omega_p t + \phi_p)
\]

where \( R_p \) is the amplitude of the \( p \)-th harmonic:

\[
    R_p = \sqrt{(a_p^2 + b_p^2)}
\]

and \( \phi_p \) is the phase of the \( p \)-th harmonic:

\[
    \phi_p = \tan^{-1}(-b_p/a_p)
\]

(see Ref. [9]). Once the series \( a_p \) and \( b_p \) are defined through the use of Eq. (2), they are used in Eq. (1) to reconstruct the Fourier description of the series using any number of harmonics. Fourier analysis offers the potential for reducing the volume of data because a long time series composed of repeated annual cycles may often be described by using only the within-year components, i.e. the overall mean and the amplitudes and phases (i.e. timing) of the annual, biannual and triannual cycles.

Further details of temporal Fourier processing are given in Ref. [10] and examples of the application of Fourier processed imagery to studying ecological patterns and processes are given in Ref. [11].

2.4. Data analysis

The reduced dimension data set produced by the methods outlined above form the set of predictor variables used to describe the field observations on vectors or
diseases. We assume that areas of vector or disease presence and absence are characterized by environmental conditions having multivariate normal distributions (partitioning of the data may need to be carried out until this criterion is approximated). Various forms and modifications of discriminant analysis are then relatively easily applied to the observational data and may be interpreted to provide biological insight into the nature of the limits to the distribution and abundance of vectors and diseases.

In its simplest form, discriminant analysis assumes both multivariate normality and a common within-group co-variance of the variables for all points defining vector or disease presence and absence. Co-variances are estimated from representative samples from reliable distribution maps, the 'training sets'. Means of multivariate distributions are referred to as centroids and are defined by mathematical vectors \([\bar{x}_n]\), where \(n\) is the number of dimensions (variables). The Mahalanobis distance, \(D^2\), is the distance between two multivariate distribution centroids, or between a sample point and a centroid, and is defined as follows:

\[
D_{12}^2 = (\bar{x}_1 - \bar{x}_2)'C_w^{-1}(\bar{x}_1 - \bar{x}_2)
\]

\(= d' C_w^{-1}d\)  \hspace{1cm} (4)

where the subscripts refer to groups 1 (e.g. for vector absence) and 2 (e.g. for vector presence), \(d = (\bar{x}_1 - \bar{x}_2)\) and \(C_w^{-1}\) is the inverse of the within-group covariance (dispersion) matrix [12]. Thus \(D^2\) is the distance between the sample centroids adjusted for their common co-variance. Equation (4) may be used in a number of ways. Firstly it may be used to assign new data points to one or other category (of presence or absence) by examining the value of \(D^2\), between each point and each of the training-set defined centroids. The point is then assigned to the group for which \(D^2\) is a minimum. Secondly, the equation may be used to calculate the probability with which each data point belongs to each of the training set groups. This involves defining the position of the point within each multivariate distribution around each centroid (most easily achieved by calculating \(D^2\), which is distributed as \(\chi^2\) with \((g - 1)\) degrees of freedom, where \(g\) is the number of variables defining each centroid). In general these measures are normalized by dividing each by the sum of all measures (i.e. the sum of the probabilities across all classes in the training set) to give posterior probabilities, defined as follows:
where \( P(1|x) \) is the posterior probability that observation \( x \) belongs to group 1 and \( P(2|x) \) the posterior probability that it belongs to group 2 [12]. \( p_1 \) and \( p_2 \) are the prior probabilities of belonging to the same two groups respectively, defined as the probabilities with which any observation might belong to either group given prior knowledge or experience of the situation. In the absence of any prior experience it is usual to assume equal prior probability of belonging to any of the groups. In the simple case of two group discrimination, \( p_1 = p_2 = 0.5 \). The normalization invoked by summing the probabilities in Eq. (5) is based on the assumption that observation \( x \) must come from one or other of the classes defined in the training-set data. Other terms of the multivariate normal distribution in Eq. (5) then cancel out [13].

Finally, Eqs (4) and (5) should be applied in modified form when the assumption of common covariances is obviously invalid. This is especially likely when a vector or disease occupies only a small fraction of a large habitat. In this case the co-variances of the variables within the occupied area are often different from those of the same variables outside the distributional limits. Equation (5) is then modified as follows:

\[
P(1|x) = \frac{p_1 |C_1|^{-1/2} e^{-D_1^2/2}}{\sum_{g=1}^{2} p_g |C_g|^{-1/2} e^{-D_g^2/2}}
\]

\[
P(2|x) = \frac{p_2 |C_2|^{-1/2} e^{-D_2^2/2}}{\sum_{g=1}^{2} p_g |C_g|^{-1/2} e^{-D_g^2/2}}
\]

(6)

where \( |C_1| \) and \( |C_2| \) are the determinants of the co-variance matrices for groups \( g = 1 \) and \( g = 2 \), respectively. The Mahalanobis distances in expressions (6), calculated from
Eq. (4), are evaluated using the separate within-group co-variance matrices [13]. With unequal co-variance matrices the discriminant axis (strictly speaking, a plane) that separates the two groups in multivariate space is no longer linear, and Eq. (6) then defines the probabilities that are also used by maximum likelihood methods applied to the same problem.

In many cases there are more than two groups to discriminate, either because areas of presence or absence are so variable that they must be split into smaller units, or because the data to be described are best dealt with as a series of categories (for example, of disease prevalence). In this case, Eqs (4)–(6) are extended to as many categories as are represented in the training set data. Given that training set data are limited in size, however, it is unwise to subdivide them into too many categories in an attempt to increase the accuracy of predictions, because the co-variance matrices necessary to generate the assignment rules must be defined adequately. Similarly, there is no obvious rule about the use of expected or observed prior probabilities in Eqs (5) or (6). Use of observed (generally, training set) prior probabilities shifts the equiprobability contours towards the smaller groups, resulting in a larger proportion of assignments to the classes with larger group sizes. This shift, however, may occasionally be large enough to reduce the accuracy of describing even the training set data.

In the application of the above methodology to tsetse distributions in east Africa, sets of training data were generated by random sampling of about 1000 points representing vector absence and some 200 points representing vector presence from imagery whose total size was in excess of 400 000 pixels (although the sea and non-African land masses were masked out so that they could not be chosen for either category). These sample points were then used to extract the digital elevation and Fourier processed NDVI, thermal (i.e. AVHRR channel 4) and cold cloud duration imagery data. In the latter cases none of the phase imagery was sampled because it was felt that the timing of phenologically equivalent events would be very different throughout the very large area sampled (which spans the equator). These sample data were then subjected to cluster analysis (using the k-means cluster algorithm in SPSS) to generate a series of up to five groupings of areas of both presence and absence in the East African region. Clustering was carried out to allow the possibility, within the discriminant analysis, to split areas of fly presence or areas of fly absence into more than a single group each, in order to ensure multivariate normality within the analysis; this is especially necessary when there is a wide range of conditions across the entire sampled area. The SPSS algorithm carries out clustering after standardizing each variable to zero mean and unit standard deviation, a device which eliminates the risk that variables (such as elevation) showing a large numerical range dominate variables (such as NDVI) with very much smaller ranges. Cluster assignments were saved back to the original data file, for use in the discriminant analysis programs.
2.5. Selection of variables and implementation

Application of discriminant analysis involved the forward stepwise selection of the processed satellite predictor variables, each variable being selected on the basis of criteria that needed to be decided beforehand. In general, it is prudent to base selection on some calculation using the Mahalanobis distances, since these are scale invariant measures of between-class separation in multivariate space. One obvious measure, which is the sum of the Mahalanobis distances between all classes (or, in the case of several groups for single attributes such as presence or absence, the sum of the Mahalanobis distances between the different categories) usually results in the most distinct pair of classes determining the variable selection. Although this improves the discrimination between these two particular classes, that between other classes is sometimes reduced. Another selection method in multiclass \((g > 2)\) discrimination is to ignore the zero class that is often more heterogeneous than the non-zero classes. Thus, in the case of disease prevalence, there is usually much more heterogeneity in areas where prevalence is zero than elsewhere. Selection can then be made on the basis of the sum of the Mahalanobis distances between the non-zero classes. A third selection criterion is to choose that predictor variable that maximizes the minimum distance between pairs of classes in each round of selection (possibly weighting the distances on the basis of sample sizes when these are quite different). The assumption here is that if the minimum is maximized, the separation between the least separable classes, and therefore the overall performance of the analysis, will be improved.

The above formulas were used on the published and field data analysed here, ensuring that outlying points were eliminated before analysis. Such points arise when using remotely sensed data, where image pixels with mask values (indicating suspect or cloudy pixels) or, in coastal or lakeland areas, pixels contaminated by the signal from water are included in the data set. In the present analyses all data were first corrected to their class mean values and standardized by dividing by the class standard deviation: points which were more than six standard deviations away from their class means were omitted from the analysis (after which class means were recalculated and checks carried out again).

2.6. Measures of predictive accuracy

As with any other diagnostic test, satellite data will be adopted only if they provide a diagnostic of fly presence or absence with a sufficient degree of accuracy. One measure of accuracy is the overall percentage of correct predictions of both presence and absence. Such a measure is affected by the overall frequency of tsetse and non-tsetse areas and is therefore rarely acceptable (the overall accuracy of
predicting the presence and absence of a rare species could be made to be very high simply by predicting its total absence!). False predictions of fly presence and absence are more useful measures of predictive accuracy, as are sensitivity (ability to identify areas of fly presence correctly) and specificity (ability to identify areas of fly absence correctly). Other measures of accuracy from the remote sensing literature include both the kappa and tau statistics that attempt to combine measures of predictive accuracy weighted on the basis of the expectation of predictive success given the overall frequencies within the training set data [14]. In each of the above examples, no greater penalty is imposed for a false prediction of presence than for a false prediction of absence, but in practice this may not be the case. When different outcomes have different costs, it is possible to weight the predictive process so that outcomes which are more 'expensive' are predicted with greater certainty than outcomes which are less expensive. Clearly the cost of not predicting an area of rinderpest when one is in fact present is potentially much greater than one of predicting its presence when, in fact, it is absent. These ideas, familiar to veterinary epidemiologists, are only rarely incorporated into Geographical Information System (GIS) modelling of pest, vector and disease outbreaks, although there are no great technical difficulties in doing so.

3. RESULTS

The results of the analysis of the East African distributions of five species of tsetse, G. morsitans, G. pallidipes, G. austeni, G. longipennis and G. fuscipes fuscipes, are shown in Table I. Maps of the predicted distributions of the five species are shown in Figs 1 to 5, using the results obtained by selecting variables that maximized the minimum Mahalanobis distances between classes (i.e the right hand side of each pair of columns in Table I). Table I shows that this method of selecting variables in general improves the overall fit to the observed distributions (percentage correct and percentage specificity figures are always increased, as are the kappa and tau statistics for all species except G. austeni), but this improvement comes at the expense of a decreased sensitivity (most marked in the case of G. pallidipes). Table I suggests there is always a trade-off between sensitivity and specificity when comparing these methods of variable selection; the end user needs to decide on an acceptable balance between predicting flies everywhere (sensitivity = 100%, specificity = 0) or nowhere (sensitivity = 0%, specificity = 100%).

Some of the reasons for the results shown in Table I and Figs 1 to 5 may be deduced by examining the relationship between the observed categories of the training-set data and those to which they were assigned by the analysis. This can be done by examining either the number of pixels assigned to each class or the posterior probabilities with which any particular assignment was made. The results
### TABLE I. KEY PREDICTOR VARIABLES USED TO DESCRIBE THE DISTRIBUTION OF FIVE SPECIES OF TSETSE IN EAST AFRICA

<table>
<thead>
<tr>
<th>Species</th>
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<th></th>
<th></th>
<th></th>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>NDx</td>
<td>NDa1</td>
<td>DEM</td>
<td>DEM</td>
<td>DEM</td>
<td>DEM</td>
<td>CDx</td>
<td>DEM</td>
<td>NDm/CDm</td>
</tr>
<tr>
<td>2</td>
<td>NDm/CDm</td>
<td>C4a3</td>
<td>C4m</td>
<td>CDm</td>
<td>C4a3</td>
<td>DEM</td>
<td>C4a3</td>
<td>CDm</td>
<td>CDx</td>
</tr>
<tr>
<td>3</td>
<td>CDm</td>
<td>DEM</td>
<td>CDm</td>
<td>NDm</td>
<td>CDm</td>
<td>NDm</td>
<td>NDa3</td>
<td>NDa3</td>
<td>NDm</td>
</tr>
<tr>
<td>4</td>
<td>NDm</td>
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<td>CDm</td>
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<td>5</td>
<td>C4m</td>
<td>CDm</td>
<td>C4a3</td>
<td>C4a3</td>
<td>Cdm</td>
<td>ATN(NDm/C4m)</td>
<td>CDm</td>
<td>NDm</td>
<td>NDp-C4p</td>
</tr>
<tr>
<td>6</td>
<td>NDa1</td>
<td>C4x</td>
<td>ATN(NDm/C4m)</td>
<td>ATN(NDm/C4m)</td>
<td>CDa1</td>
<td>NDp-C4p</td>
<td>NDx</td>
<td>CDa2</td>
<td>NDp-CDp</td>
</tr>
<tr>
<td>7</td>
<td>NDp-C4p</td>
<td>CDa3</td>
<td>NDm</td>
<td>CDr</td>
<td>CDx</td>
<td>CDa3</td>
<td>NDm/CDm</td>
<td>NDm/CDm</td>
<td>DEM</td>
</tr>
<tr>
<td>8</td>
<td>ATN(NDm/C4m)</td>
<td>NDm/CDm</td>
<td>NDm/C4m</td>
<td>NDa2</td>
<td>NDp-C4p</td>
<td>C4a2</td>
<td>CDm</td>
<td>C4a1</td>
<td>ATN(NDm/C4m)</td>
</tr>
<tr>
<td>9</td>
<td>CDx</td>
<td>NDm</td>
<td>NDm/C4m</td>
<td>NDm</td>
<td>C4a2</td>
<td>CDm</td>
<td>NDm</td>
<td>C4n</td>
<td>C4a2</td>
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<tr>
<td>10</td>
<td>NDa2</td>
<td>ATN(NDm/C4m)</td>
<td>C4n</td>
<td>C4x</td>
<td>NDp-CDp</td>
<td>C4a1</td>
<td>NDm/C4m</td>
<td>NDm/C4m</td>
<td>CDa1</td>
</tr>
<tr>
<td>% correct</td>
<td>79</td>
<td>85</td>
<td>84</td>
<td>87</td>
<td>96</td>
<td>97</td>
<td>91</td>
<td>93</td>
<td>92</td>
</tr>
<tr>
<td>% false +ve</td>
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<td>8</td>
</tr>
<tr>
<td>% false -ve</td>
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<td>1</td>
<td>3</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.965</td>
<td>0.950</td>
<td>0.819</td>
<td>0.693</td>
<td>0.989</td>
<td>0.973</td>
<td>0.955</td>
<td>0.950</td>
<td>0.980</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.756</td>
<td>0.827</td>
<td>0.838</td>
<td>0.908</td>
<td>0.954</td>
<td>0.965</td>
<td>0.901</td>
<td>0.926</td>
<td>0.902</td>
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<tr>
<td>κ</td>
<td>0.450</td>
<td>0.680</td>
<td>0.559</td>
<td>0.635</td>
<td>0.826</td>
<td>0.826</td>
<td>0.761</td>
<td>0.809</td>
<td>0.776</td>
</tr>
<tr>
<td>τ</td>
<td>0.388</td>
<td>0.652</td>
<td>0.491</td>
<td>0.603</td>
<td>0.818</td>
<td>0.817</td>
<td>0.741</td>
<td>0.798</td>
<td>0.760</td>
</tr>
</tbody>
</table>
TABLE I. (cont.)

Note: For each species the variables are ranked in order, using the selection criteria of maximizing the sum (Max $\Sigma$) or maximizing the minimum (Max min.) of the Mahalanobis distances between categories (i.e. of presence or absence). In each case, training set data for presence and absence sites were each split into two groups, to improve the overall fit, and prior probabilities (based on the training set data) were used in calculating the posterior probabilities of fly presence or absence (see text for more details).

Key to variable names: the first two/three letters refer to the channel; ND — normalized difference vegetation index (NDVI); C4 — AVHRR channel 4; CD — cold cloud duration (CCD); DEM — digital elevation. The next letter(s) refer(s) to the variable; m — mean; n — minimum; x — maximum; r — range (= max. − min.); a1−a3 — amplitude of the annual-triannual Fourier cycles; p1−p3 — phase of the annual-triannual Fourier cycles. Other variables are combinations of one or more channels, e.g. NDp-CDp is the difference between the phases of the annual NDVI and CCD cycles, ATN is the arctangent of the given ratio (a quantity reported to show less seasonal variation than either of the variables alone [15]). $\kappa$ and $\tau$ are measures of predictive accuracy (see text).
FIG. 3. Predicted distribution of Glossina austeni in East Africa, using the satellite predictor variables listed in Table I (variable selection method is to maximize the minimum Mahalanobis distance).
FIG. 4.  Predicted distribution of Glossina longipennis in East Africa, using the satellite predictor variables listed in Table 1 (variable selection method is to maximize the minimum Mahalanobis distance).
FIG. 5. Predicted distribution of Glossina f. fuscipes in East Africa, using the satellite predictor variables listed in Table I (variable selection method is to maximize the minimum Mahalanobis distance).
TABLE II. PREDICTION RELATED DATA

(a) Variables used to describe the distribution of *G. pallidipes* in East Africa

<table>
<thead>
<tr>
<th>Category</th>
<th>DEM</th>
<th>CDN</th>
<th>NDm</th>
<th>CDa1</th>
<th>C4a3</th>
<th>ATN(NDm/C4n)</th>
<th>CDr</th>
<th>NDa2</th>
<th>CDa2</th>
<th>C4x</th>
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<tbody>
<tr>
<td>C1</td>
<td>580.8</td>
<td>8.41</td>
<td>0.24</td>
<td>45.3</td>
<td>0.66</td>
<td>0.95</td>
<td>120.3</td>
<td>0.04</td>
<td>22.8</td>
<td>41.2</td>
</tr>
<tr>
<td>C2</td>
<td>1507.6</td>
<td>-10.43</td>
<td>0.37</td>
<td>86.8</td>
<td>0.78</td>
<td>0.70</td>
<td>209.6</td>
<td>0.04</td>
<td>32.7</td>
<td>36.2</td>
</tr>
<tr>
<td>C3</td>
<td>1239.7</td>
<td>10.81</td>
<td>0.41</td>
<td>65.1</td>
<td>0.90</td>
<td>0.63</td>
<td>183.1</td>
<td>0.06</td>
<td>36.0</td>
<td>34.5</td>
</tr>
<tr>
<td>C4</td>
<td>437.1</td>
<td>-18.43</td>
<td>0.35</td>
<td>87.7</td>
<td>0.73</td>
<td>0.55</td>
<td>214.4</td>
<td>0.05</td>
<td>32.3</td>
<td>34.5</td>
</tr>
</tbody>
</table>

(b), (c) Predicted category, number

<table>
<thead>
<tr>
<th>Observed category</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>607</td>
<td>86</td>
<td>19</td>
<td>48</td>
<td>760</td>
</tr>
<tr>
<td>C2</td>
<td>7</td>
<td>206</td>
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<td>1</td>
<td>238</td>
</tr>
<tr>
<td>C3</td>
<td>1</td>
<td>25</td>
<td>53</td>
<td>0</td>
<td>79</td>
</tr>
<tr>
<td>C4</td>
<td>24</td>
<td>11</td>
<td>12</td>
<td>73</td>
<td>120</td>
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</tbody>
</table>

Predicted category, mean probability

<table>
<thead>
<tr>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.95</td>
<td>0.74</td>
<td>0.72</td>
<td>0.86</td>
</tr>
<tr>
<td>0.63</td>
<td>0.91</td>
<td>0.77</td>
<td>0.91</td>
</tr>
<tr>
<td>0.63</td>
<td>0.70</td>
<td>0.84</td>
<td>NA</td>
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<tr>
<td>0.77</td>
<td>0.58</td>
<td>0.65</td>
<td>0.90</td>
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</table>

(d) % producer % consumer

<table>
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<tr>
<th>Accuracy</th>
<th>% producer</th>
<th>% consumer</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>79.9</td>
<td>95.0</td>
</tr>
<tr>
<td>C2</td>
<td>86.6</td>
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<tr>
<td>C3</td>
<td>67.1</td>
<td>49.1</td>
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<tr>
<td>C4</td>
<td>60.8</td>
<td>59.8</td>
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</tbody>
</table>

Note: C1 and C2 are categories of tsetse absence, C3 and C4 of tsetse presence. For the other notation, see Table I. NA = not applicable.
for *G. pallidipes* (a species with relatively high false positive and false negative results, Table I) are shown in Table II, together with the mean values for the predictor variables in each category. In Table II, categories 1 and 2 are for fly absence, 3 and 4 for fly presence. Table IIb shows that 67/760 category 1 areas (of known fly absence) are in fact assigned to categories 3 and 4 (i.e. predicting fly presence). Tables IIb and IIc together show that 48 training sites in category 1 are assigned to category 4 with an average probability of 0.86, higher than the probability with which a further 86 sites from this category are assigned to category 2 (the second category for absence). In other words, some areas of fly absence are more similar to areas of fly presence than they are to other areas of fly absence. Table IIa shows that sites 1 and 4 are low elevation categories (DEM, the most important variable, averages 581 and 437 m, respectively), whilst the other two categories (2 for absence and 3 for presence) are high elevation sites (1508 and 1240 m, respectively). This both supports the splitting of the training set data into more than one category for presence and absence and suggests that some areas of recorded fly absence are, in fact, highly suitable for this species of fly. Conversely, some of the sites in categories 3 and 4 (of fly presence) may be assigned to categories of fly absence. Table IIb shows that 26/79 category 3 areas are assigned to absence categories 1 and 2 areas, but never to category 4 areas. Category 4 areas may, however, be assigned to category 3 (*n* = 12), although more of them are assigned to category 1 (*n* = 24), and with a higher mean probability (*p* = 0.77 versus *p* = 0.65). Table IIId shows two more measures of accuracy referred to in the literature [14], the ‘producer’ and ‘consumer’ accuracies. The former is the overall accuracy from the viewpoint of the analysis, and compares the predicted with the observed data (these figures are calculated in the same way as those in the ‘% correct’ row of Table I). Thus, for example, of the 760 samples in the training set known to be in category 1, 607 (= 79.9%) were assigned to this category by the analysis. The consumer accuracy is calculated from the viewpoint of the user of such predictions and records the percent correct predictions of those assigned to any particular category. Thus, of the 639 samples identified by the analysis as belonging to category 1, 607 (= 95.0%) actually did so. The producer accuracy is calculated using the row totals in Table IIb whilst the consumer accuracy uses the column totals. With multiple categories for each class of observation, these accuracies represent minimum values, since a prediction of belonging to a different category of the same type (presence or absence) is an inaccurate assignment, but remains an accurate prediction!

The misclassifications discussed above may arise because the method of analysis ‘forces’ every pixel into one or other of the four categories predefined in the training set data. It is possible that other sites are similar neither to the presence nor absence sites within the training set data: such sites, nevertheless, will be assigned to one or other of the four categories defined in the analysis, through the method of calculating posterior probabilities (Eq. (5)). In order to investigate the magnitude of
FIG. 6. Predicted distribution of Glossina longipennis in East Africa, using the satellite predictor variables listed in Table I. Here the analysis concentrates only on areas of fly presence and records the Mahalanobis distance to one or other of the two groups for fly presence. These distances are coded on the grey scale shown in the legend.

This effect the analysis was rerun for *G. longipennis* (a species for which the predicted distribution includes large areas from which flies are apparently absent, Fig. 4), but with the mapped output recording the smallest Mahalanobis distance to one of the two sites for tsetse presence. This map, shown in Fig. 6, therefore records the similarity of each pixel to one or other category of fly presence, and ignores all categories of fly absence. When pixels are close, in multivariate space, to the training set data for fly presence, the calculated Mahalanobis distances are small (and given darker shading in Fig. 6); when they are farther away, the Mahalanobis distances are large (and given paler shading in Fig. 6). Figure 6 is, in places, an improvement on Fig. 4. For example, it indicates that many of the areas in Ethiopia shown as suitable for this species in Fig. 4 are, in fact, very dissimilar from any of the training set data.
for fly presence: they just happen to be slightly more similar to areas of fly presence than to areas of fly absence, and are thus assigned to the former category in Fig. 4. Figure 6 also shows, however, that there are many areas of fly absence in the eastern part of Kenya (forming a crescent of points running from near the coast to the central belts of *G. longipennis*, in the middle of Kenya) that appear to be very close (in multivariate space) to areas of fly presence. These areas would therefore appear to be suitable for the species, at least in terms of the satellite variables used to define the training set data. Taken together, these observations suggest that some of the predicted areas of fly presence (and, presumably, of fly absence) obtained through calculating posterior probabilities may be inaccurate, because the conditions at these sites are different from any in the training set data, whilst other areas of fly absence are more similar to some tsetse areas (more suitable for flies?) than these, in turn, are to other tsetse areas. The former problem could be overcome by excluding from the predictions all those areas that are very different from any training set data (based on the Mahalanobis distances). The latter problem presents a real challenge to our understanding of the distributional limits of tsetse: if these areas really are suitable for flies, why are flies not recorded from them? When planning any intervention strategy against tsetse it will be important to survey such areas very carefully, to confirm the absence of flies, and to ensure that any barriers erected to prevent re-invasion from non-treated areas are sited taking account of these areas, which may act as ecological corridors from one tsetse area to another.

4. CONCLUSIONS

Remotely sensed satellite imagery provides us with a wealth of data about areas far removed from the rather sparse network of meteorological stations in Africa. Selection of those data that are best correlated with the meteorological variables of interest to us (determined by a careful analysis of meteorological data from particular study sites) can provide us with a set of relatively high spatial resolution data on climatic conditions over large areas in the tropics, and these conditions can be monitored in near real time. In addition, certain satellite data streams provide novel ways of monitoring environmental characteristics (e.g. AVHRR channel 3, a mixture of emitted and reflected infrared radiation, which has been correlated with forest regrowth [16]).

The current research challenge is to find the best set of surrogate meteorological and novel satellite variables that may be used to predict the distribution and abundance of vectors and the diseases they transmit. This involves variable selection, processing and manipulation. Thus, for example, the NDVI may be the best of several alternative vegetation indices (variable selection); temporal Fourier processing may be the best way to process such data to reveal important, biological
characteristics of natural ecosystems (variable processing); and various forms of discriminant analysis may reveal which environmental variables determine animal distributions (variable manipulation) in ways that alternative (and possibly more powerful) methods of analysis (e.g. neural network processing) do not. Each of these three steps involves important decisions, usually affected by the past experience and predilections of the particular researchers involved. Each could be usefully illuminated by carefully planned field work, carried out to test some of the ideas that this approach reveals. Such ideas have yet to be tested within the context of an active programme for tsetse control in Africa.

There have been many changes in tsetse distributions in Africa since the Ford and Katondo reference maps were produced. Some of these changes are human induced; others arise from recent climatic events like the drought of the early 1980s. Given the increasing pace of both sorts of change in Africa, satellites provide the only viable source of data for real time monitoring of these changes now and into the future. The challenge is there. It has yet to be met.

ACKNOWLEDGEMENTS

This work was carried out within the Trypanosomiasis and Land-use in Africa (TALA) Research Group, funded by the Overseas Development Administration (ODA) through a Natural Resources Institute (NRI) extramural contract, No. X0239. The author is grateful to M. Packer and S. Hay for their support and help, and to R.M. Anderson for providing facilities within the Department of Zoology, Oxford.

REFERENCES


PRACTICAL APPLICATIONS OF GEOGRAPHIC INFORMATION SYSTEMS IN TSETSE AND TRYPANOSOMIASIS CONTROL

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Abstract

PRACTICAL APPLICATIONS OF GEOGRAPHIC INFORMATION SYSTEMS IN TSETSE AND TRYPANOSOMIASIS CONTROL.

Three aspects of geographic information systems (GISs) in tsetse and trypanosomiasis control are addressed. First is their use in planning and managing control operations, second is the use of appropriate data management systems to capture data from the field and process them in a format that facilitates their geographical analysis, and third is the use of GISs to predict some of the data layers required for planning tsetse and trypanosomiasis control activities. In planning and managing trypanosomiasis interventions, GISs are applied at a range of levels. At the policy level, it can be used to integrate data such as tsetse and trypanosomiasis distributions, livestock densities and percentage cultivation to assist in allocating resources to tsetse and trypanosomiasis control. At the management level, it can be used to combine the same sorts of data in decision support models to help prioritize areas for control. At the operational level, it can be used to help plan, manage and monitor field operations. Much of the data required for these applications come directly from the field, particularly the distributions of livestock, vector and disease. The use of bespoke software such as the disease and vector integrated database (DAVID) greatly facilitates entry, display and analysis of field data, and their integration with other data within GISs. Collecting field data relevant to planning and managing tsetse and trypanosomiasis control operations is often very expensive and time consuming, therefore preventing exhaustive ground coverage. The use of GISs to combine environmental predictor variables, using multivariate statistical models, and to predict the distribution and abundance of tsetse, trypanosomiasis, cultivation and livestock is reviewed. Finally, opportunities are discussed for developing these applications in the future. It is recommended that stronger links be forged between the research workers and those in operational programmes.

1. INTRODUCTION

Geographic information systems, or GISs, became a familiar term in the 1980s, particularly in the social sciences, where the concept was first applied. There remains a degree of ambiguity regarding what GISs actually are, which has inevitably led to distrust in this technology in the fields of tsetse and trypanosomiasis control. There
have been some GIS applications to both medical and veterinary diseases (see, e.g., reviews [1, 2]), but these applications remain underexploited and hold enormous potential. This paper reviews the range of GISs techniques that has been applied to tsetse and trypanosomiasis control and suggests ways in which these might be brought together to meet the operational requirements for controlling tsetse transmitted trypanosomiasis in Africa.

GISs include the suite of computer software applications that are concerned with the capture, storage, manipulation, interrogation, analysis and presentation of all kinds of geographically registered information [3–5]. Various definitions of a GIS have been proposed, for example Haines-Young, Green and Cousins [6] give the definition “.... any computer based system for the input, storage, analysis and display of spatial information .... including systems designed primarily to capture spatial information and also to process it, namely remote sensing systems”. GIS is often referred to as a ‘management tool’, a term that greatly underestimates the underlying analytical power. I feel that it is important not to restrict the definition of GISs to the functional set of software tools specifically designed to manage geographical data. Rather, it should also be considered a conceptual framework through which different disciplines may be brought together. GIS involves applying a spatial dimension to data that becomes implicit in their management and analysis. In this broad context, GISs must include all databases that are georeferenced and all methods that are concerned with, and can be used to exploit, this geographical component.

Different requirements of GISs in tsetse and trypanosomiasis control can be identified. At a policy level, the main requirement is to help present arguments to generate funding for, and commitment to, tsetse and trypanosomiasis control. This will often be an issue of public relations and information dissemination. At the management level, the requirements are in assistance with resource allocation decisions through the generation of priority maps. At the field/operational level, the requirements are concerned with planning and monitoring field operations and generating detailed maps that are easy to update. In this paper, three aspects of GISs in tsetse and trypanosomiasis control are addressed. First is the use of GISs to plan and manage control operations, second is the use of appropriate data management systems to capture data from the field and to process them in a format that facilitates their geographical analysis, and third is the use of GISs to predict some of the data layers required for planning tsetse and trypanosomiasis control activities.

2. GIS FOR PLANNING AND MANAGING TSETSE AND TRYPANOSOMIASIS CONTROL

The ultimate objective of GISs in tsetse and trypanosomiasis control must be to assist with planning and implementing disease intervention strategies. Three broad
levels can be distinguished at which GIS applications may be important: policy, management and operational levels. Different spatial scales and different levels of detail in the data will be appropriate to these different applications, depending on the questions that need answering. These are not exclusive categories, and the results from more detailed analyses should feed up to the higher level applications.

2.1. Policy level: allocation of resources to tsetse and trypanosomiasis control

One advantage of GISs is a clear presentation of information that facilitates demonstration of problem areas, information gaps, etc. A significant use of GISs should be to identify areas on a broad scale (e.g. at the level of the country) that require assistance, and thus to focus the attentions of regional programmes and donors. At this level, a GIS might be used to compare a group of neighbouring countries in terms of, for example, the proportion of land infested with tsetse, the land pressure in tsetse free areas or the expected gains to be made through clearing tsetse from particular areas. Comparison of such estimates may be used to apportion funding appropriately. Coarse resolution GIS at a pan-African or regional scale is appropriate for this type of application.

Though not used explicitly for this purpose, the type of analysis conducted by Rogers and Wint in Ref. [7] might be appropriate for broad scale resource allocation. Their analysis covered the east African countries of Sudan, Ethiopia, Somalia, Kenya, Uganda, Rwanda, Burundi and the United Republic of Tanzania, for which GIS data layers were generated for ecozones, tsetse distribution, cattle density and percentage cultivation. Separately for each ecozone, they made comparisons of percentage cultivation and livestock densities between areas in which tsetse were present and those from which they were absent. These comparisons were used to make inferences regarding the potential changes in cattle numbers, and in area cultivated, that might result from removal of tsetse. Spatial data of this kind could be combined with country level socioeconomic data (e.g. differing values attributed to livestock ownership, relative strengths of veterinary services) to help decide on resource allocation within the EC funded east African tsetse control programme. For example, it may help to determine the contribution that should be made to the programme by the member states and to decide how regional resources should be apportioned. Clearly, there will be no simple rule for this but a well designed GIS will help answer the questions posed by policy makers and therefore assist more explicitly reasoned decision making.

2.2. Management level: prioritization of areas for tsetse and trypanosomiasis control

A major use of GISs is to prioritize areas for tsetse and trypanosomiasis control. There is overlap here with the policy level applications, but in the present context
I refer to a more spatially refined analysis. Relatively coarse resolution GISs at a regional level may be used for broad scale strategic planning, particularly with regard to cross-border priorities, while a finer scale analysis at the country level can be used to demarcate specific areas for control, with a view to implementation.

Rogers and Wint [7] have generated data layers of tsetse distribution, percentage cultivation and cattle density, at a spatial resolution of 20 pixels per degree (about 5 km along a side) for east Africa (countries listed above). Percentage cultivation and cattle density were each divided into three classes: low, medium and high. For areas where tsetse were present, a map was produced showing the six possible combinations of classes of cattle and cultivation: cattle high, cultivation high; cattle high, cultivation medium, etc. It remains for the decision makers to decide which of these classes fall into which level of priority for tsetse control, but important areas are likely to include those where intermediate to high cattle densities and high percentage cultivation are coincident with tsetse. This analysis draws attention to locations where high priority areas straddle international boundaries and where close collaboration on planning, implementing and monitoring disease intervention is likely to be mutually beneficial.

Robinson, Hopkins and Harris [8] have adapted the methodology of weighted linear combination [9, 10] to address the problem of prioritizing areas for tsetse control in part of Zambia. In weighted linear combination the criteria variables are first made comparable by scaling them into similar numbers of more or less equivalent levels, with class values increasing with increasing suitability for tsetse control. Each criterion is then assigned a weight that reflects its relative importance in the decision making process. The suitability to the objective being considered, in this case tsetse control, is then determined by summing the weighted values of each criterion. The higher the score the higher the priority. The variables they used were (a) cattle density, (b) human population density, (c) crop use intensity, (d) relative arable potential, (e) land designation and (f) proximity to control operations. The stakeholders were represented by a group of experienced veterinarians and biologists working in the region, which provided weights for the six criteria listed. As a result of this analysis, recommendations were made to modify the boundaries of a proposed community based target control area in the eastern province of Zambia, in order to maximize the potential benefits.

Robinson, working with an FAO livestock economist, further developed the use of GISs to prioritize areas for tsetse and trypanosomiasis control, distinguishing two kinds of constraint that can be recognized and mapped [11]. The first is where land pressure is a constraint and the second is where the disease itself is a constraint.

Tsetse are a problem if they prevent access to land adjacent to areas that are overcrowded and/or overstocked. Areas where there is high pressure on the land and where there would be positive economic gains from intervention, are categorized by high percentage cultivation, high stocking rates and high human population densities.
Suitable adjacent areas where control could take place to relieve land pressure are characterized by high tsetse challenge, high agricultural potential, low environmental risk, low stocking rates and low human population levels. Robinson [11] used a GIS to map tsetse infested areas with a high ‘absorption capacity’, that were adjacent to areas where there was a high pressure on the land, in the eastern part of Zambia.

Trypanosomiasis is a problem when it constrains agricultural production through the potential for utilization of the agricultural resource base being negatively and significantly affected by the disease. Such areas may or may not be overcrowded but they are unlikely to be overstocked. Areas where disease is a constraint, and the objective is to protect the agricultural and livestock resource base, are characterized by high levels of disease incidence, high agricultural potential, low levels of arable land utilization and medium stocking rates. The higher the stocking rate, the greater the initial impact on livestock production, though subsequent overstocking is likely to erode the production benefits. If livestock numbers are low then herd buildup is slow and economic gains are minimal. Intermediate stocking levels therefore provide the optimal compromise between the speed and the sustainability of the impact. Robinson [11], for the same area in Zambia, used a GIS to map areas meeting these criteria.

2.3. Operational level: planning and managing field operations

At the finest level, a GIS can be used to plan, manage and monitor field operations. A detailed georegistered database can be established of a control area in which it may be appropriate to incorporate high resolution satellite data (e.g. SPOT or Landsat) or interpreted aerial photography to help distinguish areas of suitable tsetse habitat, agricultural activity, livestock grazing, etc. Data from tsetse and trypanosomiasis surveys can then be superimposed to help devise a plan for intervention. The GIS can be used to record and map the use of control methods such as odour baited targets, pour-on insecticides and trypanocidal drugs, and can also facilitate monitoring the progress of the intervention, by surveillance for tsetse and trypanosomiasis. This will help identify areas where interventions could be eased off, as well as areas where intervention was not proving effective, and might need to be intensified or modified to some more appropriate method or combination of methods. The database programs discussed in the next section are a critical part of this application of the GIS and are designed primarily with this in mind.

3. MANAGING FIELD DATA

If the GIS is to be used to help prioritize areas for tsetse and trypanosomiasis control and to plan and manage interventions, then reliable data on tsetse,
trypanosomiasis, livestock, human populations, cultivation, land designation, etc. need to be available, in a format that is compatible with other georeferenced data. Some of the more static of these variables (e.g. land designation) may be available as digitized maps, while others (e.g. agriculture, human population and livestock) are usually provided by government statistics departments and can be manipulated to a suitable format. It is usually the direct responsibility of veterinary departments, however, to generate data on tsetse distributions, trypanosomiasis prevalence and incidence, and often to obtain supplementary livestock data. To collect these data in GIS compatible format requires one key factor: that positional information is accurately recorded. In the past, traditional cartographic methods have been used to measure geographic position topographic maps. More recently, however, this can be achieved with greater ease and accuracy since the advent of satellite based global positioning systems (GPSs). Whilst the only real requirement for GIS compatibility is that positional information be attributed to field samples, the process of field data management is greatly facilitated through the use of bespoke software. It is important to consider data management in the broader context of field data collection since one of the great advantages of an efficient field data management system is that it focuses fieldwork activities towards a standardized, efficient methodology. This enables reliable comparisons to be made using data gathered at different times and from different places.

3.1. The integrated tsetse and trypanosomiasis database (ITTD)

The Insect Pest Management Initiative (IPMI) tsetse project in Zambia was funded by the British Overseas Development Administration (ODA) and ran from 1991 to 1995. During this project, standardized methods were developed for sampling tsetse and trypanosomes, which required the development of a computerized spatial data management system through which to manage, interrogate, manipulate and display survey and surveillance data. This resulted in the development of the integrated tsetse and trypanosomosis database (ITTD) [12], which was designed and written specifically for Zambia, but has since been modified for use in all countries of the Regional Tsetse and Trypanosomiasis Control Programme (RTTCP) of the Southern African Development Council (SADC): Zambia, Zimbabwe, Malawi and Mozambique. The database program was written in the FoxPro for Windows programming language and uses DBX database structures.

During tsetse and trypanosomiasis surveys or surveillance, field staff enter results on to specifically designed forms. Logical sampling site codes are assigned to locations (e.g. trap sites or crush pens) that are regularly sampled during tsetse or trypanosomiasis surveillance, and geographic attribute data are assigned to all sample points. The completed forms are sent to a data management office in each country, and data are typed in to the ITTD. Data entry screens have numerous checks to reduce
data entry errors and, wherever possible, data values are selected from a choice of options presented in a pop-up menu. The program is configured for each country in which it is used, and only options relevant to that country (e.g. tsetse species) are included in the pop-up menus. Ideally, the data are entered twice, and a database comparison module is invoked to highlight differences that can then be checked from the forms and rectified.

Within the ITTD, tsetse and trypanosomiasis survey data are manipulated and interrogated and the results of queries are presented in tabular, graphic and map formats. Survey data are usually presented in map format while surveillance data are usually best represented as a graph with time along the x-axis and some estimate of the incidence of tsetse or trypanosomiasis up the y-axis.

Various limitations were identified in the ITTD, particularly resulting from its original design for use only in Zambia, its restriction to only some species of tsetse and trypanosomosis and to particular sampling methods, and from the programming language and database structures used.

3.2. The disease and vector integrated database (DAVID)

Currently under development in Oxford, calling upon the skills and experience of a commercial software developer, is a revision of the ITTD, referred to as the disease and vector integrated database (DAVID). The revision is being developed in the Visual Basic programming environment using MDB (Access) database structures with full Windows compatibility. High quality graphical output is being developed in IDRISI for Windows format, though the database program can run independently of IDRISI since it has its own module for displaying results. Currently, IDRISI format output is the only option but, where there is a demand, output can be modified to include other standard GIS formats. The most important aspect of this revision is that it is generic and can be set up for use in any African country, regardless of tsetse and trypanosome species present, sampling methods used, etc. To date the system has been developed, under ODA funding, to manage and output data from tsetse surveys. The next stage of development will start in April 1997 and will incorporate the management of livestock data and data from trypanosomiasis surveys and surveillance, both from parasitological and serological tests. This new program is a direct development of the original ITTD, and as such incorporates all of its functionality but overcomes many of its limitations. The ITTD will be phased out of use in the RTTCP by the end of 1997, and DAVID will replace it in the veterinary departments of Malawi, Mozambique, Zimbabwe and Zambia. The program is also being used at the Kenyan Trypanosomiasis Research Institute (KETRI) and at the Onderstepoort Veterinary Institute (OVI) in South Africa.

There is an increasingly strong demand for database systems that manage not only tsetse and trypanosomosis field data, but data covering a wide range of vector
borne and infectious diseases of livestock. The generic nature of DAVID is such that it can be adapted to meet these requirements, though this will require close collaboration with veterinary bodies that are concerned with international livestock disease surveillance standards such as the Office international des épidémies (OIE).

4. MODELLING INPUT VARIABLES

Collecting field data relevant to planning and managing tsetse and trypanosomiasis control operations is often very expensive and time-consuming, therefore preventing exhaustive ground coverage. Many of these data, however, are dependent on, or at least correlated with, other environmental variables that are more readily available over wide areas. These correlates include information on climate, soils and vegetation, which can often be derived from remotely sensed data (see, e.g., Ref. [13]) or, in the case of climate, from interpolation of ground data (see, e.g., Ref. [14]). This offers the opportunity to extend existing knowledge spatially by deriving models in areas where data are available, within a GIS, and using these models to make predictions in areas where data are absent. This section will review the use of GISs to predict the distribution of tsetse, trypanosomiasis, livestock and cultivation.

4.1. Tsetse

Rogers and Randolph [15] showed that estimates of monthly abundance and mortality rates of *Glossina palpalis* in West Africa were correlated with the saturation deficit and with the normalized difference vegetation index (NDVI), a satellite derived index of vegetation activity. Kitron et al. [16] analysed tsetse catches in western Kenya and found that 87% of the variance of catches could be explained by using multiple regression of the seven Landsat TM wavebands. These studies raise the possibility of using interpolated and satellite derived environmental data, within a GIS, to model habitat suitability and thus to map the likely distribution and abundance of tsetse in areas where field data are not available.

Robinson, Rogers and Williams [17] analysed the distribution of *G. m. centralis*, *G. m. morsitans* and *G. pallidipes*, in part of southern Africa, with respect to single environmental variables derived from averages, maxima, minima and estimates of seasonal variability of interpolated climate (temperature and rainfall) and remotely sensed vegetation (NDVI) data. Analysing single variables had the advantage that no assumptions needed to be made regarding the statistical distribution of the data, but the disadvantage that it could miss important interactions between variables.

These limitations can be overcome by adopting a multivariate approach to the analysis of tsetse distributions. Multivariate analysis of climate and remotely sensed
data has been used to model tsetse distributions in southern Africa [18–21], eastern Africa [20–22] and western Africa [23]. A number of multivariate statistical techniques is available with which to do this [19], including classical discriminant analysis, maximum likelihood classification, tree based analysis, k nearest neighbours, projection pursuit analysis and neural networks. The first two methods are relatively easy to implement and interpret but assume that the distribution of predictor variables is multivariate normal. Linear discriminant analysis is further restricted in that it assumes common covariance for the different classes in the parameter space. Tree based analysis divides the parameter space into hypercubes but its flexibility is balanced by the difficulty of interpretation when more than two or three levels are included in the analysis. In general, it is recommended as a technique for exploring data sets before final analysis by other methods [24]. The k nearest neighbour analysis gives good classification, providing the areas of presence and absence are reasonably continuous (in predictor variable space), but provides little interpretative information. Finally, projection pursuit analysis and neural networks are very flexible and can provide excellent classifications but are almost impossible to interpret biologically.

The accuracy of the results obtained by using these techniques is improving as the multivariate models improve [18] and as the environmental data sets become more accurate and include more variables [13]. The main limiting factor now is the quality of training data, for which we usually rely on surveys that were done a long time ago, often with relatively insensitive sampling methods and limited georeferencing. These analyses highlight areas where we need to obtain more reliable field data on tsetse distributions, and through the use of data management systems such as DAVID field data will be brought directly into a format that is compatible with multivariate modelling within a GIS.

4.2. Trypanosomiasis

Rogers [25] showed correlations between the monthly incidence of trypanosomiasis and monthly NDVI data. While the correlation was positive in settlements surrounding the Olambwe Valley game reserve in Kenya, where *G. pallidipes* was the vector, it was negative in Kigulu Country on the shores of Lake Victoria in Uganda where the vector was *G. fuscipes*. This difference was thought to result from contrasting local conditions, fly ecology and feeding behaviour. Relationships such as these, between disease incidence and environmental variables that can be remotely sensed, raise the possibility of predicting the incidence and prevalence of trypanosomiasis and using the GIS to extend these predictions through space and time.

Relatively little progress has been made in predicting the distribution of trypanosomiasis, largely because there are so few data on trypanosomiasis prevalence. A project in Togo, however, has been conducting trypanosomiasis surveys over the whole country. Rogers [26] applied both linear discriminant analysis and
stepwise multiple regression methods to distribution data of *Trypanosoma vivax* and *T. congolense* in Togo. The predictor variables used were elevation, tsetse distribution and satellite derived climate and vegetation data. It was found that the regression techniques tended to identify correctly the areas affected by the different diseases, but not the levels of infection. Discriminant analysis performed well in predicting areas with prevalence above a threshold of 5%.

Williams and Robinson [27] conducted preliminary analyses on trypanosomiasis prevalence data, determined from buffy coat tests, from some 90 dip tanks in northern KwaZulu/Natal, South Africa. They predicted the trypanosomiasis prevalence using a limited set of interpolated climatic and satellite derived vegetation data. Regression analysis gave a significant fit but was only able to account for about 15% of the variance. Linear discriminant analysis was more successful and showed good agreement between observed and predicted distributions of disease. They found the two most important variables to be the NDVI and pan evaporation, indicating that vegetation and evaporation are key factors in determining the distribution of disease in this region. They concluded that more detailed analysis was not warranted at present because of the limitations of the data set. However, a more detailed analysis should be conducted when data were available for more dip tanks, and for other predictor variables such as the distribution of tsetse and proximity to game parks. This should elucidate the factors that determine the distribution of trypanosomiasis as well as improve the predictions.

4.3. Cultivation

Areas with a high level of agricultural activity can often be distinguished from uncultivated areas because they (a) exhibit different spectral responses, (b) have different textures, (c) are spatially homogenous, and (d) display characteristic seasonal variability determined by times of planting and harvesting. High resolution satellite data have been used in many exercises to map agricultural land use on the basis of spectral characteristics (e.g. using Landsat and SPOT) and textural characteristics (e.g. using radar imagery).

An excellent example for the use of high spatial resolution satellite imagery to map agricultural land use, though not from Africa, is the land cover mapping project of Great Britain [28]. An supervised maximum likelihood classification was used on Landsat Thematic Mapper (TM) data to generate a land cover map which records 25 cover types, with a minimal mappable unit of 0.125 ha (for landscape features with strong spectral features). They found that classification accuracy was substantially improved by combining summer and winter imageries. Tilled land was one of the cover types so percentage cultivation could easily be generated from the land cover map as a coarser product.

The World Food Programme's (WFP) Famine Early Warning System (FEWS) conducted an extensive study using the GIS to combine a variety of data to make
estimates of crop use intensity for Zambia, Zimbabwe, Malawi and Mozambique. They used Landsat imagery to divide the area into five classes of percentage cultivation: <5%; 5–29%; 30–49%; 50–69% and 70–100%. The methodology is described in Ref. [28] and uses a combination of colour, tone, pattern, evidence of wetness, aridity and saline conditions and, where available, soil, climate, geological, geomorphic and topographic information. While ground truth data are needed to verify the absolute values of crop use intensities, as a relative estimate of agricultural intensity these data are likely to be quite realistic [30].

At a much coarser resolution, Rogers and Wint [7] generated predictions of agricultural activity in east Africa from available data for human population, remotely sensed ecoclimatic indicators and elevation. They found that over such a large area multiple regression models could not explain the existing data very well. These models improved greatly, however, when generated individually for subdivisions of the area. Two subdivisions were used: one treating the countries individually and the other defining different ecozones that were generated by first performing a principal components analysis of the contemporary satellite imagery, followed by clustering to define ten ecozones. From this analysis, they predicted the cultivation percentage throughout the region based on the ecozone subdivision. They noted that a further subdivision of the region — treating ecozones within each country separately — could further improve the accuracy of this prediction.

4.4. Livestock

In a detailed analysis of livestock and land use, Bourn and Wint [31] conducted low level aerial reconnaissance and complementary ground studies on Mali, Niger, Nigeria, Sudan and Tchad between 1980 and 1993. They found a highly significant relationship between livestock biomass and the intensity of land use: cultivation and human habitation proved to be good indicators of livestock distribution. Given that remotely sensed correlates of cultivation have already been demonstrated, the opportunity for predicting livestock distribution in the same way presents itself.

The same method that is summarized above to predict cropping percentage was used by Rogers and Wint [7] to estimate the cattle density in east Africa. Similar results were obtained, and again it was found that treating each ecozone within each country independently produced the best predictions.

5. PERSPECTIVES

It should be clear from the above discussion that the GIS has an important role in the management of trypanosomiasis and that this role spans from the policy level down to the operational level. Some of the applications discussed above, such as the
disease and vector integrated database, are of direct and immediate operational use. Others, for example predicting the distribution of tsetse, will require more research and the provision of improved training data before they can be used at operational levels though they are already sufficiently refined to be of use for broader scale policy making and prioritizing. In this final section, I will discuss the three main subject areas above in terms of the opportunities the future may hold.

5.1. Decision support

A major limitation in the use of GIS for decision support is the lack of sufficiently accurate criteria data to build into decision rules. While a range of data is potentially important, the critical variables are tsetse distribution, trypanosomiasis prevalence, livestock density and percentage cultivation. Without reliable data on these variables it will not be possible to make rational resource allocation decisions regarding disease control. Reliable data on tsetse and trypanosomiasis can only be obtained through rigorous field surveys, from which the data are managed in an appropriate system such as the disease and vector integrated database. As more field data are collected, there will be less reliance on predicting the distribution of tsetse and trypanosomiasis in areas where data are not available. Veterinary departments may need to collect livestock data themselves, in which case the same argument for efficient data management holds. The next most direct method by which livestock distribution and abundance data can be obtained is through systematic data collection, combining low level aerial reconnaissance and complementary ground studies. If conducted over a reasonably sized area this type of survey can provide excellent data, not just on livestock but also on cultivation, at a relatively low cost. While aerial reconnaissance provides possibly the best data on percentage cultivation, cultivation can be mapped very cheaply and with a reasonable reliability by using high resolution satellite data. The high resolution radar data that are becoming increasingly available hold enormous potential for mapping cultivation. A combination of high resolution satellite data and aerial reconnaissance should be able to provide livestock and cultivation data, over significantly large areas, that can be combined with tsetse and trypanosomiasis distributions within geographic information systems, to plan and manage tsetse and trypanosomiasis control operations.

Another important aspect of decision support that must be addressed is the incorporation of reliable economic models. Whilst economic considerations are not the only factors that are important in prioritizing areas for disease control, they should provide a firm base from which to develop decision rules. When we can produce accurate maps of the costs of interventions, and the benefits that are expected to result from them, we will be a significant step closer to a rational decision making methodology.

Multiobjective decision support [10] is concerned with resolving conflicting objectives in decision making, a subject that must be addressed in the future when we
prioritize areas for tsetse and trypanosomiasis control. Conflicting objectives to tsetse control are likely to arise, for example, in areas of conservational importance, areas that are environmentally fragile and areas where certain types of tsetse control may conflict with the management of other diseases (e.g. the tick situation mentioned above). The GIS is already being used to map biodiversity [32], which raises the possibility of incorporating the results from this type of analysis directly into decision making models. It is likely that environmental degradation can be estimated from remotely sensed data, which also lends itself to geographic analysis. When the disease and vector integrated database has been extended to cover a wider range of animal diseases, this will facilitate an integrated approach to the management of livestock disease that avoids conflicts.

A critical aspect in the development of decision support for prioritizing areas for tsetse control is that it should be a participatory process, incorporating representative groups of individuals who have a direct stake in the outcome of the decision. These stakeholders include local communities, veterinary departments, technical staff, project managers, donors and wildlife and conservation organisations. Stakeholders can be advised and assisted in decision rule development by technical experts, land use experts and economists. To facilitate participatory decision making, training exercises need to be developed to bring the GIS technology to the relevant people. Exercises along the lines of those in the IDRIS/UNITAR workbook on GIS and decision support [10] are being produced in Oxford, specifically on prioritizing areas for tsetse and trypanosomiasis control.

5.2. Field data management

In the area of field data management we must look forward to an increasing number of countries adopting common data management systems so that large databases containing standardized disease and vector data start to evolve. While it seems that there is a great shortage of tsetse and trypanosomiasis data for most of Africa, data have been collected in many places at one time or another. Information gets lost, however, for a number of reasons: sometimes reports are physically lost, and at other times important supplementary information such as details of the sampling methods used, or positional data, are not adequately recorded. It is important now, however, that we begin to focus our approach to field data collection so that we start to build up an increasingly accurate and useful picture of the disease and vector situation.

The two most exciting prospects in database development in the near future are, first, the integration of tsetse and trypanosomiasis data with data from a wide range of other livestock diseases, and, second, the networking of disease surveillance. Developing a data management system that integrates information from a variety of vector borne and infectious disease is particularly important in southern Africa, where the RTTCP is proposing to broaden its mandate to cover a range of animal diseases,
under the umbrella of SADC's livestock programme. Wider developments in the
disease and vector integrated database will need to be co-ordinated by a forum of
interested parties including representatives from African veterinary departments, the
RTTCP and other emerging regional programmes in east and west Africa, the SADC,
the Food and Agriculture Organization of the United Nations (FAO), the International
Atomic Energy Agency (IAEA), the Office international des évizooties (OIE) and the
Onderstepoort Veterinary Institute (OVI). When this type of data management system
is in place it will greatly facilitate an integrated approach to the management of live-
stock diseases. An example of where this may be useful is in developing an appro-
priate strategy to control both tsetse and tick borne diseases, where they occur in the
same place such as in northern KwaZulu/Natal in South Africa, and where there may
be conflicting interests, i.e. tsetse control through animal dipping versus maintenance
of endemic stability to tick borne diseases.

Another area in field data management that will present opportunities for the
future is in the networking of disease surveillance. If efficient networking systems can
be developed the management of cross-border diseases will be greatly facilitated,
increasing our ability to contain crippling epidemics such as foot-and-mouth disease
or rinderpest.

5.3. Predicting input variables

Opportunities for developments to improve predictions of input variables,
particularly of disease and vector distribution and abundance, include using more
reliable training data, generating more accurate environmental data, using improved
statistical models and developing biological models.

Improved training data will only result from dedicated field operations conducting
systematic tsetse and trypanosomiasis surveys. This is unlikely to happen suddenly since
surveys are expensive and time consuming, but if standardized methods are used, and
data are captured in dedicated database programmes such as the disease and vector inte-
gerated database, then an increasingly accurate training data set will emerge.

Improved accuracy of environmental predictor variables is likely to be achieved
through the use of remotely sensed satellite imagery. By using satellite data at its orig-
inal resolution, rather than crudely degraded products, inaccuracies introduced during
the process of data reduction will be avoided [33]. High resolution, satellite derived,
optical and radar data remain to be exploited fully, and further options will be gener-
ated through the new wave of satellite borne sensors that are due for launch by the end
of the century [34]. Alternative indices to the NDVI have been suggested [35] and need
to be tested operationally, and improved estimates of meteorological variables from
satellite data, such as temperature, rainfall and humidity, need to be developed [13].

In addition to using more sophisticated multivariate statistical methods, incor-
porating spatial autocorrelations should improve the predictive performance of our
models. Appropriate ways to deal with spatial autocorrelation will become increasingly important as we attempt to map the distribution and abundance of tsetse using environmental data at a higher spatial resolution.

The most exciting opportunities to improve predictions of input variables lie in developing a more deterministic approach to complement the essentially empirical methods that have been used to date. For example, if we can develop process based population models that link demographic rates to variables that can be remotely sensed, then we can extend these models through space and time through the satellite data. While the empirical distribution models can provide reasonable estimates of the potential range of species, a better understanding of the processes involved in their population dynamics should provide better estimates of which part of that range is actually occupied at any time. This will lead to a better understanding of the ways in which populations are susceptible to change, both natural and human induced.

5.4. Concluding remarks

The future for GISs in the control of tsetse transmitted trypanosomiasis, and many other vector borne and infectious diseases, looks bright indeed. Many exciting opportunities present themselves for making predictions in data sparse areas and for combining important data variables to supported decision making with robust economic models and an understanding of the epidemiology of the disease. However, none of these developments will be possible without reliable field data on disease and vector. I must finish therefore with a plea for close collaboration and co-operation between field programmes in Africa and research institutes throughout the world. It is only through the provision of reliable epidemiological data, and constructive feedback from field operations, that progress will be directed towards increasing the practical usefulness of GISs for controlling tsetse transmitted trypanosomiasis.

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REFERENCES


CLIMATE CHANGE AND THE DISTRIBUTION OF VECTOR BORNE DISEASES WITH SPECIAL REFERENCE TO AFRICAN HORSE SICKNESS VIRUS

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Abstract

CLIMATE CHANGE AND THE DISTRIBUTION OF VECTOR BORNE DISEASES WITH SPECIAL REFERENCE TO AFRICAN HORSE SICKNESS VIRUS.

In the context of climate change, those components of climate that are likely to have major effects upon distribution, seasonal incidence and prevalence of vector borne diseases are described. On the basis of a predicted, mean temperature increase of the order of 1 to 3.5°C, examples are given of the sort of changes that are to be expected by using a range of internationally important human and animal pathogens. Recent dramatic alterations in the epidemiology of the OIE List “A” disease, African horse sickness, are drawn upon to put forward the proposition that climate change may already be having a major effect upon some vector borne diseases.

1. INTRODUCTION

In 430 B.C. Hippocrates said: “Whoever would study medicine aright must learn of the following subjects: First, he must consider the effect of the seasons of the year and the differences between them. Secondly, he must study the warm and cold winds, both those which are common to every country and those peculiar to a particular locality.”

We can deduce from this that the influence of climate on infectious diseases has been a topic of some interest for well over 2000 years. More recently, there has been an upsurge in this interest fuelled by the various global warming scenarios that have been promulgated and highlighted by the fact that eight of the warmest years on record have all occurred within the last decade, thereby, superficially at least, seeming to support the concept of imminent climate change. Indeed, it is now accepted by most authorities that “the balance of evidence suggests that human activities are warming the planet” (Intergovernmental Panel on Climate Change 1996) and climate models project an increase in global mean temperature of between
1 and 3.5°C during the next century. It is further predicted that maximum warming will occur at high northern latitudes and during the winter, and that night time temperatures will increase more than day time temperatures [1–3].

2. VECTOR BORNE DISEASES

In the context of vector borne diseases, these predicted temperature increases and the concomitant changes in rainfall patterns are likely to have a variable, but significant effect. By definition, vector borne diseases possess a vector stage, usually an arthropod, crustacean or mollusc, which is poikilothermic and therefore peculiarly liable to be profoundly influenced by climatic variables, particularly temperature and humidity. The components of climate that have a direct effect on vector biology include the following [4–6]:

(1) Temperature — An increase in temperature will accelerate a vector’s metabolic rate. Bloodfeeding vectors will therefore be likely to feed more frequently and hence their biting rate will increase, leading to increased egg production and population size. However, the daily survival rate of individual vectors may decrease as temperature rises. Temperature may also affect the geographical range or distribution of arthropod vectors since this tends to be limited by minimum and maximum temperature (and humidity).

(2) Humidity — High relative humidity favours most vector metabolic processes so that, at high temperatures, a relatively high humidity tends to prolong survival, although increased susceptibility to fungal and bacterial pathogens may offset this to a variable degree. Low humidity causes a decrease in the daily survival rates of many arthropod vectors, because of dehydration, but in some cases it may also cause an increase in the bloodfeeding rate, in an attempt to compensate for the high levels of water loss.

(3) Precipitation — Precipitation is an important factor with respect to many bloodfeeding groups of insects including mosquitoes, Simuliidae, Culicoides and Tabanidae since these all have aquatic or semi-aquatic larval and pupal stages. Precipitation, therefore, frequently determines the presence, absence, size and persistence of suitable breeding sites. The precise level of impact of precipitation on vector breeding sites depends upon local evaporation rates, soil type, slope of terrain and proximity of large bodies of water (e.g. lakes, ponds, rivers). Since many insect vectors breed in seepages of water from irrigation pipe leaks, cattle trough overflows and residual water from previous rains, very heavy precipitation may disrupt such sites and wash the immature stages away or kill them directly.

(4) Wind — Since winds contribute to the passive dispersal of many species of flying insects, prevailing wind directions and wind speeds may affect vector distrib-
ution. Some insect vectors, including various Anopheles, Simuliidae, Phlebotominae and Culicoides, can be dispersed for hundreds of kilometres from their source.

In addition to these direct effects, climatic variables may also have important indirect effects upon vector abundance and distribution and hence disease, whereby one vector species may be displaced by another with a different vectorial capacity in response to environmental changes such as deforestation, expansion in irrigation, increase in brackish water breeding sites due to sea level rise [3].

According to Shope [7], when attempting to predict the effect of global climate change on the distribution of vector borne diseases, the following factors must be considered:

— the current distribution of the pathogen causing the disease;
— the range of the vertebrate hosts and the vectors;
— temperature related vector and parasite development rates;
— capacity for migration or dispersion of the vectors and parasites; and
— the current seasonal incidence of transmission.

3. INSECT VECTORS

In the context of insect vectors, palaeoclimatic records demonstrate that most shifts in the distribution of insect taxa have been associated mainly with temperature change and that these shifts have apparently occurred much more rapidly than shifts in the distribution of vegetation and the higher animals [8, 9]. Considering the high mobility of insects, in particular winged ones, as exemplified by the migration of swarms of the desert locust from North Africa to the Caribbean during 1988, this should come as no surprise and strongly suggests that the current distribution of many insect species that act as vectors would change rapidly following any future climate change. In this context, it has been estimated that a 1°C rise in temperature will correspond to 90 km of latitude and 150 m of altitude [10]. Suggestions that change in the geographical distribution of insect vectors may already be occurring can be inferred from newspaper reports relating the apparent extension in the range of Phlebotomus sandflies which transmit Leishmania spp. from mainland France into the British Channel Islands [11].

The introduction, into Italy in 1989, of Aedes albopictus (the Asian ‘tiger’ mosquito), an efficient vector of yellow fever and dengue viruses, demonstrates that even winged insects do not always have to find their own way into new areas of suitable climate. This mosquito was introduced via its immature stages, in water contained in car tyres imported from the USA. Aedes albopictus is now found widely over Italy and has been recorded from over 20 locations in that country [12]. Since the distribution of Ae. albopictus is bounded, conservatively, by the mean 0°C isotherm
in the coldest month of the year [13], this suggests that in Europe, where this (January) isotherm encompasses most of western Europe and stretches as far north as southern Scandinavia, *Ae. albopictus* may already be able to expand into most or all of these areas. In the USA, where it was introduced in 1985, *Ae. albopictus* now occupies 24 southeastern States ranging as far north as Nebraska and Iowa [3]. It is also expanding southwards through Florida at the rate of 40 miles per year [10]. Should climate change result in the temperature increases projected, then the range of this mosquito is likely to extend to include most of the populated areas of the eastern USA, as well as the southern tip of Canada [13]. In Europe the projected increases could result in *Ae. albopictus* being able to occupy virtually all of western and central Europe up to and including southern Scandinavia. However, such an increase in geographical distribution by a vector need not necessarily imply an equivalent increase in areas ‘at risk’ to the pathogens which it is competent to transmit. In respect of the transmission of dengue virus by *Ae. albopictus*, the lower limiting temperatures for transmission seem to be around $20^\circ C$ [10]. It is therefore likely that transmission of this virus would be possible only over the warmer part of its vector’s range. In this context, it is important to remember that ‘warmer’ should not be interpreted in terms of latitude alone. An increase in mean temperature of $4^\circ C$ would also permit transmission of dengue virus at the altitude of Mexico City, with an additional population of some 20 million then being put ‘at risk’. These sorts of situation are likely to be repeated in respect of many human and animal pathogen–vector combinations.

4. MALARIA

The key temperatures limiting the development of *Plasmodium falciparum* in *Anopheles* mosquitoes lies between 18 and $20^\circ C$ [14], while the vectors of this species of malaria generally do not survive when the mean winter temperature drops below 16 to $18^\circ C$ [15]. Modelling suggests that, within the current transmission range and anticipating a 2 to 3°C rise in temperature, a twofold higher epidemic potential can be expected. At the margins of existing transmission areas, a much more dramatic increase, of the order of 100-fold, is predicted [16, 17]. This would give rise to an increase in case load of some 50 to 80 million over current annual projections.

5. JAPANESE ENCEPHALITIS

The key temperatures limiting Japanese encephalitis virus (JEV) transmission are around $26^\circ C$. A 2 to 3°C rise in temperature could transform the current sporadic transmissions in the equatorial regions of its range to epidemic transmission in the major population centres of South-East Asia. However, the epidemics would
probably remain restricted to rice field associated areas, which are the main vector breeding sites. Nevertheless, extensive development of rice field agriculture in Africa could render this a major geographical area for virus extension [10].

6. ENCEPHALITIS VIRUS TRANSMISSION IN THE UNITED STATES OF AMERICA

On the basis of field and laboratory studies carried out in California, Reeves et al. [18] forecast that a 3 to 5°C rise in temperature would cause a significant northward shift of both western equine encephalitis virus (WEEV) and St. Louis encephalitis virus (SLEV) in North America. WEEV may disappear from its current endemic regions, and SLSV may extend much further north into Canada, where sporadic outbreaks of disease already occur.

These predicted changes are based partly on the fact that the lowest temperature at which WEEV transmission can occur via its vector *Culex tarsalis* is 11°C while for SLEV it is 15°C [3]. But at temperatures above 30°C, the vector modulates WEEV infection so that the proportion of infected vectors decreases dramatically, thus reducing overall transmission rates. However, SLEV infection is not modulated by the vector, and so its transmission will not be similarly affected at the higher temperatures.

7. BLUETONGUE VIRUS

Bluetongue virus (BTV) is a dsRNA virus that causes a disease of such international significance in certain of its ruminant hosts, particularly sheep, that it has been allocated OIE List “A” status. The virus exists as a number of distinct serotypes (24) and is transmitted between its vertebrate hosts almost entirely by certain species of *Culicoides* biting midge [19]. In consequence of this, the world distribution of BTV is limited to those geographical areas where competent vector species of *Culicoides* are present and to those times of the year that are favourable to adult vector activity [19-24].

The present global distribution of BTV lies approximately between latitudes 35° S and 40° N. Within this area the virus has a virtually worldwide distribution, being found in North, Central and South America, Africa, the Middle East, the Far East and Australia [25, 26]. As a general rule, BTV can be considered as infecting livestock in all countries in the tropics and subtropics. Additionally, several countries that are close to the subtropics, such as the USA, have enzootically infected livestock. It is also the case that those countries whose territory spans a wide range of latitudes (e.g. Australia, USA) frequently include large geographical areas where BTV activity is apparently absent [26].
In the USA, BTV is at present enzootic in the south, central and western areas of the country and in Florida, with an epizootic band stretching from the Canadian border to the southern Atlantic coast. The northeastern states are a 'BT free' area. Recent studies, using established climate change models that predict a 4°C rise in mean temperature, suggest that the BTV enzootic area will expand north- and eastwards to include all western, central and southeastern states and that the epizootic zone will cover the whole remainder of the country; there will be no BT free zone at all in the 48 States [7].

In Australia, BTV was first identified in the northern part of the Northern Territory in 1977, the original isolate being made from a pool of mixed *Culicoides* species collected in 1975 [28]. The virus is now known to also occur in parts of Western Australia, Queensland and New South Wales [29]. Within these areas several species of competent *Culicoides* vectors are present. One of the most efficient species, *C. wadai*, provides an example of the sort of alteration in vector distribution that climate change could bring or may be bringing about.

*Culicoides wadai* is present throughout much of South-East Asia including Indonesia. However, it was first recorded in Australia only as recently as 1971, from the vicinity of Darwin just north of latitude 15° S in the Northern Territory. This location, at the northern tip of Australia, suggests that the midge may have been introduced as 'aerial plankton' by being blown on the prevailing winds from neighbouring Indonesian islands some 150 miles or so away [30]. Such movements by adult *Culicoides* fall well within the distances described by Sellers et al. [31] and Hayashi et al. [32]. Subsequent to its arrival in Australia, *C. wadai* continued to be recorded only from the Darwin area until 1977. Then, from 1978 to 1982, extensive surveys revealed that it had dramatically extended its range both southwards into the Kimberley Mountains and eastwards as far as the Queensland coast, east of the Great Dividing Range, and by 1988, it had reached so far south as the Hunter Valley in New South Wales [25, 30, 33]. This is an extension in range of some 2000 miles in a little over ten years. The known distribution of *C. wadai* now puts this species in close proximity to some of the major Australian sheep rearing areas and since sheep are the vertebrate hosts most severely affected by BTV the movements of this efficient vector continue to be monitored with some trepidation [28].

8. AFRICAN HORSE SICKNESS VIRUS

8.1. Distribution and epidemiology

African horse sickness virus (AHSV) is, like BTV, an OIE List “A” virus and also like BTV its biological vectors are *Culicoides* biting midges. In the case of AHSV, the only confirmed field vector is the species *C. imicola*. The vertebrate hosts
of AHSV include all species of equine but its effects are exceptionally severe in naive horses and mortality rates in these animals frequently exceed 80 or 90%. This makes AHS one of the most lethal horse diseases on the planet.

As its name implies, AHSV is an African disease and is enzootic in tropical and subtropical areas south of the Sahara in a band stretching from Senegal in the west to Ethiopia and Somalia in the east and extending as far south as northern South Africa [19, 34]. The Sahara desert, however, seems to provide an effective geographical barrier which has prevented the virus from establishing itself permanently in northern Africa.

Until relatively recently the virus was believed to be confined to continental Africa except for occasional excursions across the Red Sea into Arabia [35, 36]. However, from 1959 to 1961, AHSV serotype 9 exploded out of Africa and spread in a broad swathe across Saudi Arabia, the Syrian Arab Republic, Lebanon, Jordan, Iraq, Turkey, the Islamic Republic of Iran, Afghanistan, Pakistan and India [17]. Nevertheless, by the end of 1961, in the face of a massive vaccination campaign and the deaths of over 300 000 equines, the disease in Asia came to a halt [37]. The inability of the virus to persist in Asia was probably due to a combination of factors, including vaccination, vector control campaigns and adverse climatic conditions which reduced or prevented adult vector activity during the winters [38].

During 1965, AHSV-9 again spread beyond its sub-Saharan enzootic zones, appearing first in Morocco and then rapidly spreading into Algeria and Tunisia before crossing the Strait of Gibraltar into Cadiz province, southern Spain [39-44]. AHSV persisted in North Africa into 1966 before finally disappearing from the area but the extension into Spain lasted for less than three weeks [39].

Subsequent to 1966, AHSV was reported only from areas of sub-Saharan Africa for over 20 years. However, in July 1987 an outbreak of AHS due to serotype 4 of the virus was confirmed in central Spain, initially in the Province of Madrid, but spreading rapidly into the neighbouring provinces of Toledo and Avila [45]. The most likely explanation for this outbreak is related to the importation of a number of zebra from Namibia, five of which were taken to the El Rincón Safari Park (where horses were already present) at Aldea del Fresno, some 50 km SW of Madrid [45]. Zebra are susceptible to infection with AHSV but unlike horses usually show no clinical signs. This park subsequently became the site of the first 27 cases of AHS in Spain in 1987. This epizootic continued for three to four months of 1987 in central Spain and then apparently ended during October [46, 47].

Although the 1987 Spanish outbreak caused some alarm because AHS is a notifiable disease and this was the furthest north that it had ever been recorded, concern was moderated because all previous information suggested that the virus was incapable of overwintering in Europe. Consequently, the apparent end of the epizootic in October 1987 was in line with expectations and was assumed to be the finish of the matter. However sadly this was not to be the case, and further more severe outbreaks
occurred in Spain during 1988, 1989 and 1990, in Portugal in 1989, and in Morocco in 1989, 1990 and 1991. All these outbreaks were due to AHSV-4, a serotype that had never previously been seen outside southern Africa. During the course of the outbreaks, there was no other evidence of AHSV, of any serotype, within 2000 miles of Spain or northern Morocco; therefore, it does seem certain that there was only one introduction of the virus into the western Mediterranean area and that was via the zebra in 1987. Subsequent to that time, the virus had persisted in the area for at least five years, overwintering four times in the process.

This situation was unprecedented. Nowhere else and at no other time had any serotype of AHSV ever succeeded in overwintering more than twice outside sub-Saharan Africa. There was therefore much speculation as to how and why the situation had changed.

Previously, most scientific opinion had suggested that AHSV requires an ‘unidentified’ long term vertebrate reservoir and high populations of efficient vector Culicoides for its persistence. Since these factors were thought to be absent outside sub-Saharan Africa these were the reasons put forward to explain why the virus had always failed to survive in such areas.

However, the requirement for an unidentified vertebrate reservoir in order to ensure the survival of AHSV is difficult to reconcile with reality. It is well documented that AHSV causes a viraemia in horses and zebra that may persist for as long as 18 and 40 days, respectively [38, 48]. It is also the case that in much of sub-Saharan Africa the climate is sufficiently congenial for adult vector Culicoides (C. imicola) to be active throughout the year [49, 50]. Consequently, AHSV should be able to survive very well, even in its ‘sub-Saharan’ heartlands, solely through continuous and uninterrupted cycles of transmission between its vertebrate and invertebrate hosts and without the necessity for a long term reservoir. But the vital question to ask is whether this postulated ‘sub-Saharan’ survival mechanism can also explain the persistence of AHSV in Iberia and northern Morocco from 1987 to 1991?

Presumably, to answer this question one must use the same logic. First, there must be efficient vector species of Culicoides in Spain, Portugal and Morocco and, second, in at least some parts of these countries these efficient vectors must be present as active adults throughout the year, with any vector free periods that may occur being shorter than the maximum duration of viraemia in the local susceptible vertebrate population (i.e. 18 days in this case). All year round presence of adult vectors is a vital part of this mechanism because AHSV is not transmitted vertically by its Culicoides vectors.

8.2. Culicoides imicola, the only proven field vector of AHSV

The only proven field vector of AHSV is C. imicola [50]. This is an Afro-Asiatic species which is common throughout Africa and much of South-East Asia
as far east as Laos [51–53]. Until recently it was thought to be absent from Europe but in 1982 it was recorded from Cordoba in southern Spain [54] and then in 1984 from Portugal south of Lisbon [55]. Since that time we have learned that this species is widely distributed throughout southwestern Spain and most of Portugal ranging as far north as 41°5' N and including all areas from where AHS was recorded [56]. *Culicoides imicola* has also been found to be widely present throughout northern Morocco [57]. So there is an efficient vector of AHSV in Iberia and Morocco. Furthermore, we now know that in central Spain in the area of the 1987 outbreak *C. imicola* has a seasonal incidence and the adults disappear during November and December not to reappear until the following April, some three to four months later [58]. Bearing in mind the maximum duration of viraemia in horses (18 days) and the 'continuous cycling' mechanism described above, this is far too long a period of time for the virus to survive in vertebrates alone. Therefore, under existing climatic conditions AHSV would not be expected to overwinter in this part of the Iberian peninsula, and indeed the virus did not persist there. It arrived in the area in July 1987 and disappeared in October 1987, never to reappear [58]. Further south in Spain (in parts of Andalucia) and also in southern Portugal and northern Morocco, particularly in those areas which are bounded by the 12.5°C isotherm for the average daily maximum temperature in the coldest month of the year (January) [24] the situation is different: adult *C. imicola* are present throughout the year [57, 58]. As a direct result of this, these areas are potential enzootic zones for AHSV, and the virus overwintered in this geographic region until 1991 when it was finally eliminated, probably as a result of comprehensive vaccination campaigns.

The incursion of *C. imicola* from North Africa into Spain and Portugal, though apparently a recent occurrence, seems to be a permanent one, and Rawlings et al. [56] have suggested that the northwards expansion in its range is still continuing. We do not as yet know whether this northwards extension is the result of some general climatic change or whether the recent series of exceptionally warm years during the last decade are merely providing a preview of what such a change could bring about; as a result, however, south-west Europe is now a potential enzootic zone for AHSV and also for a number of other viruses that this midge is known to transmit (BTV, epizootic haemorrhagic disease of deer virus, Akabane virus).

### 8.3. Other potential vectors of AHSV

Although *C. imicola* is the only confirmed field vector of AHSV, during the outbreaks in Spain, isolations of this virus were also made from mixed pools consisting almost entirely of *C. obsoletus* and *C. pulicaris* but excluding *C. imicola* [59]. Neither of these two species had previously been connected with AHSV but as they have a distribution which is more northerly than the usual range of AHSV [60]
this is not unexpected since presumably they have had little opportunity to become infected [58]. It is therefore possible that the northerly extension in the range of AHSV to include southern Europe, facilitated by the earlier expansions in the distribution of its traditional vector *C. imicola*, could have brought the virus into contact with new, previously unsuspected vectors. Without the vigourous control campaigns mounted by the Portuguese and Spanish veterinary authorities this could have precipitated an even further northwards spread of disease, rather like passing a baton on in a relay race. The occurrence of novel vectors is always possible and is to be suspected whenever the distribution of a vector borne pathogen alters significantly.

Other preliminary work, on 'non-vector species of Culicoides' and the transmission of AHSV, which is at present under way in our laboratory, may be able to shed additional light on the AHSV isolations from *C. obsoletus* and *C. pulicaris* detailed above. In this context we have shown that northern European midge *C. nubeculosus* has a standard oral susceptibility rate for AHSV of less than 1% when the immature stages of the midge are reared at 25°C and when the adults are fed upon virus at a titre of $10^{6.0}$TCID$_{50}$ per mL [61]. However, if the rearing temperature of the immature stages is raised to 30–35°C not only does the developmental time from egg to adult decrease dramatically to produce significantly smaller adults but the oral susceptibility rate increases to over 10% [62]. Since four generations of selective breeding from susceptible parents had had no effect upon the *C. nubeculosus* susceptibility rate this is clearly not a genetically controlled, hereditable trait as is exhibited by most biological vectors of arboviruses [63]. Instead, it is likely that, at the higher rearing temperatures, individuals are produced with a higher incidence of the 'leaky gut phenomenon' [64, 65]. In such individuals virus is able to pass directly from the gut lumen into the haemocoel of the insect without the necessity of replicating in the gut cells. Once in the haemocoel it is well documented that most arboviruses will replicate and may be transmitted even by insect species that are normally non-vectors, and this is also the case with AHSV in *C. nubeculosus*. Such a sequence of events can be envisaged as a hybrid mechanism whereby infection is initiated by a mechanical event (i.e. passage of virus from the lumen of the midgut through the gut wall of the insect into the haemocoel, without replication in the gut cells) but transmission is the result of a series of subsequent biological events (i.e. virus infection of the salivary gland cells, replication of virus in the salivary gland cells and release of progeny virus particles into the salivary ducts). The AHSV isolations made from mixed pools of *C. obsoletus* and *C. pulicaris* in Spain as reported above, two *Culicoides* species which are not normally considered to be AHSV vectors, may be an example of the leaky gut phenomenon in operation, potentiated by the unusually warm conditions that were prevailing in the area at the time. Increasing temperature due to climate change will tend to increase the likelihood of such 'unusual' isolations.
8.4. Effect of temperature on AHSV infection in *Culicoides* vectors

Shope [7] suggested that temperature related vector and pathogen development rates must be considered when attempting to predict the effect of climate change on the distribution of vector borne diseases.

In the context of AHSV, work by Wellby et al. [66] and Mellor [61], has shown that AHSV replicates in a high proportion of vector *Culicoides* at 25°C and can be transmitted by nine days post infection (dpi). However, at this temperature the survival rate of the insects was low, and most insects had died within 11 days. As the ambient temperature was reduced the proportion of vectors infected also decreased and the time to transmission extended dramatically, but conversely survival rates increased significantly. At temperatures lower than 15°C, AHSV failed to replicate in, or to be transmitted by, any vectors. From these findings it can be seen that the likelihood of transmission of AHSV is a function of the interaction of these opposing trends and the optimum temperature for transmission must fall somewhere between the two extremes. Clearly, it is now important to carry out further work on virus replication in, and transmission by, vectors, involving variable temperature regimes that more closely simulate natural conditions (e.g. diurnal or nocturnal cycling) than the standard temperatures used hitherto. Preliminary studies in this area already suggest that the replication curves of AHSV in vector *Culicoides* kept under an alternating regime of 12 h at 25°C and 12 h at 15°C are similar to those exhibited in vectors kept continually at 25°C [67]. In order to gain a clear idea of the likelihood of transmission, under suboptimal temperature conditions (i.e. in ‘field situations’), it is important to discover the minimum (cumulative?) number of hours at permissive temperatures that are necessary to facilitate transmission.

Interestingly, in their 1996 study, Wellby et al. [66] also showed that, although no detectable AHSV virogenesis at all occurred in vectors kept at 10°C, by 13 days post-inoculation virtually all insects tested were free from virus. When 58 surviving vectors that had been kept at 10°C for 35 days were moved to 25°C for three days, the infection rate increased from apparently zero to 15.5%. It therefore appears that at low temperatures AHSV does not replicate in the vector but virus may persist in some midges at a level below that detectable by the traditional assay systems and when the temperature later rises to permissive levels, virus replication is able to commence and transmission may then be possible.

In the context of these studies, Wellby et al. [66] and other workers [24, 68] have demonstrated that *C. imicola*, the major AHSV vector, is active in the field at temperatures up to 3°C lower than the minimum required by AHSV. In practical terms, this suggests that *C. imicola* may be capable of AHSV transmission only over the ‘warmer’ parts of its range. While in the cooler areas (i.e. further north or at higher altitudes), even should the vector be present, transmission will be impossible or else only possible at restricted times of the year (i.e. in summer) or in climatically
favourable localities. These findings also suggest that should *C. imicola* continue its northerly expansion in range its ability to transmit AHSV will progressively decrease with increasing latitude — unless, of course, accompanied by climate moderation. However, since the survival rates of adult vectors increases dramatically at low temperatures, up to as long as 90 days in some cases [69], this set of circumstances could constitute an overwintering mechanism for AHSV in the absence of vertebrate involvement and also in the apparent absence of infected vectors (Table I).

**TABLE I. AFRICAN HORSE SICKNESS VIRUS: OVERWINTERING AND TRANSMISSION BY VECTORS**

(1) African horse sickness virus (AHSV) seems to require temperatures ≥15°C in order to replicate in, and be transmitted by, vector *Culicoides*.

(2) Adults of the AHSV vector *C. Imicola* are active down to temperatures as low as 12°C and can survive even cooler temperatures in an inactive state.

(3) Since the virus requires a higher minimum temperature than the vector this suggests that replication and transmission may be possible over only part of the vector’s range.

(4) However, during ‘cold’ periods virus can survive for extended periods at undetectable levels in adult vectors, whose own life span is extended at these temperatures, to as long as 90 days in some cases.

(5) Once temperatures rise to a permissive level (≥15°C), virus replication in the vector commences and transmission becomes possible.

(6) This situation provides an overwintering mechanism for AHSV in the absence of vertebrate involvement and also in the absence of detectable virus in the vectors.

9. **CONCLUSIONS**

Temperature dependent interactions between vectors and the pathogens that they transmit are highly complex and the outcomes can be difficult to predict. Furthermore, these interactions are likely to be profoundly influenced by climate change, whether or not this is human induced. Should the predicted alterations in our climate over the first part of the 21st century be confirmed then the changes that have recently occurred in the distribution of AHSV and its vectors may serve as a timely reminder, indeed a forerunner, of impending changes in the epidemiology of a wide range of other vector borne diseases of man and animals.
REFERENCES


CONSIDERATIONS OF IMPACT

(Session 7)

Chairperson

P. ROEDER
FAO
COST–BENEFIT ANALYSIS IN ANIMAL DISEASE CONTROL

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Abstract

COST–BENEFIT ANALYSIS IN ANIMAL DISEASE CONTROL.

Animal health economics is a relatively new discipline which is progressively develop­
ing a solid framework of concepts, procedures and data to support the decision making process
in optimizing animal health management. Research in this field deals primarily with three
interrelated aspects: (1) quantifying the financial effects of animal disease, (2) developing
methods for optimizing decisions when individual animals, herds or populations are affected,
and (3) determining the costs and benefits of disease control measures. In the paper the four
most common economic modelling techniques in animal health economics (i.e. partial
budgeting, cost–benefit analysis, decision analysis, and systems simulation) are described and
applied on three levels of veterinary decision making: the animal, herd and national level.
Outcomes so far are summarized, and shortcomings indicated and discussed. The importance
of a close link between economics and epidemiology is stressed for future development, as well
as the need for, and possibilities of, an international exchange of models and procedures.

1. INTRODUCTION

Economics is sometimes qualified as the discipline that simply measures things
in monetary units, while everyone else uses physical units. This view, however, is far
too simple and is inappropriate. Economics — as a science — deals primarily with
decision making, in which money is only one of the elements of the system. Animal
health economics, therefore, can be described as the discipline that aims to provide a
framework of concepts, procedures and data to support the decision making process
in optimizing animal health management [1].

Controlling the costs of production is becoming critically important in modern
livestock farming. Improving animal health and fertility can play a major role in
achieving efficient and economically rewarding production. Current veterinary
services are evolving to meet the need for a service targeted tightly to the needs of
farmers through planned disease prevention and control programmes and manage­
ment for optimal health. The application of these services is rarely an all-or-nothing
affair. Usually several measures or programmes are available, each of them offering
a different degree of protection and requiring a different level of investment.
Determining the optimal input level, therefore, is to a large extent a matter of economic decision making. This is not only the case for the individual livestock owner, but also for a national government that must determine the optimal policy against specific infectious diseases.

The scientific foundation for the discipline of animal health economics was laid about 25 to 30 years ago in Australia [2] and England [3]. The two authors successfully introduced a simple but essential economic principle in making veterinary decisions: disease control input should be increased to the level where the cost of an additional input equals the return from the additional output. This so-called 'equimarginal principle' can be considered a fundamentally different approach from the previously more common opinion of disease control being an all-or-nothing affair. Since then, increasing effort has been made to apply this principle in the various areas of animal health economics.

In this paper a basic economic framework of livestock disease is first presented. Then the most common modelling techniques for this type of analysis are summarized and illustrated with applications on three levels of veterinary decision making: animal, herd and national levels. Future directions in animal health economics are also discussed.

2. ECONOMIC FRAMEWORK OF LIVESTOCK DISEASE

The basic conceptual model underlying economic analyses includes three major components: people, products and resources [4]. It is people who want things and make decisions, therefore providing the driving force for economic activity. Products are goods and services that satisfy what people want, and may be regarded as the outcome of economic activity. Resources are the physical factors and services that are the basis for generating the products and, as such, the starting point of economic activity.

Animal disease in this context can be considered an influence which affects the transformation process of resources into products and causes extra resource use and/or less production than before. These effects may be immediately visible (death, abortion) or obscured (reduced weight gain). To express the physical effects in economic terms, the 'value' of products and the 'cost' of resources are required. The idea of value is not intrinsic in any product or service but is determined by people's request for the products, and is relative to its availability ('supply and demand'). Economics attempt to deal with the 'real value' of any product, which may or may not be accurately captured by its recorded price. Similarly, the idea of cost comes from the resources that are used in making a product available. This underlies the definition of the 'real cost' (or 'opportunity cost') of resources, which reflects the potential benefits that are given up because one option is chosen
over another, and again may not be adequately reflected by financial expenditures incurred in its production. Both ‘real value’ and ‘real cost’ — and hence the losses due to one and the same disease — may differ considerably across the various economic levels to be considered, i.e. the individual farmer, the joint livestock owners, the consumers and the national economy [5].

In the case of the more common (and endemic) diseases that the individual farmer can control, such as mastitis, supply and demand force the market prices of animal products to move over time with the average disease level. Thus the resulting losses are transferred to the consumers, and conversely it is the consumer who benefits from improved animal health. In a sufficiently large market (such as the European Union) there is hardly any relationship between the extent and seriousness of these diseases on the one hand and the average income of the joint livestock owners on the other. However, for the individual farmer this linkage does exist. A particular farm may suffer more (or less) from disease than is compensated by the average ‘disease margin’ included in the market price. To a lesser extent this also applies to a group of livestock owners.

In the case of an epidemic of infectious disease (e.g. foot-and-mouth disease; FMD), market prices of outputs will depend primarily on whether or not foreign trade restrictions apply. When an outbreak does not lead to export bans, the market prices may temporarily rise a little, depending on the spread and duration of the outbreak. If exports are restricted, however, prices in countries with large export markets will drop substantially owing to an oversupply of the domestic market. This fall in prices causes losses which may greatly exceed the direct losses of the disease due to mortality, for instance. Unaffected farms also suffer from this drop in market prices. Consumers will benefit, however, making the losses for the national economy considerably less than those for the joint livestock owners. Income transfers between consumers and producers, namely, are not relevant to the assessment of national economic losses [6, 7].

The calculation of the losses is, in itself, not very important but can help provide a better overall view of the impact of disease and can contribute to estimating the extent of the losses to be avoided. The latter is particularly the case if the spread of losses between farms (or countries) is indicated in addition to the losses in the average situation. The accuracy of the outcome depends to a large extent on the availability and reliability of the underlying data. But even with enough data available it is not a simple task [8], because the effects of disease: (1) are not always obvious and pronounced, (2) are influenced by other factors such as nutrition and housing, (3) have a temporal dimension which adds to the complexity of determining their impacts at different stages in time, and (4) often manifest themselves in combination with other diseases. This may help explain why the outcome of calculations often differs so much, even for similar farm and price conditions [9].
3. COMMON MODELLING TECHNIQUES IN ANIMAL HEALTH ECONOMICS

There is a wide range of modelling techniques available to help perform economic analysis of animal diseases and their control [10]. The choice of a modelling technique will depend on a number of factors, such as (1) the nature of the problem, (2) the resources available (time, money and analytical tools) and (3) the availability of data on the problem. A first choice to be made is between static and dynamic models. A static model does not contain time as a variable and, therefore, cannot analyse or simulate the effect over time, as opposed to a dynamic model. A model that makes definite predictions for quantities (such as milk production and live weight) is called deterministic. A stochastic model, on the other hand, contains probability distributions and/or random elements to deal with uncertainty in prices and performances. With random elements, repeated runs of the model ('replicates') are necessary to provide insight into the variation in outcome. A final difference to consider concerns optimization versus simulation. An optimization model determines the optimum solution given the objective function and restrictions, whereas a simulation model calculates the outcome of predefined sets of input variables (scenarios, strategies).

If the proposed analysis concerns a simple economic comparison of disease control measures on a farm, and the outcome does not involve a specific time pattern nor a great degree of chance (i.e. being neither dynamic nor stochastic), then partial budgeting is the method of choice. Partial budgeting is simply a quantification of the economic consequences of a specific change in farm procedure and requires the simplest data collection of all the methods. It is particularly useful in analysing relatively small changes within the production process, e.g. the effect of dry cow therapy to control mastitis. The general format for a partial budget is made up of four sections [11]: (1) additional returns realized from the change, (2) reduced costs as a result of the change, (3) returns foregone as a consequence of the change, and (4) extra costs incurred owing to the implementation of the change. The change should be adopted if the sum of (1) and (2) is greater than that of (3) and (4). An example of a partial budget is presented in Table I, quantifying the economics of a Caesarean section in dairy cattle [12]. The net result is negative in this case. Would the calf otherwise die, then its value should be included as additional returns and the net result may become positive.

If the subject of research deals with more long term disease control programmes at regional or national level, then cost–benefit analysis is typically the analytical structure of choice. Cost–benefit analysis is a procedure for determining the profitability of proposed courses of action over an extended period of time [13]. Since the time at which costs or benefits occur may differ between the alternatives, it is important that these future costs and benefits are 'discounted' to make them...
TABLE I. ECONOMICS OF CAESARIAN SECTION IN DAIRY CATTLE
(amounts in US $)

<table>
<thead>
<tr>
<th>Additional returns</th>
<th>25</th>
<th>Returns foregone</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>— heavier weights of calves</td>
<td></td>
<td>— drop in milk production</td>
<td></td>
</tr>
<tr>
<td>Reduced costs</td>
<td>35</td>
<td>Extra costs</td>
<td>160</td>
</tr>
<tr>
<td>— feed (less milk production)</td>
<td></td>
<td>— surgery and culling</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>Total</td>
<td>190</td>
</tr>
</tbody>
</table>

Net result: $35 - 190 = -155$

completely comparable, which results in the present value of costs and benefits. The reason for discounting is the time preference of money. A benefit of US $100 to be received in one year has less value today than a benefit of US $100 received immediately, because of (potential) interest yields. The formula used to calculate the present value (PV) of a future cost or benefit (FV), where r is the annual ‘interest rate’ (in %) and n is the number of years in the future, is given by

$$PV = \frac{FV}{(1 + \frac{r}{100})^n}$$

The ‘interest rate’ used in cost–benefit analysis is called the discount rate, since it makes future values smaller than present values. The higher the discount rate the more a programme with high initial costs and a low level of benefits over a long period of time will be penalized. Conventionally, the discount rate does not allow for inflation of prices, and future prices are calculated at current prices instead of inflated prices. This avoids the difficulty of predicting future inflation rates, which would in any case have no effect on the real rate of return from the programme under consideration. This discount rate then includes the so-called real rate of interest, being the difference between the market rate of interest and the inflation rate. An example of the cost–benefit approach is presented in Table II, using a real interest rate of 5%. As can be seen from Table II, the undiscounted benefits exceed the undiscounted cost in this example, whereas discounting induces the opposite.

If there are multiple possible outcomes of the proposed courses of action and chance is an important factor in determining which outcome occurs, then one of the various forms of decision analysis is the approach of choice, with riskiness of decision being taken into account [14]. Decision tree analysis is probably the most frequently used technique of decision analysis. A decision tree is defined as a graphical method of expressing, in chronological order, the alternative actions available to
TABLE II. EXAMPLE OF COST–BENEFIT ANALYSIS

<table>
<thead>
<tr>
<th>Year</th>
<th>Discount factor</th>
<th>Undiscounted Costs</th>
<th>Undiscounted Benefits</th>
<th>Discounted Costs</th>
<th>Discounted Benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28</td>
<td>0</td>
<td>26.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>0.91</td>
<td>15</td>
<td>10</td>
<td>13.7</td>
<td>9.1</td>
</tr>
<tr>
<td>3</td>
<td>0.86</td>
<td>10</td>
<td>20</td>
<td>8.6</td>
<td>17.2</td>
</tr>
<tr>
<td>4</td>
<td>0.82</td>
<td>0</td>
<td>25</td>
<td>0.0</td>
<td>20.5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>53</td>
<td>55</td>
<td>48.9</td>
<td>46.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> 0.95 = 1/(1 + 5/100).
<sup>b</sup> 26.6 = 0.95 x 28.

the decision maker and the choices determined by chance. An example is presented in Fig. 1. Choices such as whether or not to vaccinate are represented by squares called decision nodes. Chance events or states of nature, such as response to treatment, are represented by circles called chance nodes. The branches following each decision node must be exclusive, that is, they must include all possible outcomes, and the outcomes must be mutually exclusive. After each chance node, there is a probability (P<sub>j</sub>) that an event occurs. The probabilities following a chance node must add up to 1.00, and can be taken from literature, experimental data or expert opinion.

**FIG. 1.** Hypothetical decision tree representing action choices (strategies A1, A2, A3), states of nature (outbreaks in north, south, east and west), associated probabilities and monetary values of outcome.
The expected value of outcome for each action \((V_{ij})\) is entered at the far right of the tree branches. The decision is then usually based on the expected monetary value (EMV). The EMV for each action \((A_i)\) would be \(EMV(A_i) = \sum_j (P_j V_{ij})\), with the highest EMV being preferred. In the case of the example in Fig. 1 the EMV of strategy \(A_1\) is the highest, i.e. 98 against 66 for strategy \(A_2\) and 57 for strategy \(A_3\).

If there are complex feedback loops whereby the effect of one decision about the control of the disease flows through to influence some aspect of animal production, which in turn flows back to influence a variable further back in the production system, and there is substantial uncertainty about the precise effects, then the problem is becoming sufficiently complex that systems simulation will be the method of choice. This method can best be described as creating a mathematical model of the system under consideration (e.g. animal, farm, population), which can then be manipulated by input modification. It is especially attractive where real-life experiments would be impossible, costly or disruptive (e.g. with highly infectious diseases), and to explore strategies that are not applied yet ('what ... if' calculations). Special attention has to be paid to the correspondence between the model and reality to obtain meaningful results for real-world situations. Dent and Blackie [15] consider six critical — and interlinked — steps to be involved in applying modelling in general and systems simulation in particular. A clear description of the system and statement of the reasons why the system simulation work is being carried out is an essential first step, as indicated in Fig. 2. In the modelling context, a system is commonly described as a set of related components which exist within some predefined boundary and react as a whole to external and internal stimuli. Placing the boundary is considered the key issue in defining and structuring any system and should depend primarily on the function the model has to fulfil. Validation is another very important but difficult step in the modelling procedure. The key issue here is to judge whether or not the model mimics the real system sufficiently well to fulfil the purposes for which it has been developed. If a model is considered ‘valid’, then the outcome of the model should be similar to those resulting from physically experimenting with the system (if possible). If a model is not valid, then any conclusions derived from it will be of doubtful value. One of the most powerful techniques in using systems simulation is sensitivity analysis, in which the values of relevant parameters are systematically varied over some range of interest to determine their impact on the results. Good knowledge of sensitive parameters should be available and entered into the model. If not available, sensitivity analysis can help to set priorities for further (empirical) research. In this way a valuable interaction between systems simulation and field data analysis is possible. Systems simulation may be used to quantify the significant gaps in (veterinary) knowledge, while knowledge obtained from field data research increases the contents of economic models. This interaction is considered fundamental to the study of animal diseases and their control.
4. APPLICATIONS IN THE AREA OF ANIMAL HEALTH ECONOMICS

An increasing number of applications of the various economic modelling techniques are becoming available in the area of animal health economics. Three levels of veterinary decision making are being addressed in these analyses: the animal, herd and national level. Major type of outcome and issues for further research will now be indicated for each of these levels.
4.1. Animal level

Decisions as to whether or not to treat individual animals are most often evaluated using decision-tree analysis [16, 17]. This technique can easily include probability distributions of possible outcomes as indicated before, e.g. with respect to failure or success of a treatment. The difficulty, however, is that the technique itself is not able to determine the economic value to include in case of premature disposal of the animal. Applications differ widely in this respect, making it difficult to interpret and compare their outcomes. Many applications take the slaughter value of the animal to be included, while others choose to use the cost of purchasing a pregnant replacement, sometimes adjusted by the salvage value. What should be included is the value that equals the difference between (1) the income that the animal could earn during her remaining expected life, had the reason for replacement not presented itself — given normal probabilities of disposal due to other reasons — and (2) the expected income over the same period of time of replacement animals with normal productive qualities and normal probabilities of disposal [18]. This value is called retention payoff (RPO), and enables ranking of individuals within the herd on their expected future profitability: the higher the RPO, the more valuable the animal. A value below zero means that replacement is the most profitable choice.

From an economic point of view the RPO value is important to know, as health and production control decisions at the animal level all include replacement as one of the alternatives. Dynamic programming is considered the appropriate method to calculate these values, as it provides a systematic procedure for determining the combination of decisions (as expressed in a decision tree) which maximizes overall effectiveness [19]. With respect to applications in livestock economics it allows nonlinear relationships, genetic improvement, seasonal variation and variation in expected performance of both the present and all subsequent replacement animals to be included. Extensive applications have been made in dairy cows [20–22] and sows [23]. RPO values can now be made available for animals that differ in age, productive capacity and reproductive status. These values represent the maximum amount that should be spent in trying to keep the animals in the herd. The analyses also provide insight into the critical production level below which an animal should be culled and replaced anyway, apart from any disease (i.e. RPO < 0). For Dutch conditions it was found that such a selection on insufficient productive capacity should be significantly stronger in cows than in sows (see Fig. 3). The key factor here is the repeatability of performance across parities, which is much higher for milk production than for litter size. Reproductive performance, on the other hand, is economically far more important in sows than in cows. The cost of a one day delay in conception, for instance, reduces annual sow income by about 1% against 0.1 to 0.3% in cows [24]. So, culling on reproductive failure should be significantly stronger in sows than in cows. This means that fewer breedings are allowed in sows before the RPO index falls below
FIG. 3. Critical production level at which cows and sows should be culled, apart from any disease (i.e. $RPO < 0$).

zero and replacement becomes the more profitable option. Research is under way to actually integrate health problems into the dynamic programming approach. Mastitis in dairy cattle, commonly recognized as the cause of considerable losses in many countries, is the first health factor that has been included [25].

4.2. Herd level

Throughout the world, several field trials have been conducted to investigate the economic attractiveness of what is called herd health and management programmes. These programmes usually include a wide range of herd health and fertility management issues, and are built around farm advisory visits. Data that have become available from these trials were often not very detailed with respect to disease incidence and economic performance, making it impossible to go beyond partial budgeting analysis. Moreover, very few trials were designed so as to be able to conduct a sound economic analysis. Such an analysis requires data from both the 'with' and 'without' groups, both by collecting data from 'before' (b) and 'after' (a) application of the intervention programme and by collecting data on farms participating in the
programme (P) and on comparable control farms (C). When available, these data make it possible to estimate the causal effects of the programme more precisely, i.e. \((P_a - P_b) - (C_a - C_b)\), especially when particular herds with obvious health problems take part in the programme.

In the Netherlands, the most appropriate field trial for an economic analysis was carried out on dairy farms from 1974/1975 to 1976/1977 [26]. A total of 30 programme farms and 31 control farms were included, none of them showing specific herd health problems. In the preparatory year (1974/1975), the two groups showed no large differences in economic results. After two years of programme application statistically significant improvements were found, both regarding fertility (calving interval) and replacement due to reproductive failure and ill health. Regarding udder health (i.e. cell count) no significant effect was found. The average increase in gross returns minus feed cost per cow turned out to be US $256 in the programme group, which was US $98 more than in the control group. Additional veterinary costs per cow were estimated to average US $20 at the most, indicating this herd health programme to be a sound investment. From these farms data were gathered for 10 years after initial participation in the programme experiment to see whether the effect on income had been sustained [27]. The necessary data were not available on all 61 farms and hence two new groups were formed, consisting of 15 programme farms and 20 control farms. Results are summarized in Table III. In the new groups the short term programme effect was smaller (US $65) and not statistically significant, but had the same tendency. In the first few years after the programme, gross returns minus feed cost per cow increased significantly more on the control farms, as a result of both higher milk production and lower feed cost. In the period 1980/1981 to 1985/1986 the

<table>
<thead>
<tr>
<th></th>
<th>Initial farms</th>
<th>Remaining farms</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>31C</td>
<td>15P</td>
</tr>
<tr>
<td>1976/1977</td>
<td>1426</td>
<td>1351</td>
<td>75</td>
</tr>
<tr>
<td>1980/1981</td>
<td>599</td>
<td>575</td>
<td>24</td>
</tr>
</tbody>
</table>

\(a\) \(p < 0.05\).
increase in income for both groups was almost the same, i.e. US $599 and US $575 per cow per year, respectively. So, the effect on income had disappeared over time, which may lead to the conclusion that such programmes had better continue to maintain profitability.

To really optimize this type of veterinary input on farms according to the equimarginal principle mentioned before, much more detailed information is required [28]. Actually, the current single-point type of results (i.e. with and without intervention) needs to be extended to include a wider range of possible contents and frequencies for the various programmes and/or control measures. Field trial alone will not provide this kind of information, being too costly and time consuming. Systems simulation is an appropriate alternative. PC based models have become available that are able to imitate closely real farm conditions, using either Monte Carlo simulation [29, 30] or dynamic probabilistic modelling techniques [24]. These models are able to simulate the extent and dynamics of the production process in considerable detail, and can account for uncertainty in herd performance and prices. By entering farm specific data into these models, it is possible to explore management decisions and strategies tailored to individual farm conditions. Increasing efforts should also be made to model farms within a production chain as a whole and optimize the overall chain results [31]. This becomes especially important where consumers put an increasing demand on product quality, food safety and animal welfare.

4.3. National level

Outbreaks of infectious animal diseases are understandably feared, especially in major exporting countries. Control of this type of disease goes beyond the range of influence of the individual farmer, and needs to be carried out at the national or even international level. Decisions on what strategy is best to apply are highly subject to uncertain conditions, especially with respect to the risk of outbreaks and foreign trade restrictions. Empirical data (if any) are scarce, making computer simulation the obvious method of economic analysis for this type of disease. To make economically sound decisions, an integrated modelling approach is required that simulates the effects of different conditions and scenarios considering (1) the spread of the disease, (2) the direct cost of prevention and eradication and (3) the indirect effects due to export bans.

Economic analysis at the national level is not a simple addition of analyses performed at the farm level. The approach is entirely different, and based on principles that come from what is called ‘macro-economics’. A key issue in this approach is the theory of demand and supply and the way these interact [32]. Figure 4 shows the demand curve (D) and the supply curve (S) for a country exporting a certain product. At the basic price level $P$, producers supply amount $Q_s$ while consumers demand amount $Q_d$, with the difference $(Q_s - Q_d)$ being exported. When export bans occur, a
A new equilibrium will arise at a lower price level, influencing the welfare of both producers and consumers. Assuming producers strive for maximum profits in competitive markets, the supply curve (S) is the same as the rising part of the so-called marginal cost curve, indicating the costs of an additional unit of output. The return to fixed inputs, therefore, is formed by the gross returns (quantity times price) minus the variable costs (the area under the supply curve) and is commonly called producer surplus. Consequently, the losses for the producers due to a drop in price from $P$ to $P'$ is the reduction in producer surplus (area $PFCP'$). In the short term, a large part of the costs are fixed and the supply curve will be steep. With short lived disease outbreaks, therefore, the vertical supply curve ($S'$) can be used to quantify the losses in producer income. Actual losses for the producers are reduced by any compensation paid by the government. Consumers gain from a drop in price, indicated by the increase in consumer surplus. The consumer surplus is defined as the difference between the willingness to pay for a certain quantity and the amount actually paid, illustrated in Fig. 4 by the area under the demand curve and above the price line (area $PGBP'$). From the alternative demand curve ($D'$) it can be seen that the slope of the curve (i.e. the price elasticity of demand) is not very important for the calculation of an increase in consumer surplus. Within the theory of welfare economics, there is discussion about the aggregation of benefits and costs at the national level [33]. Simple aggregation of these effects presumes an equal weight of benefits and costs for each group and individual, which is usually not the case. It is, therefore, recommendable to report both the separate effects and their equally weighted total, leaving policy makers the opportunity to include their own weights.

This basic principle for determining the indirect losses of export bans has been worked out for FMD, and focused on the decision whether or not to stop annual vaccination of cattle [6]. The various markets for meat and breeding cattle were described by the volume of export, the level of consumption, the price elasticity of demand and
the transport costs per unit of product. In calculating the indirect effects, it was also necessary to specify what reactions to expect in importing countries in case of an FMD outbreak in the Netherlands. Moreover, the possibility of temporarily increasing export to other markets as well as the storage capacity and behaviour of traders had to be specified. Results showed that the indirect losses, indeed, are many times greater than the direct costs, although they did not influence the economic ranking of the various strategies in this case (see Table IV). Strategies without annual vaccination were found to be the most preferable for the Netherlands, despite their higher outbreak costs. These higher outbreak costs were outweighed by savings on the yearly vaccination, a reduction in expected frequency of primary outbreaks (i.e. once every five years in a vaccinated population and once every ten years in a non-vaccinated population) and extra profits of export to markets which because of vaccination would have remained closed. These findings support the decision of the European Union to cease the annual vaccination against foot-and-mouth disease from 1 January 1992. Such a non-vaccination policy increases the importance of reducing the risk of introduction [34] and of optimizing the control measures when an outbreak does occur [35].

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Vaccinated population</th>
<th>Non-vaccinated population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ia</td>
<td>Ib</td>
</tr>
<tr>
<td>Number of herds removed</td>
<td>33</td>
<td>27</td>
</tr>
<tr>
<td>Direct losses (million US $)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Weeks with market disruption</td>
<td>60</td>
<td>58</td>
</tr>
<tr>
<td>Indirect losses (million US $)</td>
<td>124</td>
<td>113</td>
</tr>
<tr>
<td>of which:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>— producer losses</td>
<td>195</td>
<td>179</td>
</tr>
<tr>
<td>— consumer losses</td>
<td>-71</td>
<td>-66</td>
</tr>
<tr>
<td>Total losses (million US $)</td>
<td>128</td>
<td>117</td>
</tr>
<tr>
<td>Annual losses (million US $)</td>
<td>39</td>
<td>37</td>
</tr>
</tbody>
</table>

*Stamping-out of infected farms (Ia and Iia); stamping-out of infected farms plus ring vaccination (Ib and Iib); stamping-out of infected and serious contact farms (Iic).*
The management of animal health is becoming increasingly important in modern livestock farming. A critical aspect of good management is to make the right decisions. The process of decision making is commonly described in five steps [11]: (1) define the problem or opportunity, (2) identify alternative courses of action, (3) gather information and analyse each of the alternative actions, (4) make the decision and take the action, and (5) evaluate the outcome. Current animal, herd and national disease information systems are restricted mainly to data recording and analysis, which especially support step 1 and partly step 2 of the decision making process [36]. Research in animal health economics focuses on the development of models that allow for the evaluation of alternative decisions and strategies, as illustrated in this paper, supporting primarily steps 3 and 5. Economic models can in the first place be helpful at a very early stage, when almost no data are available yet. Calculations have then to rely on estimates of input values, to be obtained from experts and/or practical experiences, but these can systematically be varied to determine their impact on the results (sensitivity analysis). Such preliminary outcomes can be very helpful in setting priorities for further veterinary research. Once available, more reliable input values can be entered into the model to increase the realness of the outcome. This is an on-going process until no further significant improvement is made. A major challenge in future research is to integrate these models (where appropriate) into existing information systems, making them more accessible for actual use in veterinary decision making at the animal, herd and national levels. These systems in general and the economic models in particular should be flexible in their structure, and suitable to be tailored to individual farm and price conditions. That also opens the possibility for a sound international exchange and application. Once available, they can provide a solid and uniform basis for a mutual comparison of the economics of animal diseases and their control, and may also be a starting point for further (and joint) research. Experience gained so far in this respect with Dutch models is promising [37].

REFERENCES


VETERINARY SCIENCE IN THE CONTEXT OF SUSTAINABLE LIVESTOCK PRODUCTION

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Abstract

Sustainability of current livestock production systems, including the influence of livestock on the environment and the preservation of animal genetic resources, has been a constant debate over the last decade. For the purpose of the paper six elements that offer a useful approach to characterize sustainability have been identified: continuation through time, system oriented, quantitative, predictive, stochastic and the use of an integrated measure of sustainability to both identify and prioritize technical, social and economic constraints.

1. INTRODUCTION

With sharply increasing demands for animal products in many developing countries and the potential pressure of livestock production exceeding the carrying capacity of the environment, it is of critical importance that future production sustains the quality of the global land, water, air, and plant and animal genetic resources [1–3]. To provide a basis for further analysis, this paper starts by looking at the requirements that must be met in defining sustainability, given the fact that at least 15 definitions exist [4].

A systems approach is a prerequisite for the study of sustainable livestock production, because sustainability can only be used as a relevant criterion for evaluating technology when the system is clearly defined, including its boundaries, components, temporal and spatial scales and context in hierarchy [4]. Steinfeld and Mäki-Hokkonen [5] classify 11 different livestock systems, based on agro-ecological zones and land use. Systems are, however, not static and the interaction among systems is often complex and without properly fixed boundaries [6–8]. Predictions about the direction in which systems will evolve in time are, therefore, difficult [8].

Several factors have led to misconceptions about the roles of livestock and will be briefly discussed in this paper. We will not discuss in detail all the strategies and technologies for sustainable livestock production that have been described in recent
years. Where appropriate, we will indicate the contributions that could be made by veterinary science.

A great challenge for animal scientists is to express sustainability in quantitative terms, especially because in addition to ecological criteria also social and economic criteria should be included [9].

2. SUSTAINABILITY

2.1. Definitions of sustainability

'Sustainability' has become a 'buzz-word' and is used in many connections. If not precisely defined it loses much of its meaning, certainly if it is used as a strategic tool. The FAO definition [10] states that sustainability comprises: "The management and conservation of the natural resource base, and the orientation of the technological and institutional change in such a manner as to ensure the attainment and continued satisfaction of human needs for present and future generations. Such sustainable development conserves land, water, and plant and animal genetic resources, is environmentally non-degrading, technically appropriate, economically viable and socially acceptable."

This definition is rather broad. Recently, sustainability in relation to agriculture has been discussed by Hansen [4]. He classified the meaning of sustainability, as applied to agriculture, according to the motivating concern:

(1) Sustainability as an ideology. In this context sustainability is often described by its contrast to conventional agriculture. Some aspects of ethno-veterinary medicine will fall within this category.

(2) Sustainability as a set of strategies. Specific strategies are suggested by ideological interpretations of sustainability. The strategy most frequently linked to sustainability is reduction or elimination of the use of chemotherapeutics and insecticides. Traps and targets for tsetse control would positively fall in this category.

(3) Sustainability as a set of goals. These goals generally include some expression of maintenance or enhancement of the natural environment, e.g. game exploitation and provision of human food needs: food safety and security. Economic viability and social welfare are also important goals and closely linked with the socioeconomic role of livestock [8].

(4) Sustainability as the ability to continue. This interprets sustainability as a system's ability to continue through time. Veterinary science could contribute in this respect by identifying constraints (diseases and disease risk factors) and evaluating proposed approaches for their control.
Using this classification, Hansen [4] identified 15 different definitions of sustainability. He selected six elements that offer a useful approach to characterizing sustainability of agricultural systems, i.e. continuation through time, system oriented, quantification, prediction, stochastic and the use of an integrated measure of sustainability to identify and prioritize technical, social and economic constraints.

It is interesting to note that the ideas of Hansen [4] largely coincide with the concept of sustainability as developed within the section Animal Production Systems (APS) Section of the Wageningen Institute of Animal Sciences.

2.2. Agro-ecosystem health

An illustrative way to elucidate the role of veterinary science in the discussions on sustainability is through the ecosystem health approach. Not because it gives a completely new, 16th definition of sustainability, but because of its strong veterinary and medical methodology [11, 12].

Waltner-Toews [13] states that the language and concepts of health and health care provide us with the most appropriate framework for implementing sustainability in agricultural systems. Ecosystem health tries to accommodate “the social aspirations and goals of a system together with the biological flourishing of species sharing the system and integrates agricultural economics with social dimensions”.

This approach may have a great appeal for research workers and students trained in a medical discipline who are familiar with the links between epidemiology and economics, or the relation between social customs, food security and foodborne diseases [14]. In our opinion, however, it is just as well represented by the FAO definition, Hansen’s analysis and the system concept. The plea of agro-ecosystem health to integrate value judgements from the social and economic sciences with objective technical measurements is valid and necessary within the agro-ecological framework [15]. A similar plea was held in the 1970s in promoting Farming Systems Research [16].

3. SUSTAINABILITY AND LIVESTOCK PRODUCTION SYSTEMS

Sustainability should be discussed in relation to specific livestock production systems. The central idea of the systems approach is that one must understand a system before it can be influenced in a predictable manner. The relevance of the interventions has to be assessed within the context of the production system. Zero grazing may be excellent for the control of ticks and internal parasites, but the increased labour requirements may conflict with existing cropping patterns and household activities. Conflicting interests in and among systems (e.g. centrally organized disease control) can so be made explicit by a systems approach including disease surveillance [17].
In the tropics, we find a wide range of agro-ecological and socioeconomic production environments. The majority of farmers in the tropics are found in the low external input systems and only ask from the veterinary services 'risk avoidance', i.e. prevention and possible cure of diseases.

A systems approach further implies that an entity (usually the farm household) is studied on the basis of different interacting components. Each household is not treated as an independent isolated entity but as part of a regional system and certainly with livestock production influenced from outside the system boundary, e.g. infectious diseases, supply of fodder, price policy, etc. [8, 17].

Peters [7], in discussing the trends in livestock production in the tropics, foresees an expansion of non-ruminant production and a shift from backyard to intensive systems. This development may be restricted by socioeconomic and physical constraints, and each step requires specific technologies and policy measures.

With intensification of the production, management or production diseases become increasingly important. They are multifactorial in aetiology, and solutions are based on the application of known technical solutions such as from epidemiological studies [17].

Steinfeld and Mäki-Hokkonen [5] describe eleven different livestock systems based on agro-ecological zonation. The most important health constraints in the five ecological zones in the tropics are given in Table I [17, 18].

Animal health, mainly the vector borne diseases and helminths in sub-Saharan Africa, is still a top priority in this area, but less so in other regions of the tropics [7].

Zwart and de Jong [17] indicate that the level of detailing herd performance indices for arid and semi-arid pastoral and mixed systems is much lower than for specialized dairy systems. The former therefore require far less veterinary inputs, but when the systems exist side by side this can lead to controversies in disease control.

It also may be rather difficult to collect sufficient data that are reliable or representative for a particular livestock system of smallholders. Nevertheless, performance

<table>
<thead>
<tr>
<th>TABLE I. ANIMAL HEALTH CONSTRAINTS IN DIFFERENT ECOLOGICAL ZONES</th>
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<tbody>
<tr>
<td>Constraints</td>
</tr>
<tr>
<td>Epidemic infectious diseases</td>
</tr>
<tr>
<td>Vector borne diseases</td>
</tr>
<tr>
<td>Parasitic diseases</td>
</tr>
<tr>
<td>Production/management diseases</td>
</tr>
</tbody>
</table>

+ — some importance; ++ — moderate importance; +++ — high importance.
data from small farms can be related to the level of input of veterinary services, dairy societies, management practices, etc. [17].

Technology alone, however, is not the ultimate answer. Peters [7] suggests that in the future there will be a global shift from the primary emphasis on a technology towards an institutional and organizational process awareness. This should lead to identification of system constraints on the basis of which more appropriate technologies will be developed, delivered and applied.

4. MISCONCEPTIONS AND ALTERNATIVES IN LIVESTOCK PRODUCTION

The difficulty in operationalizing sustainability, the conflicting interests within and among systems, the change in time of the systems and the often missing link between technical and socioeconomic innovations have led to many misconceptions about the role of livestock in the world and the role of various disciplines in the sustainability debate. The underlying concepts have evolved in the last years in the APS Section in Wageningen, and the possible alternative strategies for sustainable livestock production are given here to provoke comments and discussion.

4.1. Food security and other roles of livestock

The animal protein gap is not relevant for the 800 million people suffering from hunger. The major world food problem is an energy (carbohydrates) deficiency. Animal protein is not the first necessity of life, except for small children and nursing mothers. The demand for animal protein is very much related, however, to economic development, especially in the growing urban centres [7, 17].

It is even doubtful whether animal products will improve the nutritional status of the farming household, although the household members sometimes are forced to consume their home produced eggs or milk owing to market failures.

The important function of livestock lies in the fact that it provides more than only food: draught power, manure and fibres. Another important motive for keeping livestock is its function of capital asset, as illustrated by Bosman et al. [19] for goat keeping in south-western Nigeria. The financial returns from labour invested in goats were far below those from cocoa or even cassava. The goats, however, enabled farming households to meet unexpected expenditures. On that basis Bosman et al. [19] developed a concept to value the insurance and financing benefits. It then became clear why farmers kept goats: because in Nigeria these functions were four times more important than the meat production function so that the benefits from goats per unit of labour were comparable to those from crops.
TABLE II. VALUE OF THE PRODUCTION OF CATTLE PER FARM HOUSEHOLD, MALANG AREA, INDONESIAa

<table>
<thead>
<tr>
<th></th>
<th>Value per annum (Rp. x 10^2 b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progeny</td>
<td>133</td>
</tr>
<tr>
<td>Insurance</td>
<td>57</td>
</tr>
<tr>
<td>Manure</td>
<td>44</td>
</tr>
<tr>
<td>Weight gain</td>
<td>44</td>
</tr>
<tr>
<td>Draught power</td>
<td>17</td>
</tr>
<tr>
<td>Financing</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Expenditure</td>
<td>-3</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Total livestock income</td>
<td>305</td>
</tr>
</tbody>
</table>

a Taken from Ref. [8].


Similar conclusions were reached when Ifar [8] studied the role of cattle in mixed farming systems in East Java, Indonesia (Table II). Weight gain is here even less important than insurance.

The various goals can even be conflicting: selling an animal for urgent cash needs may not coincide with the optimal moment from a meat production or breeding perspective.

Research, development, education and training are almost exclusively focused on biological production. The alternative (manure, draught power, weed control) and intangible (finance and insurance) benefits, on the other hand, are very much neglected, while all these support human welfare.

These various functions of livestock are affected by the prevailing socio-economic conditions, and any change in the latter will influence the livestock system.

The socioeconomic environment has relatively more impact on livestock than on crops because livestock represents a much stronger buffering capacity than crops for adverse physical environments in developing countries.

4.2. Measuring productivity

Productivity refers to the relation between inputs and outputs. Exotic animals and technologies from industrialized countries very often do not give the desired results in developing countries because of the high inputs required, and the hidden
costs are often borne by the taxpayer. However, animal systems with hardly any inputs cannot be automatically classified as low productivity systems, because the relation between inputs and outputs is the relevant criterion. De Wit et al. [20], therefore, consider productivity as a poor criterion to compare different systems, because each system requires its specific unit, e.g. production per animal, per kilogram metabolic weight, per hectare, per kilogram nitrogen, unit of energy, etc.

Within a system, productivity depends upon many determinants and it is epidemiology which studies and quantifies their interrelation. Moreover, epidemiology can help in making explicit the costs for the control of disease risk factors.

Hursey and Slingenbergh [21] used a Geographic Information System (GIS) to describe the effects of the tsetse fly on agriculture in sub-Saharan Africa. They state that it is now generally appreciated that strategic planning for tsetse and trypanosomiasis (or any other disease for that matter) cannot be undertaken within the narrow framework of animal health, nor, indeed, confined to the livestock sector alone. It requires an understanding of resource potential, environmental implications and farming systems and constraints, as well as consideration of the dynamics of population growth and food demand over time.

A weak point of productivity assessment always remains the expected economic benefits, because intermediate and intangible benefits are usually not included and the price of the products can vary greatly in time. It is generally assumed that the control of infectious and epidemic diseases is a prerequisite for economic livestock development. In recent articles on the strategy of the FAO for animal health care, on the other hand, very little attention is given to the economic returns of disease control [22, 23].

4.3. Natural environment versus external inputs

Another misconception is that all environmental constraints can be removed by either housing, vector control, vaccination, drugs or improved nutrition. This may be true for high external input systems. However, in low external input systems it seems more promising in the future to make increasing use of the inter- and intra-breed variations to match animals with the environment. The study of genetic markers may greatly enhance the use of disease resistant animals [24]. This should go hand in hand with simple ‘bedside tests’, for either disease diagnosis, epidemiological surveys, disease control or eradication campaigns.

There is also growing pressure on the abundant use of vaccines and even more so of drugs, which is partly due to market demands and public concern, but also has a strong veterinary basis. Wentink [25] even expects that for intensive dairy herds in the European Union, vaccines will be completely abolished, so that the herds must live without a solid immunity against most infectious agents. Strict hygiene, health certification procedures and a closed herd should make the use of (marker) vaccines
necessary only in extreme circumstances [25, 26]. For the near future, however, vaccines and drugs, but more and more in combination with genetic resistance, will still play a major role in the tropics.

The trend described in the European Union can, however, hamper the trade in animals and animal products between developing and developed countries.

4.4. Prime movers

Another controversy is whether technical innovations are the prime movers for higher animal production, or whether producer friendly policies initiate the demands for new technologies.

De Jong [27] in his study on dairy development in developing countries considers that price policies and investment support, as part of an overall livestock policy, are in this respect more important than technological innovations. For the Sahel region, Breman [28] concludes that efforts to create more favourable socio-economic conditions will be more useful than those directed towards the direct adoption of technical options by farmers.

A free market system, although preached by many donor agencies, is certainly for the time being not favourable for livestock development [27].

Adoption of new technologies requires time, as seen, for example, in Indonesia, where despite a favourable milk price for the producers it takes time before they have adopted all the ins and outs for profitable milk production [29]. There can be no doubt, however, that a favourable price policy determines the success or failure of technical innovations [7].

The IAEA, which is strongly technology driven in its research, should therefore, in our opinion, carefully consider whether their findings are truly relevant for the livestock systems it wants to address.

4.5. The role of extension

The ideal symbiosis among research, extension and farmers is mainly found in high external input systems or when really new technologies are introduced, e.g. vaccinations. The majority of the farmers in developing countries by contrast operate in a wide range of low input systems. We have often produced interventions that farmers find unprofitable, too risky, too labour intensive and hence impossible to implement. Our first priority should be to understand the farmers with their goals and planned strategies [30] before we try to transform their systems.

The animal health workers are in this respect in a unique position, provided an appropriate infrastructure exists, because generally their interventions work within days and so gain the confidence of the farmer, whereas animal husbandry workers may have to wait for months or even years before their results are visible.
5. MEASURING SUSTAINABILITY

Sustainability indicators have to be included in considering possibilities for livestock development, but hardly any research has been done on how sustainability should be measured.

De Wit et al. [20] argue that identification of livestock specific criteria is impossible given the heterogeneity of livestock production systems plus the fact that they are part of a broader agricultural system. Moreover, there is very often not a linear relation between livestock specific and agro-ecological criteria. They recommend a system specific analysis to assess the overall effect of livestock in agricultural systems. Identification of livestock specific criteria eventually becomes impossible, as the indicators for sustainability act as feedback mechanisms, which (partly) control the pressure of animals on the natural resources. Reducing mortality (an indicator often used in animal health studies) can, in some instances, be a tool to promote sustainability, but if the number of animals kept alive is too high it may exceed the capacity of the feed resources. Farmers with more resources may even sooner profit from veterinary interventions but, with more of their animals surviving, may contribute to a greater depletion of natural resources than small farmers.

Preston [31] expressed the sustainability of a system in terms of its effects on the economy, environment, the need for fossil energy, animal welfare and food security and quality.

De Wit et al. [20] confined themselves to a list of technical sustainability criteria including N, P and K balances, soil erosion, fossil energy utilization, etc.

Table III illustrates criteria for measuring sustainability as far as health, reproduction and welfare balances are concerned, and the effect of livestock on these characteristics [32].

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Importance for sustainability</th>
<th>Factors affecting animal production in an agricultural system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Health, reproduction and welfare balances</td>
<td>Optimal functioning of animals</td>
<td>Health hazard and risks in animal sectors (structure)</td>
</tr>
<tr>
<td></td>
<td>Efficient production and use of resources</td>
<td>Public health, safety and product(ion) quality</td>
</tr>
<tr>
<td></td>
<td>Public health and safety</td>
<td>Environmental effects of residues (risk assessment)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Level of intensification and farm style variation</td>
</tr>
</tbody>
</table>
Preston [31], according to his criteria, comes to the conclusion that ranching as practised in Central and South America fails on almost all criteria. Hence, it is a rhetoric question whether any veterinary intervention in this region contributes to sustainability.

Ba et al. [33] studied the impact of vaccinations and deworming on goat mortality and off-take in the semi-arid area of Mali and found that in goats older than 6 months these treatments reduced mortality significantly but had no significant effect on either weight gain or increase in off-take. The net result was an increase in numbers, and ultimately it is the system that will determine whether this increase has a positive or negative effect.

ILRI [24], in its draft Animal Health Improvement Programme, expects that improved control of animal diseases through the use of vaccines, diagnostic tools and enhanced genetic resistance to diseases will reduce the cost of animal production and increase the supply of animal products. More cost efficient production and more livestock products on the market are expected to increase food security for the urban and peri-urban populations as well as for some smallholder livestock producers, and the latter also gain greater economic security. ILRI [24] also sketches another possible scenario that could result in a negative impact from the use of their research efforts, i.e. an oversupply of livestock products leading to reduced farm prices and decreased smallholder incomes, and an increase in animal numbers that aggravates feed constraints, which in turn may lead to increased environmental degradation. Moreover, in our opinion, increased food production by the smallholders does not lead automatically to a better food security for them.

This again shows the difficulties in identifying sustainability based on livestock criteria only.

In Table IV, adapted from ILRI [24], a semi-quantitative impact assessment of the programme on genetic disease resistance has been made.

Seifert [34] discusses extensively the relation between animal health management and animal production systems. He mentions only one typical example where veterinary indicators can be used as an assessment for sustainability. In this respect he considers the increase in soil borne diseases, in particular botulism, as an indicator of a beginning collapse of the balance between the exploitation and maintenance of the ecosystem used by the nomads. High mortality among game animals can also act as an indicator for the collapse of an ecosystem.

The greatest challenge remains, however, to quantify the different criteria. Van der Ven [35] formulated this as a mathematical optimization problem using interactive multiple goal linear programming (IMGLP) as the optimization technique. With that, she constructed ‘iso-labour income lines’, as a function of both nitrate leaching and ammonia volatilization for dairy farming on sandy soils in the Netherlands. The analysis showed that a reduction in nitrate leaching did not influence labour income over a wide range. The model presents the end of a possible
TABLE IV. GOALS, INDICATORS AND MEANS OF VERIFICATION FOR GENETIC DISEASE RESISTANCE

<table>
<thead>
<tr>
<th>Goals</th>
<th>Indicators</th>
<th>Means of verification</th>
</tr>
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<tbody>
<tr>
<td>Sustainably improved (livestock) productivity at national and regional levels in disease challenge conditions, with reduced reliance on non-genetic control options</td>
<td>— Increased agricultural production in disease challenge environments — Reduced expenditures on drugs, pesticides and other non-genetic control options — Increased trade in disease resistant breeding stock — Increased livestock numbers in disease challenge environments</td>
<td>— National agriculture production statistics — International agricultural production statistics FAO/World Bank/OECD — Importation statistics — NARS reports — Livestock breeding and trade statistics — Livestock distribution statistics</td>
</tr>
</tbody>
</table>

development path but does not predict future developments. It explores, however, the possibilities for future developments from which improvements in sustainability can be derived, provided suitable criteria have been identified.

Udo et al. [36], using simulation studies with the ‘intervention impact assessment’ method, predicted that proposed interventions — such as bull fattening, the use of improved feeding strategies and the introduction of dairy cattle — would not be sustainable for the marginal upland area in East Java considered, because of the limited economic and physical resource base. This approach is still semi-quantitative but they hope to develop this further for a whole production system, based on the extent to which the various criteria are satisfied [37].

6. ROLE OF VETERINARY SCIENCE IN SUSTAINABILITY

The control of animal diseases is — next to eradication and prevention — a paramount strategy in animal health care. However, the disadvantage is that action is taken after problems have occurred and focused on product level (i.e. the animal). For more appropriate preventive orientation, it would be much more attractive to put emphasis on disease risk control instead of disease control.

Veterinary epidemiology provides the tools to identify the respective disease risk factors and to quantify the contribution of those factors to disease occurrence. By its very nature, epidemiology is oriented towards the control of the disease risk
factors throughout the whole production process and hence towards preventing product deviations (i.e. diseased animals) from occurring or at least reducing the risks.

Veterinary epidemiology can make use of other tools to achieve certain goals. For example, in the case of trypanosomiasis it is highly attractive to apply GIS in conjunction with epidemiological databases to facilitate the identification of high risk regions and vector distribution. Linking such a GIS to meteorological conditions enables the development of predictive disease models in support of sustainable livestock production [21].

In the western situation, developments are proceeding whereby consumer demands with respect to product quality (meat, milk, eggs), production method (animal welfare, environmental issues) and public health or food safety are becoming increasingly important to livestock producers. For exporting countries these conditions strongly determine market access and market maintenance. The key word has become 'quality'. Many attributes may refer to quality: it is related to intrinsic product features but also to animal health, welfare and environmental characteristics.

Animal health may be regarded as 'just another quality feature' and hence animal health care could be executed according to quality control methods. Particularly in situations where livestock producers form part of an integrated food production chain this has become important. Quality control could be performed according to good manufacturing practice codes (such as those observed in feedmills) or good veterinary practice or good farming practice codes, which are written directives about how to operate a certain process. These codes refer to a quality attitude rather than to control activities. Another quality control method involves hazard analysis critical control points (HACCPs), where the hazard in animal production refers, for example, to the disease and the critical control points (CCPs) refer to conditions or points in the production process where risks can be monitored and lost control can be restored. HACCPs closely relate to the epidemiological approach of risk identification and quantification. They have the advantage that preventive actions and control of CCPs are demonstrable to third parties. The latter facilitates herd health certification and disease risk insurance [26]. Ultimately, HACCP can be incorporated into ISO certification throughout a whole production chain, comprising at the end both animal health and public health safety, animal welfare issues and environmental issues (ISO 14001) next to regular quality control (ISO 9002).

It is obvious that such developments will either not take place at all in non-western situations or at least not at the same pace. On the same time axis there are further arrears in animal health care, followed by a lower priority for public health and environmental issues in the non-western situation as compared to the western situation. When developing countries desire to keep pace, this could mean that they induce socioeconomic problems by enforcing livestock production intensification, hence reducing the number of small farmers.
Epidemiology is closely linked to animal health economics. Disease control and disease risk management are economic processes, but it is open for debate whether all this should be paid out of private or public funds or a mixture of the two [38]. Disease control and risk management are, however, prerequisites for a viable livestock development. Not only from a point of view of preventing disease losses but also from a point of view of disease hampering trade, as is clearly demonstrated by the BSE affair. Furthermore, cost–benefit estimations are associated with veterinary intervention programmes and even with quality control programmes. Monitoring and surveillance programmes are the basis for disease control and disease risk control; they are closely associated with the need for appropriate, rapid and reliable diagnostic tests.

Ethnoveterinary surveys, i.e. surveys based on local knowledge systems, can provide useful information on local perceptions of livestock diseases and their control. As such, these make disease control programmes socially more acceptable [17]. Veterinary science can offer a limited number of quantifiable indicators for the sustainability of a system: level of use of medicinal products and insecticides; degree of disease tolerance in animals; risk factor analysis for disease risk control and quality related issues; animal welfare impairment and even environmental quality assessment.

In conclusion it can be said that veterinary science is just one player in the interdisciplinary team addressing sustainability.

REFERENCES


OVERCOMING ANIMAL HEALTH BARRIERS TO TRADE: THE ROLE OF OIE

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Abstract

OVERCOMING ANIMAL HEALTH BARRIERS TO TRADE: THE ROLE OF OIE.

The Office international des épizooties (OIE) is the world organization for animal health. Its activities are supported by four specialist commissions and a number of working groups, made up of international experts in particular fields. The paper outlines some of the key activities of the organization, in particular focusing on its role in setting international standards for trade in animals and animal products, and for infectious disease control programmes. In the area of laboratory science the work of the OIE Standards Commission is highlighted. This has a threefold role: (a) the preparation of written standards for laboratory diagnostic tests and vaccines for infectious diseases of animals; (b) the designation and co-ordination of a network of reference laboratories and collaborating centres; (c) the development of international standard reagents for diagnostic tests and vaccines.

1. INTRODUCTION

The Office international des épizooties (OIE) is the world organization for animal health and comprises delegates from the official veterinary services of over 140 member countries, supported by a central bureau. Since its founding in 1924, the three principal aims have been:

(a) the provision of information on infectious animal diseases, worldwide;
(b) international promotion and co-ordination of studies on the surveillance and control of infectious diseases of animals;
(c) the harmonization of international agreements and regulations for disease control, including the facilitation of trade in animals and animal products.

The work of the OIE has assumed a new prominence in the 1990s through recognition of its role in providing standards, guidelines and recommendations for animal health and zoonoses within the Sanitary and Phytosanitary Agreement of the World Trade Organization (WTO). The activities of the OIE are supported by four specialist commissions and a number of working groups, made up of international

* The author is the Secretary-General of the OIE Standards Commission.
experts in the relevant fields. OIE does not normally initiate or operate disease control programmes; rather it seeks to provide guidelines and set standards so that both individual member countries and other international organizations may operate such programmes in accordance with common criteria. This, in turn, can lead to mutual recognition of veterinary services [1], together with the associated animal health certification, and data from laboratory testing.

2. ORGANIZATIONAL STRUCTURE OF THE OIE

OIE operates on a basis of consensus between the member countries, and in particular through resolutions passed by the International Committee of member country delegates (chief veterinary officers or their representatives) at the general session held annually in May. This gives the opportunity for discussion and review of the activities and recommendations made by the various specialist Commissions and groups which operate under the auspices of the OIE. The International Committee also appoints the Director General of the organization and the members of the OIE commissions. As well as the administrative commission and the five regional commissions (Africa, Americas, Asia/Far East/Oceania, Europe and Middle East), there are four specialist commissions, entitled international animal health code, standards, foot-and-mouth disease (FMD) and other epizootics, and fish diseases, respectively. The fish diseases commission deals separately with this specialized area and publishes a separate code and a diagnostic manual, which complement the equivalent volumes for mammalian, avian and bee diseases. The other three specialist commissions work closely together to provide an internationally co-ordinated approach to the prevention and control of infectious diseases of animals.

The code commission develops recommended procedures which will facilitate trade in animals and animal products without compromising the health status of trading partners. The OIE International Animal Health Code [2] sets out animal health conditions for trade, including definitions of the criteria by which individual animals, herds or areas may be considered free from a particular disease. Frequently, these conditions call for laboratory diagnostic tests for which purpose the Code refers to its sister publication the OIE Manual of Standards for Diagnostic Tests and Vaccines [3]. The FMD and other epizootics commission supports international efforts to control major epizootic diseases by developing appropriate strategies and by collaborating with other international organizations in regional programmes for disease control. Again these activities may call for mutual recognition of laboratory diagnostic tests between member countries, as well as requiring common standards for the production of vaccines. The OIE Manual addresses both of these issues.

The regional commissions identify animal health issues which are of concern to countries in the particular region, either because of a localized incidence of a specific
disease or due to animal production or trade aspects which are specific to the region. The regional commissions also organize regional conferences focused on technical items and on regional co-operation for animal disease control. They may also develop regional programmes for surveillance and control of major diseases.

The OIE commissions are supported by working groups on biotechnology, wildlife diseases, informatics and epidemiology, and veterinary drug registration, and by ad hoc groups which are formed as required to address specific issues.

3. INTERNATIONAL SPREAD AND CONTROL OF CONTAGIOUS DISEASES: THE NEED FOR INFORMATION

Infectious diseases of international significance are categorized into Lists A and B. List A comprises 15 diseases which are considered to have a high risk of epizootic spread, and for which member countries generally have official control, eradication or prevention schemes. These are diseases of high economic importance, which generally have short incubation periods and a strong propensity to spread within and between countries. Much of the activity of OIE, as of member countries' official veterinary services and of other international organizations, is devoted to the prevention and control of the List A diseases, and the importance of such infections as foot and mouth disease, rinderpest, swine fever, and Newcastle disease is well recognized and has been re-emphasized at this symposium. It is the international disease notification system for these diseases which lies at the hub of the OIE information systems. The OIE has established a warning system which enables member countries to act rapidly should the need arise. Within 24 h of the first outbreak of a List A disease, or any other disease which may have serious repercussions on public health or on the economy of animal production, the affected country reports the incident to the OIE Central Bureau in Paris. This information is then transmitted immediately to member countries directly at risk, by fax or telex, together with a weekly publication of Disease Information to all other countries. This system has been greatly enhanced in recent years through the development of a computer system which provides rapid processing of data in the central office.

The information system is supplemented by the bimonthly Bulletin giving summary tables of disease statistics and other relevant information. In addition, an annual publication, World Animal Health, is produced, which provides a comprehensive tabulation of the international animal health situation for Lists A and B, including data on disease occurrence and on the control methods employed in different countries. The weekly report of List A diseases, as well as an abstract of the previous year's epidemiological situation and countries' control measures on more than 100 reportable diseases, are now on the World Wide Web (http://www.oie.org).
In addition to this current disease information, the OIE is actively involved in the dissemination of scientific information, both research results and the outcome of expert consultations. This is achieved through participation in scientific conferences, and through production of a range of publications including the OIE’s own peer reviewed journal the Scientific and Technical Review.

4. CONTROL OF EPIZOOTIC DISEASES: THE NEED FOR AGREED INTERNATIONAL STRATEGIES

The OIE FMD commission was established in 1946 to study control methods for this most contagious of animal diseases, and in 1988 the mandate was widened to include other epizootics. The mission is to develop improved strategies for the control of animal diseases. To this end the commission collects all available information on the subject, from research workers, from disease experts and from epizootiological data in the field. There is a close collaboration with the other OIE specialist commissions and working groups. The responsibilities now include the preparation of guidelines for the surveillance of the most serious international epizootic diseases. The commission also provides expert support to the OIE in evaluation procedures, at the request of the countries concerned. This again contributes to the effort towards liberalization of trade without engendering unnecessary animal health risks.

As well as the ongoing work to control FMD worldwide, another significant achievement has been the development of the ‘OIE Pathway’ [4] to take a country or a region from rinderpest enzootic status through various stages of control to freedom from this disease. This has provided a framework for those international organizations that are involved in animal disease control programmes (notably FAO) to foster international co-operation, leading towards the prospect of global eradication of rinderpest early in the next century. More recently, the commission has been active through its subgroup on spongiform encephalopathies in collating the scientific data available on these diseases and making recommendations for future research and epidemiological studies which could be pursued in the different regions of the world [5].

Usually, OIE does not itself intervene in field operations. Nevertheless, through its information gathering activities and its role in setting standards and guidelines for disease prevention and control, the organization plays an important role in international decisions concerning epizootics. Accordingly, a working agreement with FAO was signed in 1953. Similar agreements were signed with WHO in 1961 and with the Inter-American Institute for Co-operation on Agriculture (IICA) in 1981. The OIE also enjoys close relations with numerous international organizations in economic, technical and financial matters, including the World Bank, the Asian Development Bank, the Organization of African Unity, the European Union, Pan-American Health Organization and many others. Most recently, the WTO Agreement on the Application
of Sanitary and Phytosanitary Measures has defined the relevant international standards, guidelines and recommendations for animal health and zoonoses as those developed under the auspices of the OIE [6].

5. INTERNATIONAL TRADE: THE NEED FOR ANIMAL HEALTH STANDARDS

The OIE Code Commission prepares draft texts for the International Animal Health Code [5]. Once ratified by the international committee of the OIE, these texts become recognized international standards providing health guarantees required of trading partners, so as to avoid the risk of spreading disease through trade in live animals and animal products.

The OIE Code is regularly updated in the light of information from highly qualified experts, supported by views and comments from member countries. The Code sets out animal health conditions for trade, including definitions of the criteria by which individual animals, herds or areas may be considered free from a particular disease. Movement of animals between areas of equivalent disease status may be done with minimum regulation, whereas when moving from a lower to a higher disease status area there would be a need for certain guarantees (depending on the disease) which might include diagnostic tests on the animals themselves or those in contact, together with specified periods of quarantine. Conversely, when moving from a higher to a lower status area, it might be advisable to protect the animals by quarantine, isolation or vaccination. These procedures are complicated by the need to consider all possible disease risks to the animals in both source and destination countries.

6. DIAGNOSTICS TESTS AND VACCINES: THE NEED FOR UNIFORM LABORATORY STANDARDS

6.1 The OIE Standards Commission

The OIE Code often specifies diagnostic tests which an animal or product should pass before being acceptable for trade. It is clearly important that these tests are done to uniform standards regardless of the laboratory where they are carried out. Likewise, the disease control schedules defined by the FMD commission may in some cases require vaccines of defined quality and performance. The Standards Commission, which meets twice per year, is supported by permanent staff from the OIE scientific and technical department, and by international experts in various fields such as diagnostics, vaccinology, disease control or specific disease experts. This support may include participation in meetings of the commission, or the provision of
written advice. Special acknowledgement should be made of the great assistance pro-
vided in recent years by guest delegates from the laboratories of the Joint FAO/IAEA
Division of Nuclear Techniques in Food and Agriculture at Seibersdorf, Austria. The
principal activities of the standards commission are:

(a) Preparation of the OIE Manual of Standards for Diagnostic Tests and Vaccines
[3];
(b) Organization of OIE Reference Laboratories for List A diseases, and selected
diseases on List B;
(c) Organization of a programme for the preparation of OIE standard reference
reagents.

6.2 The OIE Manual of Standards for Diagnostic Tests and Vaccines

This work was first published in loose leaf form in three volumes between 1989
and 1991 as the OIE Manual of Recommended Diagnostic Techniques and
Requirements for Biological Products. These were updated and collated for the per-
manently bound second edition in 1992 which adopted the more memorable title
Manual of Standards for Diagnostic Tests and Vaccines, frequently abbreviated to
OIE Manual. The third edition, again in permanent binding and in an enlarged format,
was completed at the end of 1996 [3]. With the advent of the WTO and the definition
of OIE’s role in setting standards, the OIE Manual has assumed a new importance,
and it is worth reflecting on the philosophy which lies behind its production.

The OIE Manual comprises (a) an introductory section dealing with general
issues such as sampling methods, tests for sterility and freedom from contamination,
and laboratory safety; (b) specific chapters on the List A diseases; (c) specific
chapters on List B diseases (including diseases of bees) and a few other diseases of
importance to trade; (d) a list of reference laboratories. The disease chapters contain
a summary, an introduction to the disease, diagnostic test procedures and require-
ments for biological products for that disease.

Within the area of diagnostic tests, the principal purpose is to provide well
validated, widely accepted and available methods, which are relevant to the determi-
nation of an animal’s health status before international trade. In particular, ‘prescribed
tests’ are provided wherever possible. These are tests which the commission considers
(based on advice from disease experts) are most appropriate for international trade
purposes. A second category of ‘alternative tests’ provides alternatives which may be
relevant to local situations, or which may be newer tests that have not yet received full
international acceptance for trade purposes. Finally, other tests are often described
which may be of local application, or which may be useful for other purposes (such
as disease diagnosis), but are not generally appropriate for international trade.
Surprisingly, there are still many infectious diseases for which reliable tests for trade
certification do not exist. Information is also provided on the application of laboratory
tests to disease surveillance and control programmes. The OIE Manual represents a
set of internationally agreed written standards; the intention is not to be overly
prescriptive in providing rigid standard operating procedures, but rather to provide a
reference method against which other tests and newly developed techniques may be
evaluated. Considerable progress has been made, and the quality of the standard test
procedures in the latest edition of the OIE Manual (1996) has been much improved.
Nevertheless, deficiencies remain in certain areas, and the standards commission is
always seeking better test methods which are robust, reliable, reproducible and
recognized internationally. It is important to recognize the global scope of the OIE
Manual and therefore to provide methods suitable for differing levels of laboratory
sophistication, and also to avoid methods which are favoured locally but do not
receive widespread recognition. Where new tests are developed which depend on spe­
cific reagents (such as monoclonal antibodies), OIE would require assurances as to
the general availability of the reagents before accepting the test as a new standard. A
particular problem can arise when a new test is developed which performs better than
the existing test. If the existing test is used as the ‘gold standard’, this can unfairly
count against the newer test because of an apparently poor relative specificity or sen­
sitivity. Assay validation is a matter of great concern, and a new chapter on the topic
has been commissioned for the introductory section of the latest edition of the OIE
Manual.

There are also new introductory chapters on good laboratory practice and on the
general principles applied in the production of veterinary vaccines. Each disease
chapter has a section dealing with vaccines (or other biological products) except
where none exist for the particular disease. In the past, the balance of the commis­
sion’s work has been weighted towards standardization of diagnostic tests; however,
the focus on vaccines is now gaining greater prominence as the global market
develops. Again the aim is to produce a set of written standards of international accep­
tance. The latter is achieved by circulating drafts of each chapter to all OIE member
countries for comment. The aim is to provide a harmonized approach to vaccine
standardization which is acceptable to the often widely differing licensing and
regulatory requirements of different countries or regions. This is not easy to achieve,
and further difficulties arise because of the commercial confidentiality of much
vaccine validation and production data. Efforts are also made to ensure harmony with
existing published methods, as for example in the European Pharmacopoeia.

6.3 OIE reference laboratories and collaborating centres

The OIE Standards Commission, and indeed the whole of the organization,
depends heavily on the advice of international experts for individual diseases, many
of whom are located in the designated OIE reference laboratories. The commission
EDWARDS recommends the designation of reference laboratory status on the basis of the achievements, expertise and current activities of the laboratory. A list of functions and responsibilities is supplied to reference laboratories, although not all are expected to carry out all functions. These include providing a centre of expertise, consultancy and laboratory training, not only for OIE but also for individual member countries; preparation, storage and distribution of standard reference reagents; and research and development on the disease, diagnostic tests and control.

In addition, a number of OIE collaborating centres have been designated, which provide expertise in a particular technology or, in some cases, for a particular region (e.g. ELISA and molecular techniques in diagnosis; diagnostic methodology surveillance and control of animal diseases in Africa; veterinary drugs).

The OIE is not a laboratory accreditation service nor does it operate official quality assessments. The individual reference laboratories are encouraged to undertake programmes for interlaboratory comparisons at the international level. This helps provide confidence to trading partners in the results emanating from different laboratories, as well as promoting harmonization of diagnostic techniques between laboratories. Furthermore, sets of guidelines have been developed to assist member countries wishing to evaluate each others' laboratory services [7], and for organizations wishing to operate laboratory proficiency test schemes [8]. These guidelines are based on the standards in the ISO 9000 series and ISO/IEC Guides 25 and 43, but tailored specifically towards veterinary laboratories.

6.4 Standard reference reagents

The OIE Standards Commission has encouraged OIE reference laboratories to prepare and distribute reference reagents for diagnostic tests. A number of key diseases were identified where there was an urgent need for such reagents, and programmes were initiated. To facilitate a uniform approach a set of guidelines was prepared, giving advice on the type of standard reagents required and offering guidance on their preparation. The philosophy was to provide well characterized, fully documented primary reference materials (including, where possible, a negative, a weak positive and a standard positive), which could be distributed to national veterinary laboratories. Thus laboratories carrying out different types of assay would have a common standard against which to evaluate their own particular test protocol. The ability of any particular test protocol to give correct results on the standard reagents would increase trade confidence between countries. It is hoped that national laboratories will develop their own secondary standards, equivalent to the OIE standard reagents, which they could then distribute to local laboratories within their country.

Most has been achieved so far with standard reference sera for diagnostic tests, although in some cases these may also be applicable to vaccine testing. Development of standard reference reagents for veterinary vaccines lags behind human medicine,
although it should be noted that the WHO already designates a number of standard preparations for veterinary vaccines. National standards exist in some countries, while progress at the international level is being taken forward especially by the European Pharmacopoeia. Since the OIE and the Standards Commission have limited resources an agreement has been made to collaborate with the European Pharmacopoeia wherever possible, so that European standards could also be accepted, where appropriate, as international standards by OIE. Nevertheless, it is recognized that many globally important diseases will not be included owing to their lack of relevance to the European situation.

7. CONCLUSIONS

The OIE was founded in the 1920s as a response to a rinderpest outbreak in Europe arising from cattle undergoing international shipment. Since that time it has become a truly global organization which provides a vehicle for rapid disease information exchange between countries, which continues to promote and co-ordinate international research efforts into infectious diseases and which sets international animal health standards for trade in animals and animal products and for diagnostic laboratory tests and vaccines.

REFERENCES

THE IMPACT OF EMERGING TECHNOLOGIES ON DISEASE DIAGNOSIS AND CONTROL

Summing up the Symposium

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Abstract

THE IMPACT OF EMERGING TECHNOLOGIES ON DISEASE DIAGNOSIS AND CONTROL: SUMMING UP THE SYMPOSIUM.

The paper primarily addresses the issue of the application of technology in managing disease control programmes.

1. GENERAL INTRODUCTION

Much has been achieved in the past by being able to see clearly, both physically and metaphorically, and this has never been truer than it is today when there is a tendency to neglect observational skills in disease diagnosis in favour of a slavish reliance on advanced technology. J. Hilton raised another issue at the symposium relevant to the wearing of spectacles. He suggested that the information technology revolution has resulted in the loss of many secretarial jobs. We were told that there has been a dramatic reduction in secretarial assistance whilst the remainder of the secretarial cadre have had their skills upgraded. So who does the secretarial duties now? The scientist, who now spends much of the time in front of monitors using word processors and spreadsheets. And what is the result of the skill upgrade? It is inordinate demands for justifications and work plans which require even more word processing. However, the more serious point is that a most valuable tool is identification of disease through the application of clinical skills.

2. ELEGANT DIAGNOSTIC TECHNOLOGY CANNOT REPLACE CLINICAL, EPIDEMIOLOGICAL AND ANALYTICAL SKILLS

A disturbing trend today is that we are becoming increasingly more reliant on laboratory diagnostic confirmation through the use of high-tech laboratory tests to the detriment of clinical recognition even in the face of severe problems demanding an
immediate response. The trend is to delay action until such confirmation is received as if this were the only criterion. Action may even be totally subservient to diagnostic confirmation, leading to inordinate delays. Had we waited for this in January 1997 in the United Republic of Tanzania, rinderpest could well have been burning through Zambia by now. However elegant, the tools are just that — tools —, often invaluable support for decision making but not an end in themselves.

This symposium has illustrated most clearly the elegant modern diagnostic and, possibly, vaccine technology available for use in fighting livestock disease constraints to production. We live in a time when the developing world has an élite corps of highly trained laboratory personnel such as never existed before and a nascent network of laboratories with highly proficient personnel capable of employing immunological techniques and now the introduction of PCR technology. There can be no doubt about the calibre of scientists able to operate on a TCDC (Technical Co-operation between Developing Countries) basis. Recently, African veterinary scientists have shared their expertise with colleagues both within and outside the continent. Yet, at the same time, it must be pointed out that veterinary services and their capability to implement control has never been weaker. We must ask ourselves, why?

N. Ferris’s presentation on the valuable contribution to diagnosis made by ELISA contained another message — the vision of the intellectually challenged veterinarian out of depth in the world of science — but this sentiment illustrates a very real problem we face now and which will probably increase in the future, namely the denigration of veterinary skills.

3. VETERINARIANS ARE NOT SIMPLE COUNTRY FOLK WITHOUT SKILLS WHOSE ONLY USE IS TO COLLECT SAMPLES FOR EXAMINATION BY SCIENTISTS

Throughout the developing world we are witnessing the progressive dismantling of veterinary services in the guise of privatization. One cannot support the status quo with overstaffed public veterinary services consuming all available financial resources in salaries, leaving little for operational activities. Even then the salaries are generally inadequate and activities are targeted to clinical service delivery which is more rewarding financially whilst failing to address the public sector needs of epidemic disease control. It is not surprising, therefore, that structural adjustment is a fashionable priority of development agencies. Yet, some of the zeal with which it is pursued owes more than a little to the derogatory perception of veterinarians and an appreciation of veterinary activities viewed from the perspective of companion animal practice in the developed world and analogy with human general practitioners.
4. VETERINARIANS ARE NOT ONLY SYRINGE WIELDERS

Arguably, the concept that veterinarians can largely be replaced by technicians, supported by laboratory technologists, might be appropriate in the developed world where livestock production is an industrialized process and the threat of epidemic diseases is diminishing steadily. Quite rightly such mechanical work could and should be delegated to technicians. However, this concept of the role of veterinarians involves a very naive appreciation of the tasks involved in disease control. M. Thrusfield alluded to this in his description of the changing nature of disease spectra with development over time and illustrated some of the reasons why this understanding may have arisen. He made it clear that infectious diseases were the major constraints to livestock production, by analogy with human medicine, in the western world until recently and that, although the situation may have changed there now, this situation still prevails in the developing world. Consequent on the change in disease constraints away from major epidemic infectious diseases, qualitative epidemiology is now denigrated as passé in the industrialized nations in favour of quantitative analysis, just as are veterinary skills. However, as he indicated, the situation in developing countries resembles more closely the past situation in the industrialized world and demands the same mix of skills as was applicable then. It demands well trained veterinary investigation staff and disease control strategists benefiting from the application of newly available technology and operating within a veterinary management environment with executive power.

J. Crowther suggested that we must be careful not to “throw away the baby with the bath water”. He was indicating that we should not reject old established techniques just for the sake of adopting more fashionable and charismatic technology. The concept should be endorsed and extended to being careful not to discount the need for sound veterinary disease control strategists in favour of promoting attractive technology.

If there has been a deficit of this meeting, apart from the limited discussion of recombinant vaccine technology, it has been the lack of a discussion of the veterinary infrastructure required to apply practically the valuable information to be gained from the new technology. Let us not lose sight of this. Who will undertake the control of the major epidemic diseases in developing countries — molecular biologists? As A. Diallo pointed out, a good molecular biologist is not equivalent to a good diagnostician, let alone a disease control strategist. All are required to work together in synergy. Technology is fashionable, focused and relatively easy to transfer, yet the new technology cannot contribute effectively in isolation. Ensuring its use as a management tool is a challenge which must be addressed more vigorously than it has been to date. Understanding a disease problem is the first step but it is not the goal; its resolution must follow, and this requires an educated and active veterinary service addressing issues of the public good. No amount of exquisite technology will replace
the need for ensured early warning and early reaction capability for epidemic diseases and this needs veterinary skills as well as elegant laboratory technology. The technologies we have been focusing on here can be likened to the tail on the statutory veterinary service dog. It used to be a small tail wagged by a very large and fierce dog. We now have a very large tail trying to wag a small cowed dog and in the near future we could end up with no dog at all. For the effective control of epidemic diseases there is no substitute for an experienced veterinary manager who appreciates the value of available technology in assisting his or her veterinary service to function dynamically and effectively.

5. APPLICATION OF NEW TECHNOLOGY

In 1989 in Ethiopia, rinderpest was a major problem and occurred in an apparently haphazard, random and sporadic manner throughout the country. Using 'shoe leather' disease investigation, what M. Thrusfield would describe as qualitative epidemiology, we set about attempting to understand what was going on. The tools we had were mobility, clinical skills and neolithic diagnostic methodology (and a good pair of spectacles). It took nearly three years of investigation before we were able to discern patterns within the disease occurrence and to define some of the determinants of that occurrence. The result was that we were able to define discrete areas of endemic disease persistence, high risk areas for epidemic extension from them and areas of low risk. The practical outcome was to devise and implement an eradication strategy which required the targeted conventional vaccination of some three million cattle, to replace the totally unrealistic objective of blanket vaccination of the entire country cattle population of some 30 million cattle, complemented by participatory approaches to eliminating the endemic foci. Mariner's thermostable rinderpest vaccine, a most valuable technical innovation in its own right, was an essential component. The result was freeing of resources for disease surveillance and virtual elimination of rinderpest within three years.

What has this to do with the new technologies? With respect to rinderpest, we now have available:

— immunological techniques for the detection of antibodies as a diagnostic tool, a means of quality controlling vaccination campaigns and as a means of verifying disease absence;
— immunological techniques for specific and sensitive detection of rinderpest virus;
— molecular techniques which can detect virus and when combined with sequencing provide information of epidemiological value of direct use for decision support in control/eradication.
But no mention was made of any of these or other new technologies in describing the developments in Ethiopia, other than a passing allusion to the application of participatory rural appraisal and disease control techniques. Ethiopian colleagues have now had the opportunity to start disease mapping in their country using GIS as described by T. Tobinson, and it is clear that if we had had the capability to look at disease occurrence spatially and temporally with topography, land use and livestock movements earlier it would have greatly speeded up the process of understanding. Looking at East Africa in this way one can relatively easily visualize the pulsed epidemic extensions of rinderpest from endemic foci. Georeferencing greatly enhances the value of disease occurrence and surveillance data and must be promoted.

Diagnosis was difficult although The Universal Penside Diagnostic Aide backed up by the Agar Gel Immunodiffusion Test worked well. This antigen detection test is a test of good specificity but poor sensitivity. However, it works and by maximizing the numbers of animals examined and samples taken we were able to confirm most outbreaks. Serology for diagnosis was out of the question as at that time the use of ELISA tests was in another organization's hands and dedicated to a retrospective evaluation of vaccination campaign efficacy. Recourse to immunocapture ELISA, serology as a surveillance tool and molecular characterization of virus strains, all of which are now available, would have greatly facilitated the clarification of events as they are now doing. Penside tests, as described by J. Anderson and J. Reddington, in the hands of field veterinary officers would have made a major contribution in allowing rapid confirmation of outbreaks to avoid the need for visits from headquarters.

In the last few years Ethiopians have used to great effect:

— disease mapping (as described by T. Ndegwa, T. Robinson and D. Rogers);
— rapid and sensitive primary diagnosis by immunocapture ELISA (as described by A. Diallo);
— participatory approaches to epidemiological analysis (as mentioned by M. Thrusfield), supported by:
  — serology as an investigative tool;
  — seromonitoring for quality assurance of vaccination campaigns (as described by J. Anderson) as a tool in managing control campaigns;
  — molecular characterization of virus strains (as described by T. Barrett) as an epidemiological tool.

Application of these tools is helping to clarify epidemiological understanding, most notably in Africa, and has an important role to play in guiding strategies. Indeed, translation of the results into cost effective plans for action is almost certainly the major challenge we are faced with in eradicating rinderpest. T. Barrett illustrated the concept of molecular epidemiology with respect to rinderpest. It has been a most useful tool in developing an understanding of rinderpest epidemiology
FIG. 1. Global rinderpest risk related to molecular virus characterization. The zones depicted indicate the geographical areas involved in endemic rinderpest persistence together with those at high risk from epidemic extensions from the foci. Asian lineage — zones 1, 2, 3, 4, 5, 6; African lineage 1 — zones 7, 8; African lineage 2 — zone 9.

which provides a sound basis for control — the essential synergy referred to earlier. T. Barrett’s dendrograms can be translated into applied epidemiology to construct a putative map of global rinderpest risk (Fig. 1) allowing countries and regions to focus on surveillance and disease control strategies extending beyond the point of incidence. That was why an early EMPRES initiative was to designate the Institute of Animal Health Pirbright Laboratory as the FAO World Reference Laboratory for rinderpest.

Given the understanding that rinderpest is now restricted to fairly well defined endemic foci, an FAO Expert Consultation has recommended that any occurrence of rinderpest outside the known endemic foci must be considered an international emergency and this policy is is now being pursued by FAO in co-ordination of the global rinderpest eradication strategy. Molecular epidemiology aids control of such emergencies through facilitating the tracing of epidemic extensions from the endemic foci.

The lesson to be learned is that the technology available is of great value in eradicating the disease provided that it is used: to define the determinants of disease
occurrence; to elaborate sound targeted control strategies; to manage control/eradication campaigns.

The challenge for the Global Rinderpest Eradication Programme is to apply this technology in a similar manner to design and implement eradication campaigns in the other theatres in which the war against rinderpest is waged — namely South Asia, the Near East and the Arabian Peninsula.

Similar lessons are evident in FMD. The presentation by N. Knowles again illustrated the power of molecular epidemiology in enhancing the total epidemiological understanding. Much of this work is aimed at protecting Europe from the threat of invasion but we must use it to guide the implementation of control strategies leading to eradication in selected subregions of the world such as South-East Asia. It is obvious that the concept of FMD has to be replaced by foot-and-mouth diseases not at the serotype but at the topotype level to generate the epidemiological understanding necessary to elaborate sound control strategies. J. Bashiruddin showed that there are similar encouraging developments with CBPP.

This catalogue of successes is very commendable but it is important to indicate some of the problems.

6. PROBLEMS

6.1. The need for a dedicated research base

Introducing new technology provides a potent means of answering questions; these are powerful tools but that power itself may raise new questions, and events in East Africa today are illustrating this.

A strain of rinderpest which is very different from the classical cattle plague strains is currently a hot issue in East Africa. This virus has been difficult to detect clinically in cattle because it generally produces only mild disease and its detection in the laboratory is proving difficult in all aspects — antigen detection, nucleic acid detection, genetic characterization and serology. Many questions are raised which are not yet answered by, or may even be the result of, the new technology. Let us not forget that viruses can change (especially RNA) and, as K. Nielsen warned, “if everything seems to be going well, something has been overlooked”.

Is there a risk of adoption of new technology before we have fully digested the information it can provide? Is it possible that we have initiated new technology at the expense of traditional methods prematurely? For example, RT-PCR in a virgin epidemic situation is sufficient for primary diagnosis but in a situation where vaccination may be occurring in the face of field infection a positive PCR result is obviously not always conclusive and sequencing becomes the definitive diagnostic tool. This is time consuming, expensive and laborious and limits
its use as a primary diagnostic tool. Laboratory scientists using these techniques are pressed to make early judgements, sometimes prematurely, which are open to overinterpretation, and results may even exceed our ability to interpret them at present. A full evaluation of the role of molecular techniques in primary diagnosis, rather than epidemiological analysis, is needed. Again, J. Crowther warned that we must be careful not to discard old established techniques just for the sake of adopting more fashionable and charismatic technology.

The message is that validation of a new technique goes far beyond its laboratory and experimental field validation. Full validation requires a dedicated and sustained research base to answer the inevitable questions generated during application. With new technology it appears also that we may have to re-evaluate our understanding based on earlier less sensitive technology. Who will provide the necessary research base to do this? Increasingly we see that the developed countries are not prepared to support the required work now that they are distanced in time from colonial responsibilities and the major epidemic diseases are perceived to present a reduced domestic threat. Not only is the research and expertise base dwindling but the ever increasing emphasis on molecular biology, as highlighted in the discussion on global warming following P. Mellor's paper, restricts options further. Redressing this is a challenge to international agencies and others concerned with disease control on a global basis. The international community, particularly the Joint FAO/IAEA Division, needs to extend its concepts of technology validation and quality control to take this on board as a prerequisite for technology transfer.

6.2. Scientists or kit users?

Kit development for rinderpest and other diseases, with its potential for standardization and quality assurance as described by B. van der Eerden, has been a most useful development. It has overcome many of the problems of standardization and reliability. But, there is a down side. We are in danger of establishing a kit user mentality with built-in dependency and a lack of ability to do the basics as indicated by A. Diallo. The Joint FAO/IAEA Division's research networks have been very valuable in assisting the professional development of many scientists in developing countries yet, in their own countries, the majority are restricted to the use of test kits prepared well away from their country. They have little opportunity to practise and apply the panoply of tools used in investigating infectious diseases. This lack creates a reliance on international reference laboratories for real research work and, as indicated, this facility is threatened. Again, it is our challenge to find a way to support this kind of work within countries and in regional reference laboratories. A related aspect must be to ensure that expertise is spread around a little more than at present; relying on one expert in each country, as is often the case, is a very vulnerable situation.
6.3. Sustainability

We have seen from the presentations by J. Anderson, T. Barrett and A. Diallo what the new technologies offer with respect to rinderpest within a strong network. We have come a long way in terms of getting the job done, but are we any nearer sustainability to reduce the need for continuing external support?

We may have established centres of excellence but they are largely reliant on externally funded project support. What are the prospects for sustainability? In Africa we recently witnessed the rapid collapse of the rinderpest seromonitoring network when there was a temporary hiatus in external inputs. How can we ensure that the basic requirements of disease diagnosis and surveillance once established are absorbed into core government functions? There is little sign of this happening at present. Once project support terminates who will maintain infrastructure, pay for utilities, maintain equipment, provide consumables, purchase reagents and provide backstopping expertise?

Perhaps we have not examined our own objectives clearly enough. We must discriminate between doing a job (i.e. eradicating rinderpest) and attempting sustainable development of diagnostic expertise and services so that countries can address disease issues on their own. If the former is our sole goal, we should ask ourselves if the path we are embarked on is the most cost effective way of achieving the goal. Political correctness aside, conceivably, it might be more cost effective for the donor community to support and quality control centres of excellence in the developed world to provide the services required. But, rinderpest is just one disease which threatens food security; when we add the host of others into the equation, surely it becomes more cost effective to strengthen local capacity. The rinderpest networking arrangements have demonstrated clearly the potential that exists for experience gained with rinderpest to strengthen diagnostic and surveillance capacity in a generic manner.

The cost savings resulting from structural adjustment programmes should be applied to sustainable development of the services required to address the priority constraints to livestock production in which epidemic diseases must feature strongly. This not only applies to national capacity but also includes groups of countries supporting their own regional reference and research laboratories as was once the case in the East African Community. Both the national authorities and those organizations promoting structural adjustment have a responsibility to promote such concepts if they are serious about strengthening food security in the developing world.

6.4. From activity to action

The transfer and establishment of technology has been very successful although in reality so far it has been a case of only partial technology transfer with built-in
dependency. We have been much less successful in getting the results obtained to have any effect on decision making in control campaign management as opposed to providing a retrospective analysis of past events. With surveillance and vaccination monitoring, results are of value when they are fed rapidly into the analysis of progress and impact on the management of control programmes. So far this has not been achieved to anywhere near the extent required and the reasons for this must be analysed and addressed. However, perhaps, the tide is turning. There have been some major achievements, for example in the Islamic Republic of Iran, Ethiopia and the United Republic of Tanzania. Meeting the challenge of moving from activity to effective action must be a priority issue for those promoting the technology.

6.5. The future — Producing new technology

One of the most exciting prospects for epidemic disease recognition is the prospect of penside tests and biosensors as indicated by J. Anderson, J. Reddington and I. Tothill. Yet it is difficult to see how these will be developed to meet the needs of disease control in developing countries.

We were told that their development is market driven and that they are amenable to mass production. The former implies the requirement for a major market but our market requires tests for diseases which would not be a priority for the industrialized countries and cannot, or will not, afford the cost anyway. The main thrust for this technology in the industrialized world is as an adjunct to clinical diagnosis and therapy prescription. Rather than being amenable to mass production is it not the case that the technology is completely dependent on mass production to avoid prohibitive production costs? It was surprising to hear G. Uilenberg describe the global acaricide market as small! If that is considered small in commercial terms then what prospect is there for a penside diagnostic test for, say, African Swine Fever or Newcastle disease in village chickens, both of major significance for food security?

We must differentiate clearly between clinical service support activity (clinical decision making), analogous to much of the human market developments, and major nationally and internationally co-ordinated programmes of disease control. In the commerce driven 1990s, as well as into the foreseeable future, and with the prevailing insular attitudes of self-interest, it is difficult to see where the funding will come from to develop methods applicable to diseases which no longer preoccupy industrialized nations. Relatively modest inputs into research and development for specific priority needs can be remarkably successful, as the support of the UK Overseas Development Administration for development of the rinderpest penside test has demonstrated. However, once developed, should a benefactor be found, the vogue for commercial exploitation raises the price to a level where the product is only in reach of externally funded projects and programmes. The simplest possible technology is required so that test kit assembly can be performed at the point of use.
7. CONCLUSIONS

There is no doubt that the transfer of technology to developing countries is starting to yield results and that new technologies are emerging which have much to offer in the future. However, let us ensure that we get as much value as possible out of the technological tools available, keeping in sight the need for technology use to be reflected in improved management of disease risk. This can only be achieved by strengthening veterinary management as well as laboratory technology. This requires a strengthened public veterinary sector working in unison with a strong and responsible private veterinary sector.

The progress made in the developing world provides an indication of the potential; the challenge is to see this reflected in improved animal health status. M. Jeggo referred to the need for performance indicators in monitoring the progress of disease control programmes. We also need performance indicators to monitor the application and use of technology.

It is clear that we face many challenges in animal health, one of which is to ensure that the fruits of technological advances are applied for the benefit of all the human race. This was the theme of the World Food Summit, which made a global commitment to world food security, and it is implicit in the Plan of Action Commitment Number 3, Objective 3.2, which includes:

“(j) Seek to ensure effective prevention and progressive control of plant and animal pests and diseases, including especially those which are of transboundary nature, such as rinderpest, cattle tick, foot-and-mouth disease and desert locust, where outbreaks can cause major food shortages, destabilise markets and trigger trade measures; and promote concurrently, regional collaboration in plant pests and animal diseases control and widespread development and use of integrated pest management practices.”

In the same Plan of Action Commitment, Objective 3.3 is particularly apposite in the context of this symposium:

“To promote sound policies and programmes on transfer and use of technologies, skills development and training appropriate to the food security needs of developing countries and compatible with sustainable development, particularly in rural and disadvantaged areas.”

For the industrialized and developing nations to work together to diminish the threat of transboundary animal disease epidemics and improve food security in the developing world is not benevolence, it is enlightened self-interest.
POSTER PRESENTATIONS
SERO-EPIDEMILOGICAL INVESTIGATIONS OF RINDERPEST IN JORDAN

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1. AIMS

Evaluation of the serological status of vaccinated and non-vaccinated ruminants in Jordan (from 1988 to 1996) before the halting of rinderpest (RP) vaccination and the declaration of provisional OIE free status.

2. METHODS

Serum samples were collected from RP vaccinated and non-vaccinated cattle, sheep and goats before vaccination and between 6 and 36 months later. The sera were tested at the Veterinary Laboratory or the Vaccine Center, Amman, Jordan, or at the Institute of Animal Health, Pirbright, United Kingdom, by the Competitive ELISA method, using the FAO/IAEA test kits.

3. RESULTS

The results of tests in cattle (1988–1996) are summarized in Table I. All the seropositive animals had been previously vaccinated. A trial, conducted in 1990, of 184 cattle in 11 herds showed 59% (range 16–100%) seropositive at 4–10 months post-vaccination.
### TABLE I. RESULTS OF TESTS CARRIED OUT ON CATTLE (1988–1996)

<table>
<thead>
<tr>
<th>Year</th>
<th>Total cattle</th>
<th>Number of cattle vaccinated</th>
<th>% cattle vaccinated</th>
<th>Number of sera</th>
<th>% seropositive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1996</td>
<td>73 000</td>
<td>7 821</td>
<td>11</td>
<td>348</td>
<td>25</td>
</tr>
<tr>
<td>1992</td>
<td>63 800</td>
<td>17 560</td>
<td>28</td>
<td>822</td>
<td>32</td>
</tr>
<tr>
<td>1991</td>
<td>64 150</td>
<td>29 654</td>
<td>46</td>
<td>219</td>
<td>39</td>
</tr>
<tr>
<td>1990</td>
<td>42 400</td>
<td>5 533</td>
<td>13</td>
<td>260</td>
<td>21</td>
</tr>
<tr>
<td>1989</td>
<td>25 500</td>
<td>4 329</td>
<td>17</td>
<td>539</td>
<td>1</td>
</tr>
<tr>
<td>1988</td>
<td>29 500</td>
<td>4 826</td>
<td>16</td>
<td>353</td>
<td>7</td>
</tr>
</tbody>
</table>

From 1992 to 1996, over 3000 sera from more than 200 sheep and goat flocks throughout Jordan were tested, and only RP vaccinated animals were found to be seropositive. The response to RP vaccination (12–18 months post-vaccination) was generally very low, varying from 3 to 20% in vaccination trials in the local Awassi sheep and to 41% in Merinos imported from Australia.

### 4. CONCLUSIONS

The last recorded cases of RP occurred in 1971 (during the 1969–1973 Near East panzootic). The above results indicate that there has been no serological evidence of disease since 1988. There were no reports of clinical cases of RP during this time either.

The percentage of cattle found seropositive showed a general correlation with the number of animals that had been vaccinated. Although only 59% responded to vaccination, this was higher than the 3–41% in sheep and goats, which was possibly due to immunosuppression caused by intercurrent disease.

As a member of the West Asia Rinderpest Eradication Campaign Coordination (WARECC), Jordan is committed to attaining the OIE conditions for the eradication of RP and, as a result of these studies, has decided to halt vaccination and declare provisional RP free status.
An important restraining factor to camel health, welfare and productivity is the range of potential infectious diseases. Brucellosis is an infectious zoonotic disease of worldwide importance in domestic ruminants, and the causative bacteria (Brucella abortus in cattle and B. melitensis in sheep and goats) are transmitted to humans through contact with infected livestock or by consumption of contaminated dairy products. Because of the economic importance of cattle in developed countries, means for B. abortus diagnosis and prophylaxis have been widely investigated, and several serological tests have been developed for cattle brucellosis. By contrast, although camels are crucial in the economy of developing countries, the brucellosis of camels has received comparatively little attention. There are some limited reports on brucellosis in camels in many parts of the world; however, epidemiological studies have not been extensive and have been limited by the diagnostic procedures available. So far, it has only been diagnosed by conventional serological tests (RBPT, SAT, CFT), which were shown to be inadequate in camels. Enzyme linked immunosorbent assays (indirect ELISAs) were developed for the detection of total and specific IgG and IgM antibody responses (using for the first time monoclonal antibodies
specifically generated for camel IgG and IgM) to *B. abortus* and *B. melitensis* in *Camelus dromedarius* sera. These assays have been used in epidemiological studies of camel herds in the Libyan Arab Jamahiriya; and results were compared both to a competition ELISA, developed for use in a range of other animal species, and to RBPT and SAT. The overall positivity rate resulting from testing 520 camel sera by RBPT and SAT was 1.4% and 1.2%, respectively. The total antibody response to *B. abortus* measured by indirect ELISA was 3.5%; no significant difference was seen with *B. melitensis*. A 3% positivity rate was recorded by the competition ELISA. Linear regression analysis showed that rabbit anti-camel IgG strongly correlated with mouse monoclonal anti-camel IgG (n = 520, r = 0.784, p = <0.001). The rabbit anti-camel IgG had a good correlation with the mouse monoclonal anti-camel IgM (n = 520, r = 0.674, p = <0.001). The least correlation was between IgG and IgM responses (n = 520, r = 0.622, p < 0.001). In conclusion, this study shows that *Brucella* ELISA is a reliable, rapid, sensitive and specific assay with a superior performance over conventional tests; it provides a profile of *Brucella* specific IgG and IgM for the diagnosis of acute or chronic camel brucellosis and is suited for mass screening. It is hoped that this method will contribute to improving diagnosis, eradication and control of *Brucella* infection in camels.

**IAEA-SM-348/3P**

**COMPARISON OF ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA), INDIRECT HAEMAGGLUTINATION TEST (IHA) AND SLIDE AGGLUTINATION TEST (SAT) FOR SCREENING OF *Pasteurella multocida* ASSOCIATED WITH HAEMORRHAGIC SEPTICAEMIA IN CATTLE AND BUFFALO OF PAKISTAN**

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1. **AIMS**

Haemorrhagic septicaemia (HS) is an acute infectious epidemic disease of cattle and buffalo caused by *Pasteurella multocida*. The disease is prevalent sporadically throughout the year but may occur in epizootic form during the rainy season. The organism associated with this disease is heterogeneous in its characteristics.
Many attempts have been made to subdivide these organisms into types which bear some relation to host species. Some isolates obtained from the clinical cases have been identified as Carter's type B. However, no systemic studies of the organism as it occurred during the subsequent years has been made hitherto. The knowledge of occurrence of various serotypes of *P. multocida* is important for the determination of reservoirs of infection and the geographical spread of this organism. This is also important for the production of autogenous vaccine and the recognition of the prevalence of new serotypes. Keeping in view the above considerations, the present work was planned to categorize the strains of *P. multocida* isolated from cattle and buffalo in order to learn about the prevalence of serotypes. In this investigation, an attempt was also made to compare the status of efficiency and sensitivity of SAT, IHA and ELISA.

2. METHODS

The evaluation system is one of the critical factors. The comparative studies were carried out to predict the sensitivity of three evaluation systems, and screening was planned to be done by SAT, IHA and ELISA. A total of 45 strains of *P. multocida* were tested during these studies.

SAT: SAT was carried out according to the procedure described by Namioka and Murata in 1961.

IHA: IHA test was performed according to Carter's method in 1955.

ELISA: ELISA was done according to the procedures described by Kemeny and Challacombe in 1989.

3. RESULTS

SAT: The behaviour of all field isolates was not uniform. The antisera against Robert's type I showed strong agglutination, except for seven strains which were less agglutinated. As Robert's type I is equivalent to Heddleston's type 2, Carter's type B and Namioka and Murata's types 6:B and 6:E, which cause HS in cattle and buffalo, all strains of *P. multocida* collected from various sources fell in Heddleston's type 2.

IHA: Of the 45 strains tested, 84% were type B, 9% were type D and 7% were untypeable. By the IHA test, the capsular antigen of type B strain reacted with the whole antigen. They did not react at all to the type E antiserum. However, the capsular antigen of type E strain reacted with both types E and B antisera.

ELISA: The results of ELISA were parallel to that of IHA and clearly indicated that all the field isolates belonged to capsular type B.
4. CONCLUSIONS

In Pakistan, HS is generally regarded as a peracute or acute disease often associated with sudden death. Consequently, it has been confirmed that HS in this area is caused by Carter’s type В of *P. multocida*. In comparing efficiency and sensitivity of the three tests, ELISA was found to be the most sensitive.

IAEA-SM-348/4P

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) USED FOR THE DETECTION OF *Cysticercus bovis* INFECTION IN CATTLE

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1. AIMS

Sensitivity and specificity of an ELISA for the detection of circulating antibodies in the sera of cattle naturally infected with *Taenia saginata* cysticercosis by using *T. hydatigena* cyst fluid antigen were studied in order to allow the detection of the infection prevalence and prescreening of the suspected animals at slaughterhouses.

2. METHODS

Antigens used for the ELISA were prepared form *T. hydatigena* cyst fluid. A pooled collection of cyst fluid, after filtration and centrifugation, was used as a crude fraction antigen or concentrated and fractionated by ammonium sulfate saturation. Both a crude fraction (ThCF-C) and an ammonium sulphate soluble fraction (ThFAS-L) of *T. hydatigena* cyst fluid were further evaluated for use in the ELISA of bovine cysticercosis. Sensitivity and specificity of the ELISA were examined by using naturally infected animal sera and hyperimmune sera.
Antigenic fractions were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to compare the protein patterns. After electrophoresis, the separated bands were transferred to nitrocellulose membrane by western blotting.

3. RESULTS

The crude *T. hydatigena* cyst fluid antigen (ThCF-C) cross-reacted with common cattle parasites (*Fasciola hepatica* and *Echinococcus granulosus*) and was found to be unsatisfactory in the ELISA. An ammonium sulphate soluble fraction of *T. hydatigena* cyst fluid (ThFAS-L) was shown to have high specificity in the ELISA for the detection of bovine cysticercosis. With hyperimmune serum samples, it was found that the intensity of the viable metacestode burden had a direct effect on the first appreciable rise in the ELISA values around four weeks post-infection. The minimal detection level was 100 live cysticerci in cattle. Among the naturally infected cattle, the sensitivity of the ELISA was poor, only 9% of the 44 proven cases were diagnosed. No negative sera gave false positive results in the ELISA.

SDS-PAGE indicated that both ThCF-C and ThFAS-L fractions were composed of high (MW 45 000 to 66 000) and low (MW 13 000 to 29 000) molecular weight proteins. Protein immunoblot analysis showed that a low molecular weight protein (MW 21 800) of ThFAS-L had a diagnostic significance. ThCF-C did not show any specific reaction during the immunoblotting procedure.

4. CONCLUSIONS

Sensitivity and specificity of the ELISA for the diagnosis of *T. saginata* cysticercosis were studied. The specificity of the ELISA, by using ThFAS-L antigen, was found to be quite high when the hyperimmune serum samples of animals contained relatively high numbers of metacestodes. There was a direct relationship between the intensity of infection and the ELISA values of hyperimmune serum samples. Although ThFAS-L did not cross-react with *F. hepatica* and *E. granulosus* and gave no false positive reactions, it is not possible to regard this test as sensitive enough to detect naturally and weakly infected animals. Sensitivity and specificity of the ELISA may be improved by elution of the protein of MW 21 800, which showed a diagnostic significance during immunoblot analysis. At present, the ELISA using ThFAS-L antigen has limited use in the diagnosis of cysticercosis infections in cattle.
1. AIMS

The aims are to develop a sensitive and specific assay for the routine diagnosis of the porcine reproductive and respiratory syndrome (PRRS) in field samples using RT-PCR amplification of conserved genomic regions and to establish the sensitivity of the RT-PCR for the detection of PRRSV in pig sera experimentally infected with PRRSV and PRRSV infected cell culture by comparing different extraction methods.

2. METHODS

Four commercial RNA extraction methods (Trizol LS, Ultraspec-3 RNA, QIAamp viral RNA QIAamp tissue kits) were compared. The sensitivity of the methods was determined by extraction of RNA from 10-fold dilutions of PRRSV diluted in PRRSV negative serum and in Eagle minimal essential medium, followed by the amplification of extracted nucleic acids by RT-PCR.

Twenty-five isolates of PRRSV from different regions of the United States of America were analysed by using different sets of primer pairs from ORF 4, 6 and 7 of the American strain (ATCC VR 2332) and ORF lb of the Lelystad strain of PRRSV. The reverse transcription of RNA was carried out at 42°C for 15 min, at 99°C for 5 min, and at 5°C for 5 min and stored at 4°C. For the single and first stage of nested PCR, after an initial denaturation step at 93°C for 4 min, the amplification was performed by using 30 cycles of 93°C for 45 s, 56°C for 30 s, and 72°C for 10 min.
Nested PCR was achieved by amplifying 3 µL of the first stage under the reaction condition using inner primer pairs. The specificity of the amplification was demonstrated by the size of the PCR products on gel electrophoresis and confirmed by southern blot hybridization. Gels were blotted onto a nylon membrane, denatured, cross-linked by UV light and hybridized with a digoxigenin labelled internal probe at 42°C for 2 h.

3. RESULTS

The QIAmp viral RNA was found to be the most sensitive, detecting an equivalent of 10 TCID₅₀ of the virus in 100 µL of the sample. The specific amplification from all the isolates was obtained with primers from ORF 7, the gene encoding the nucleocapsid protein. Oligonucleotide primers from ORF 4 and 6 detected 92% of the isolates, whereas primer from ORF 1b detected 88% of the isolates.

4. CONCLUSIONS

The results of this study indicate that RT-PCR is a suitable assay for the detection of PRRSV in swine sera, the sensitivity of the test being 10 TCID₅₀ by direct gel visualization. The sensitivity of the test was affected by the extraction method used and should be considered in the optimization of the assay. Pairs of primers derived from ORF 7 may be used as a potential target for the detection of PRRSV strains by RT-PCR. An analysis of a large number of field isolates from different parts of the world should be done to confirm these observations.

IAEA-SM-348/6P

RECONSTRUCTION AND REHABILITATION
OF VETERINARY SERVICES IN SOMALIA

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1. INTRODUCTION

Most parts of Somalia have experienced a total collapse of all governmental services, and particularly that of the veterinary services, well before the physical destruction by the late military regime and the civil wars. For many years, the more
affluent and independent livestock traders have been the target of a wide range of physical and economic destruction by the military regime. The most striking effect was the strangulation of livestock trade, which had been the backbone of the economy and the only source of income for many millions of Somalis. At present, and like other essential services, the veterinary services and the related infrastructure such as veterinary clinics, diagnostic laboratories, veterinary quarantine, etc., are virtually non-existent.

2. BACKGROUND

Owing to their physical and climatic conditions, the Somali rangelands sustain well over 60% of the country’s population and a large population of livestock comprising 4.6 million cattle, 6.3 million camels, 19.5 million goats and 11 million sheep.

3. CURRENT SITUATION

At present, more than 60% of the population is living on nomadic and semi-nomadic range livestock production systems. Because of the total collapse of the entire infrastructure, this system experiences huge problems which may be summarized as follows:

- Total collapse of vaccination cover;
- Total collapse of drug supply and distribution systems;
- Lack of control of vector borne diseases;
- Lack of quarantine facilities and services near the major ports;
- Total collapse of marketing health control;
- Poor port and port related facilities;
- Short supply of livestock fodder.

As a live animal exporting country, Somalia faces great economic problems, resulting from ever-growing international live animal trade restrictions as are imposed on all countries without adequate veterinary services.

4. PROGRAMME JUSTIFICATION

According to the livestock population figures given above, there are huge resources which must be developed and adequately utilized. On the other hand, the regions under review can rarely sustain agricultural production. The proposed
programme will take the above mentioned points into consideration and undertake problem identification and analysis. Emphasis will be put on the rehabilitation of the veterinary infrastructures and on ways to improve livestock export facilities and reintroduce marketing health control systems.

**Major components of the programme**

1. Review all previous veterinary services;
2. recommend rehabilitation and restructuring programmes in accordance with regional needs;
3. recommend ways to improve livestock health services;
4. introduce nomadic animal health care systems;
5. work out guidelines for establishing veterinary quarantine facilities;
6. work out a reconstruction programme for marketing centres, export holding pens, auction grounds and quarantine facilities at major ports;
7. identify the location and number of veterinary clinics, laboratories and veterinary quarantine facilities needed in various regions.

IAEA-SM-348/7P

**APPLICATION OF C-ELISA FOR THE SERO-SURVEY OF RINDERPEST VIRUS ANTIBODY IN CATTLE AND GOATS IN BANGLADESH**

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1. **AIMS**

The aim is to study the applicability of C-ELISA for seromonitoring of antibodies against RP virus in ruminants in Bangladesh. As no outbreaks of RP have been
reported since 1957, a broader belt rinderpest vaccination programme is being carried out in order to protect cattle from rinderpest originating from neighbouring States of India. However, the emergence of rinderpest-like epidemics in goats has been noticed for the last three years. Thus, the use of the rinderpest competitive ELISA technique will be helpful to measure the current status of rinderpest serosurveillance as well as to confirm the rinderpest-like epidemics in goats as peste des petits ruminants (PPR) in Bangladesh.

2. METHODS

Sera: A total of 1500 sera from cattle and goats were tested. One thousand randomized cattle sera were collected during 1990 and 1991 from four different selected regions of the country. Five hundred goat sera were also collected from the western districts of Bangladesh during the epidemics of rinderpest-like disease in goats. All serum samples were preserved at -20°C until use.

Serum testing: The rinderpest competitive ELISA (C-ELISA) was used for the analysis of serum samples at the virology laboratory of the Bangladesh Livestock Research Institute. The test protocol was performed exactly as detailed in the C-ELISA kit manual. The C-ELISA results were read by computer controlled Immunoskan Plus and analysed by using an EDI version 2.11 software package supplied by the Joint FAO/IAEA Division, Vienna. The data/results were further analysed by using Lotus 123 graphic (Release 5).

3. RESULTS

One thousand cattle sera collected during 1990 and 1991 were tested and only 15 (1.5%) of them were shown to have immunity against rinderpest. This indicates the absence of circulating rinderpest virus in cattle. Three hundred goat sera were tested by using C-ELISA collected during rinderpest-like epidemics in goats and none of them showed the presence of rinderpest specific antibody. Later, PPR virus was confirmed to be the cause of goat epidemics in Bangladesh.

4. CONCLUSIONS

Results obtained from a limited number of serum analyses indicate that very few animals are seropositive; most of these animals were found in the areas close to the border with the Indian States of West Bengal, Assam and Meghayla. It should be
acknowledged that there is limited control of livestock movements from India. Thus, it is strongly believed that these animals have been brought into Bangladesh by farmers for draft or milk purposes. However, only 15 samples out of 1000 cattle were positive, which represents 1.5% of 1000 cattle sampled or 0.004% of the total cattle population in Bangladesh. At this stage, it is important to establish whether such seropositivity is due to the circulating rinderpest virus or to seropositive animals brought into the country. However, the last epidemic of rinderpest was in 1957, with no signs and/or symptoms of rinderpest; then, the presence of rinderpest virus in Bangladesh would seem to be very unlikely. On the other hand, severe epidemics of PPR in goat and the absence of rinderpest antibody in goat sera are further evidence of the absence of rinderpest virus. However, before a provisional declaration of freedom from rinderpest in Bangladesh, a well designed nationwide sero-survey is needed.

IAEA-SM-348/8P

PROTECTIVE EFFICACY
OF A Pasteurella haemolytica Serotype A2
OUTER MEMBRANE PROTEIN POLYSACCHARIDE (OMP-PS)
COMPLEX VACCINE IN SHEEP

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1. AIMS

The aim is to study the immunogenicity of the crude and purified OMP-PS complex in conventional sheep and to determine the possible contribution of this candidate antigen to Pasteurella vaccines. This allows improvements of the existing vaccines for an effective control of pneumonic pasteurellosis in small ruminants.
2. METHODS

The vaccines contained a lyophilized crude or purified OMP-PS complex of *P. haemolytica* A2, resuspended in distilled water to contain a standard concentration of 10 µg/mL sialic acid. Two vaccine preparations consisting of a crude and a purified OMP-PS vaccine complex were administered to two treatment groups in each age group. The remaining group of lambs and ewes were sham vaccinated and served as controls. The sera collected were later used for mouse protection tests. Indirect haemagglutination (IHA) test, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed on all sera of the tested sheep and mice.

3. RESULTS

The purified OMP-PS vaccine stimulated a highly significant (*P < 0.05*) IHA antibody response to *P. haemolytica* A2 in adult sheep when compared to the crude OMP-PS vaccine and sham vaccinated group. The superior serological response in the group of ewes which received the purified material compared to the crude complex vaccine group was maintained throughout the duration of the experiment. The mean serum antibody titres of the sheep in the sham vaccinated control groups remained almost constant (<1:8) throughout the experiment. The viable counts in the livers of mice immunized with the purified OMP-PS complex vaccine were significantly lower (*p < 0.004*) than the counts in the other two groups, indicating good protection afforded by the vaccine. The result of immunoblotting showed that the sera from vaccinated animals recognize numerous antigens in the A2 whole cells, indicating successful immunization. There are strong reactions with all the sera from the vaccinated group against the protein in the 42 kDa region. The result also indicated that the polysaccharide material present in the purified complex was important in this protection.

4. CONCLUSIONS

The trial has indicated that the purified preparation of OMP-PS complex vaccine was most successful in enhancing the serological response. The complex was immunogenic in adult sheep, with induction of humoral anticapsular antibody in the IHA test and with OMP in immunoblotting. The sera from adult sheep immunized with OMP-PS passively protected mice against A2 challenge. The study has demonstrated that immunity and protection in sheep are not confined to vaccination with live organisms, and that the OMP-PS can act as very effective vaccines in mice and sheep.
Comparative Evaluation of Competitive ELISA Test in Colombian Cattle

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1. Aims

The main purpose of this study was to contribute to the definition of the best ELISA test to be used both for screening and differential diagnosis of Brucella abortus to be applied for control programmes.

2. Methods

A total of 2971 sera from Colombian cattle were tested for brucellosis using conventional agglutination tests, the buffered plate antigen test (BPAT), the Rose Bengal (RB) and the complement fixation test (CFT) [1]. The radial immunodiffusion test (IDR) was also performed [2]. The sera were also tested by using four different ELISAs: the indirect ELISA from the Joint FAO/IAEA Division, the indirect ELISA modified by Nielsen et al. [3] and two competitive ELISAs; one competitive ELISA used B. abortus o-polysaccharide as the antigen and an enzyme conjugated monoclonal to the o-polysaccharide for competition and detection [4]. The second competitive ELISA used lipopolysaccharide as antigen, a different monoclonal antibody specific for the o-polysaccharide and a commercially available goat anti-mouse IgG enzyme conjugate [5]. The sera were analysed on the basis of the population status; 987 positive sera were obtained from Brucella abortus infected herds based on clinical and/or bacteriological evidence of Brucella abortus and a high prevalence of brucellosis; the CFT percentage of positive animals in the herd was greater than 5%. 866 negative sera were obtained from non-vaccinated cattle from a brucellosis free area of the country, and 1118 negative sera were obtained from appropriately vaccinated cattle from areas in a free herd programme. Initial cut-off values
were derived by using negative serum samples [3]. The diagnostic sensitivity and specificity were defined from frequency histograms based on these cut-off values and using $2 \times 2$ tables, and confidence limits (95%) were calculated. The data were also analysed by using signal detection analysis (ROC).

3. RESULTS

The local cut-off values were defined as 30% for the FAO/IAEA Joint Division indirect ELISA and as 50% for ADRI, at which values the sensitivity was between 97.84 and 100% and the specificity was between 99.11 and 100% for both tests. For the competitive tests, the cut-off values were 30 and 29%, respectively, for CELISA1 and CELISA2. As expected for the vaccinated population in areas where vaccination is commonplace or mandatory, the specificity values were lower for all tests since ELISA detects more vaccinated animals than CFT. The competitive ELISA CELISA1 confirmed its capacity for differentiation between vaccinated and infected animals because of its high sensitivity of 99.08% compared with the lower sensitivity of 93.02% and a specificity of 100% of the well accepted radial immunodiffusion (RID). The CELISA2 sensitivity and specificity values were 98.62% and 97%, respectively, and 94.88% for vaccinates. The comparison between ELISAs yielded high kappa agreement and reliable confidence limits. The accuracy estimates are over 0.95 for all ELISA tests. Frequency distribution and ROC analysis were plotted and compared for optimizing the definition of the cut-off values.

4. CONCLUSIONS

The ELISA tests were standardized and validated and the cut-off values defined for local conditions. The indirect ELISA demonstrated a higher diagnostic specificity than BPAT, RB and CFT without compromising diagnostic sensitivity. The test offers a distinct diagnostic advantage as a laboratory based screening assay. The competitive ELISA is capable of discriminating between infected cattle and those who have been vaccinated or exposed to a cross-reactive organism. Lower values than expected for CELISA2 could be explained on the basis of aberrant results from sera of recently vaccinated animals stemming from areas in which adult vaccination could not be excluded. The data presented permit us to continue supporting the initial hypothesis that the ELISA methodology as designed for brucellosis will provide a more precise and standardized method for diagnosis and support of control and eradication campaigns.
REFERENCES


IAEA-SM-348/10P

DETECTION OF BLV INFECTION BY ELISA AND PCR

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1. AIMS

The aim of the present study was the detection of bovine leukemia virus (BLV) infection by the serological test (ELISA, agar gel immunodiffusion (AGID)) and direct detection of proviral DNA in peripheral blood mononuclear cells (PCR test). Sixty cows from a herd naturally infected with BLV and 24 animals from a healthy herd were selected for this study.

2. METHODS

*Indirect ELISA*. BLV for the ELISA test was isolated from the supernatant of cultured fetal lamb kidney (FLK) cells by the sucrose gradient ultracentrifugation method. The microplates were coated with purified BLV antigen and incubated
overnight in a refrigerator. Serum samples diluted 1:100 were tested in duplicate. Rabbit antibovine IgG_I conjugated to horseradish peroxidase was used.

Detection of proviral DNA. The DNA from the whole blood cells was extracted with tetradecyltrimethylammonium bromide. The primer sequences used were designated to amplify DNA targets in the env gene specific for BLV (Murtaugh et al., 1991). Each sample was amplified in a final volume of 50 µL for 35 cycles in a DNA thermal cycler. In order to visualize the yield, 20 µL was examined in agarose gel electrophoresis at 110 V for 1.5 h.

3. RESULTS

Thirty-three per cent (20/60) of cattle from two BLV infected herds were seropositive by ELISA, and, among them, 12 cows were found seropositive by AGID and ten cows (10/20) were found provirus positive by PCR. However, proviral DNA was detected in two cows from a healthy herd which were negative by ELISA and AGID.

4. CONCLUSIONS

This study presents the data of methods for the diagnosis of BLV infection: AGID, ELISA and PCR. It may be inferred that ELISA detects infected cattle earlier and with higher sensitivity than AGID. The presence of antiviral antibodies is a reliable indicator of BLV infection, but represents only indirect evidence of past or current infection. Bovine leukemia is an oncogenic retrovirus associated with enzootic leukosis. The disease is characterized by persistent lymphocytosis occurring in more than 30% of BLV infected cattle. The virus is silent in the majority of infected cells in vivo; however, infected cattle develop antibody to BLV antigens. These results suggest that, depending on the stage of the infection, the pathogenesis of BLV in cattle may involve fundamental differences in the host–virus relationship, including the number of cells infected or the number of copies of integrated provirus per cell, the regulation of the expression of viral antigens and the induction of the antiviral immune response.

Detection of integrated proviral DNA using PCR may be an alternative to serological methods of BLV diagnosis.
CONGLUTINATING COMPLEX FIXATION TEST (CCFT)
AS A METHOD ALTERNATE TO
ENZYMELINKEDIMMUNOSORBENTASSAY(ELISA)

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1. AIMS

An enzyme linked immunosorbent assay (ELISA) has proved to be the most versatile and sensitive method for the serological diagnosis of infectious animal diseases. However, other assays offer similar advantages. This has been shown by our research through the development of a conglutinating complex fixation test (CCFT). This is analogous to the complement fixation test (CFT) and is based on the phenomenon of conglutination. The limiting factor in this test is the source in cattle blood.

2. METHODS

For testing by the CCFT, test bovine sera are inactivated at 60°C for 30 min. Brucella antigen and conglutination serum are then added, after which they are maintained at 37°C for 30 min. The indicator system consisting of sheep red blood cells and haemolysin is added. The mix is maintained for one hour at 37°C and for 2 h at room temperature. Conglutination of red blood cells represents a negative result whereas absence of this phenomenon is regarded as a positive result.

3. RESULTS

The CCFT was used to examine brucellosis in 1186 samples of cattle serum from an infected herd. Other serological tests were also made on these sera. Positive results were obtained in CCFT in 88 and on ELISA in 86 cases, agreement occurring in 82 cases. Brucellosis was confirmed on LA in 39, on CFT in 54 and on RBPT in 63 cases; total results of these tests were obtained in 79 cases.
4. CONCLUSIONS

CCFT compared favourably to ELISA in the diagnosis of bovine brucellosis in terms of sensitivity and was superior to LA, CFT and PBPT.

IAEA-SM-348/13P

SEROEPIDEMIOLOGY OF RINDERPEST
IN BOVINES IN SRI LANKA
USING THE ENZYME LINKED
IMMUNOSORBENT ASSAY (ELISA) TECHNIQUE

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1. AIMS

The aim was to calculate the prevalence percentage of antibodies against rinderpest virus ('prevalence') in bovines (cattle and buffalo) in Sri Lanka and to study the factors associated with seroprevalence.

2. METHOD

Approximately 0.2% of the bovine population (n = 4397) in 23 of 25 districts were sampled by using a stratified random sampling procedure, during a 39 month period beginning from June 1992. The number of bovines sampled from each district was statistically large enough for the presence of rinderpest antibodies to be detected, assuming a 5% island wide prevalence at 95% confidence level. Serum antibodies were detected by using the competitive enzyme linked immunosorbent assay (ELISA) supplied by the IAEA. A bovine with a percent inhibition value of >50% was considered positive. Data were summarized by cross-tabulating the test results with potential risk factors and calculating the odds ratios.
3. RESULTS

The median number of farms visited per district was 36 and varied from 15 to 122. The overall prevalence was 8% and varied from 0% in eight districts to 40% across other districts. The prevalences in non-vaccinated (n = 4101) and in vaccinated (n = 296) bovines were 5 and 14%, respectively. Bovines in nine of the districts sampled had never been vaccinated against rinderpest. Surprisingly, the seroprevalence was relatively low in the vaccinated bovines sampled. Most seropositive bovines (69%) were more than four years old and were at four times higher odds of being seropositive compared to less than one year old animals. Bovines from the dry zone (annual rainfall: 20–35 in. (about 50–90 cm)) low country (DL) were at ten times higher odds of being seropositive than those from the intermediate zone (annual rainfall: 35–85 in. (about 90–220 cm)) low country. High seropositivity in DL may be attributed to the higher bovine density (0.3–0.5 bovines/hectare) in that region. A majority of bovines in DL were reared under an extensive management system, where they grazed freely during the day and were kept in open paddocks during the night. None of the bovines from intermediate zone up-country and the up-and mid-countries of the wet zones (annual rainfall: >55–125 in. (about 140–320 cm)) or from the lower bovine density (<0.3 bovines/hectare) areas were seropositive.

4. CONCLUSIONS

This is the first island wide study on seroprevalence of rinderpest in Sri Lanka. This study shows the advantages of using a serologically sensitive test, such as ELISA, in studying the seroepidemiology of a disease with low prevalence. The prevalence was highest in the northern, eastern and north-central provinces. The spread of rinderpest from the first location of the outbreak, which was in the eastern province in 1987, is attributed to the movement of bovines for slaughtering purposes. It appears that the spread of rinderpest could be reduced by controlling animal movement. Apparently, rinderpest had shifted form an epidemic form in the 1987–1989 period to an endemic form from 1990 onwards, towards areas with high bovine density (>0.3 bovines/hectare). Furthermore, the extensive management system mostly practiced in the DL regions, in which animal-to-animal contact is more frequent, had contributed to the spread of rinderpest. The prevalence was higher in older bovines, probably because of exposure to natural infection during the last epidemic.
EVALUATION OF AN
ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)
IN THE SERODIAGNOSIS OF SWINE TRYPANOSOMOSIS

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1. AIMS

Trypanosoma evansi antigen was prepared for the detection of trypanosomal antibodies infected with *T. evansi* in swine using an enzyme linked immunosorbent assay (ELISA). This antigen had to be specific for *T. evansi* and showed no cross-reactions in pigs infected with *Toxoplasma* sp. and *Eperythrozoon* sp., which are the important parasites causing abortion in pregnant sows in Thailand.

2. METHODS

Trypanosomal antigen was prepared from *T. evansi*, NIAH-2, originally isolated from naturally infected cattle from Patoomthani, Thailand, in 1993. The parasites were separated from mouse blood by ion exchange chromatography on diethylaminoethyl (DEAE) cellulose. The separated trypanosomes were washed and then sonicated for 5 min. The material was centrifuged at 36,000g for 1 h at 4°C. The supernate was used as partially purified somatic antigen. The working dilutions of the antigen, serum and conjugate were selected by determining the maximum ratio of the positive and negative optical density (OD) values in a test system. Recombinant protein G peroxidase conjugate was used in the assay. The assay was evaluated by using sera and EDTA treated blood from a pig experimentally infected with *T. evansi*, nine pigs from Thailand naturally infected with *T. evansi*, 204 pigs imported from countries free from trypanosomosis, 61 pigs from various farms in Thailand that were positive for *Toxoplasma gondii* by the latex agglutination test and three pigs naturally infected with *Eperythrozoon* sp.

3. RESULTS

In the experimentally infected pig, the assay could first detect antibody against *T. evansi* on day 17 post-infection and the highest OD value was on day 73. By comparing the parasitological detection of *T. evansi*, it was found that the parasites were
detected by blood smear only on day 13 and by mouse inoculation on days 2 to 31 post-infection. However, *T. evansi* would be found in mouse blood on days 16 to 21 after inoculation. In addition, the assay detected antibodies in all pigs naturally infected with *T. evansi*. The specificity of the test, based on assays with imported sera, was 94.7%. These was no evidence of cross-reactions with sera from pigs infected with *Toxoplasma* and *Eperythrozoon*.

4. CONCLUSIONS

In this study, the *T. evansi* antigen used in the ELISA was specific and sensitive for the detection of antibodies against *T. evansi* in pigs, the antibody being detected in an experimentally infected pig on day 17 post-infection and until the end of the study. In pigs naturally infected with *T. evansi*, the assay yielded also positive results with high OD values. In uninfected pigs from countries free from trypanosomosis, it only gave a low false positive value. There were no cross-reactions in pigs infected with *Toxoplasma* and *Eperythrozoon*. Even though peroxidase recombinant protein G conjugate, instead of specific anti-IgG swine conjugate, was used, it was still effective in ELISA. The antigen was characterized by western blot, and it was found that the predominant polypeptide band was a 67 kD protein. In Thailand, trypanosomosis is found not only in pigs, but also in cattle, buffalo, horses and dogs. Trypanosomosis is widespread in Thailand and difficult to eliminate, so that Ab-ELISA will be an important tool for the diagnosis and epidemiological study of the disease in different animal species, including pigs.

IAEA-SM-348/15P

MOLECULAR CLONING AND CHARACTERIZATION OF DUCK HEPATITIS VIRUS cDNA

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1. AIMS

The aim was to determine the complete nucleotide sequence of duck hepatitis virus (DHV) and compare this sequence with that of the other picornaviruses. The
DHV cDNA has been cloned, which will enable us to demonstrate the genome structure of the DHV RNA and the translation mechanisms of the viral protein. Also, we can use the cDNA probes to detect DHV in clinical samples.

2. METHODS

The DHV RNA was extracted by magnetic separation from virion purified by affinity chromatography from the allantoic fluid of infected chicken egg embryos. After ethanol precipitation, the RNA served as a template from cDNA synthesis using alfalfa mosaic virus (AMV) reverse transcriptase. After treatment with T4 DNA polymerase to flush the ends, the double stranded cDNA was ligated to an EcoR I adaptor and the excess adaptor removed with a sephacryl S\textsubscript{400} spin column. The adaptor cDNAs were ligated to EcoR I, cut plasmid PUC19, which was dephosphorylated. The resultant combination molecules were used to transform \textit{E. coli} strain DH\textsubscript{5a} by standard procedures. Clones containing putative DHV sequences were screened by cleaving recombinant plasmid preparations with EcoR I and were sized by using gel electrophoresis. Cloned cDNA inserts isolated from the low melting point gels were labelled with digoxigenin and used as probes in hybridization (1) to electrophoretically separated RNA bound to nitrocellulose paper and (2) to DNA bound to nitrocellulose paper after lysis of bacterial colonies in situ for further screening.

3. RESULTS

Double stranded cDNA was synthesized from DHV RNA with oligo (dT)\textsubscript{15} primer and AMV RT. We harvested 112 clones with the insert fragments of 0.5–0.7 kb. The clones were authenticated hybridization to RNA extracted from DHV infected chicken embryos. Also in this experiment, we found that the efficiency of the \textit{E. coli} strain DH\textsubscript{5a} transformation was greatest for cells from cultures at optical densities (OD 600) of 0.9.

4. CONCLUSIONS

DHV cDNA has been cloned with a size of the insert fragments of 0.5–0.7 kb. The cDNA clones were authenticated by northern hybridization. The availability of cloned DHV cDNA should make it possible to deduce RNA and amino acid sequences, both for use as a probe of clinical specimens and as to the presence of DHV genome.
DIAGNOSTIC EVALUATION OF PCR IN GOATS INFECTED WITH Trypanosoma brucei brucei

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1. AIMS

The aim was to evaluate the efficacy of the polymerase chain reaction (PCR) for the diagnosis of Trypanosoma brucei infections in goats, as compared with four parasitological methods, i.e. classical wet blood film, sodium dodecyl sulphate (SDS) haemolysed wet blood film, microhaematocrit buffy coat wet film and direct examination.

2. MATERIALS AND METHODS

Seven female Saanen goats, 12 to 18 months old, were kept in a flyproof stable near Antwerp. White Swiss mice were intraperitoneally inoculated with a cryostabilate of a pleomorphic clone of T. brucei brucei AnTat 1.1 E. After three days, infected mouse blood was diluted with PSG (phosphate, saline, glucose) to a concentration of about $10^4$ viable trypanosomes per millilitre. Each of the seven goats was subcutaneously inoculated with 1 mL of this trypanosome suspension. One goat died after four weeks. Six weeks post-infection, the six surviving animals were treated by a single 1 mL injection of 7 mg/kg diminazene aceturate (Berenil*, Hoechst). During six weeks before and three weeks after treatment, blood from each animal was examined weekly for the presence of trypanosomes by using two versions of wet blood film and two versions of microhaematocrit buffy coat technique. At each sampling, 30 µL of heparinized blood was spotted onto Whatman No. 4 filter paper, allowed to air dry, packed in plastic bags with silica gel and kept at -20°C for up to two months. DNA extraction for PCR was made according to Ref. [1], using Chelex 100 Biorad. Three different primer sets were used: a trypanozoon specific ORPHON5J, a T. vivax TVW [2] and a T. congolense GOL set (i.e. modified TCN of Ref. [3]).
3. RESULTS

All pre-infection samples yielded negative results. After one week of infection the PCR with ORPHON5J primers was positive in six of the seven goats while no trypanosomes were detected at that time. Later on, this PCR consistently gave positive results with all blood samples collected before drug treatment, thus yielding an overall sensitivity of 97%. The first trypanosome findings were made after two weeks of infection. Parasitaemia was currently very low. The overall sensitivity of the combined parasitological tests was 74.3%. The sensitivity of the individual techniques was, respectively, 49% for the classical wet blood film, 72% for the SDS clarified wet blood film, 69% for the buffy coat wet film and 69% for the buffy coat in situ examination. The PCR with the *T. vivax* TVW primers was always negative. The PCR with the *T. congolense* GOL primers yielded some non-specific amplification bands corresponding to short length DNA sequences.

4. CONCLUSIONS

PCR was clearly the most sensitive technique of all (97%), allowing early detection of the infection. The protocol used is relatively simple and merits further evaluation in animals under field conditions.

REFERENCES


DIAGNOSTIC EVALUATION OF PCR IN GOATS EXPERIMENTALLY INFECTED WITH *Trypanosoma vivax*

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1. AIMS

The aim was to evaluate the efficacy of the polymerase chain reaction (PCR) for diagnosis of *Trypanosoma vivax* infections in goats, as compared with two conventional methods, i.e. microhaematocrit buffy coat and wet blood film examination.

2. MATERIALS AND METHODS

Six healthy female Saanen goats, about one year old, were kept in a flyproof stable near Antwerp. The goats were inoculated both 1 mL and intravenously with 1 mL cryostabilate of *Trypanosoma vivax* stock EATRO 1185 containing antilog 6.6 viable trypanosomes per mL. After 15 weeks of infection, all the animals were treated by a single intramuscular injection of 7 mg/kg diminazene aceturate (Berenil®, HOECHST). During a total observation period of 18 weeks, the evolution of parasitaemia was monitored weekly by wet blood film (matching method [1]) and microhaematocrit buffy coat examination [2]. At each sampling, about 30 mL of heparinized blood was spotted onto filter paper Whatman No. 4 for PCR assays. Filter papers were air dried, packed in plastic bags with silica gel and stored at −20°C for up to two months. DNA extraction was made according to Ref. [3] using Chelex 100 Biorad. The TVW primers used have been described in Ref. [4].

3. RESULTS

All tests were intermittently positive for a varying period of time before drug treatment but never thereafter. PCR yielded twice as many positive results as the parasitological techniques. On one occasion only (one goat, week 4), PCR was negative and the parasitological tests were positive. A paired sample t-test (p = 0.0004) revealed a 25 ± 7% increased sensitivity for PCR in comparison with the combined
parasitological techniques. The overall sensitivity of the combined parasitological tests was 21%. The sensitivity of the individual techniques was, respectively, 18% for the classical wet blood film, 21% for the buffy coat wet film and 44% for the PCR.

4. CONCLUSIONS

The present experiments suggest that it is possible to develop a relatively simple PCR protocol for detection of *T. vivax* DNA in dried blood samples. As compared with wet blood samples, dried blood spots can more easily be collected under field conditions and dispatched to the laboratory. Moreover, a better stability of the DNA is to be expected.

However, the diagnostic sensitivity of the present assay was not entirely satisfactory. This could be related to either the small volume of blood processed, i.e. about 10 μL only, or the type of primers used. Towards further improvement of the test system, it will also be useful to examine the relative contribution of whole trypanosomes and DNA fragments thereof in the test sample.

As regards specificity, preliminary results obtained in Belgian goats are encouraging. Apart from the here described negativation of the assay after drug treatment, completely negative results have also been obtained in seven other non-infected goats.

REFERENCES


VALIDATION SCHEME FOR DIAGNOSTIC TESTS

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1. AIMS

The aim was to develop a scheme for the validation of assays developed in the research laboratory for the successful transfer and implementation in the diagnostic laboratory.

2. METHOD

After an assay has been developed and optimized in the research laboratory, the next logical step before implementation in the diagnostic laboratory is validation. This is the verification of performance characteristics of the assay and the determination of point estimates of diagnostic sensitivity and diagnostic specificity. This validation process includes the definition of reference negative and reference positive samples which can be defined according to culture status or, if culture status is unavailable, according to serological reactions on a combination of other serological assays. After the samples have been defined the new assay is compared with the reference standard and/or the in-use assays. The results of the comparisons are transferred to a database for analysis. By using the reference negative data, a tentative cut-off value is determined and then used to initially analyse the reference negative data for diagnostic specificity and the reference positive data for diagnostic sensitivity. Frequency distributions of the reference positives and the reference negatives are then plotted for visual confirmation of the cut-off. Finally, receiver operating characteristic (ROC) analysis of the data is performed to determine the optimal diagnostic specificity and diagnostic sensitivity of the data set. In addition, ROC analysis provides estimates of sensitivity and specificity for a range of cut-offs. A final decision on the cut-off and the resulting diagnostic sensitivity and diagnostic specificity is then recommended for the assay based on intended application. Before final implementation, the assay is transferred to the diagnostic laboratory, where the validation is continued by testing samples routinely submitted for analysis (sometimes referred to as beta site testing).
3. CONCLUSIONS

Although logistically, temporally and economically challenging, proper validation minimizes the chance of failure and increases the possibility of successful transfer and implementation in the diagnostic laboratory. A properly validated assay, which includes good design, good quality control and good documentation, reduces the chances of liability and increases confidence in the assay both nationally and internationally.

IAEA-SM-348/22P

DETECTION OF BOVINE RESPIRATORY Syncytial Virus (BRSV) BY PCR AND PHYLOGENETIC ANALYSIS AT THE GENETIC LEVEL

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1. AIMS

The aims were to develop polymerase chain reaction (PCR) assays for the specific detection of bovine respiratory syncytial virus (BRSV), the agent involved in the respiratory syndrome of cattle, and to study the use of PCR coupled with rapid sequencing for phylogenetic analysis of BRSV strains.

2. METHODS

The primers for specific detection of BRSV by RT-PCR were selected from F (fusion) gene (PCR-F) and from G (attachment) gene (PCR-G). PCR products were analysed on 2% agarose gels stained with ethidium bromide. The amplicons from
PCR-G were sequenced by the Sanger method with primers in both directions. The phylogenetic study was carried out by using the computer program package PHYLIP (neighbour joining method).

3. RESULTS

A nested PCR-F assay detected all BRSV strains tested providing a 481 bp DNA fragment. All strains were also positive by PCR-G assay providing a 371 bp band. The specificity of the PCR-F assay was confirmed by the cleavage of PCR amplicons using Sca I. Two visible electrophoretic bands (257 bp and around 100 bp) were detected only with BRSV strains. The sensitivity of the PCR-F assay achieved 0.1 TCID$_{50}$. Of 35 nasal swabs collected from naturally infected bulls, 31 were positive by both PCR assays, and 23 by immunofluorescence assay. However, none of the tested clinical samples was positive by virus isolation.

The results of phylogenetic analysis in the G gene region have shown that BRSV strains are grouped in two lineages represented by European and American strains.

4. CONCLUSIONS

Two nested PCR assays were developed for specific detection of BRSV with primers selected from the gene encoding the F fusion protein (PCR-F) and the gene encoding the G attachment protein (PCR-G). The PCR assays were more sensitive than virus isolation and immunofluorescence assay. BRSV strains were phylogenetically clustered according to their geographic origin.
IAEA-SM-348/23P

DETECTION OF CLASSICAL SWINE FEVER VIRUS (CSFV) IN CLINICAL SAMPLES BY RT-PCR ASSAY USING DIFFERENT PAIRS OF PRIMERS

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1. AIMS

The aim was compare the efficiency of RT-PCR assays using four pairs of primers selected from different regions of the CSFV genome for the detection of CSFV in clinical samples of swine and wild boars.

2. METHODS

Twenty clinical samples prepared from 17 swine and 3 wild boars which had died during the outbreaks of CSF were tested. The samples were prepared as 20% homogenates from liver, spleen, kidney and lymph nodes. RNA was isolated by a phenol–chloroform technique. The synthesis of cDNA was carried out with Moloney murine leukaemia virus reverse transcriptase and random primers. PCR primers were selected from the 5'-noncoding (5'-NC) genomic region and then from the E2, NS2 and NS5B regions. PCR products prepared from the 5'-NC region were cleaved by restriction endonuclease Bg/II. The amplicons were detected on 2% agarose gels stained with ethidium bromide.
3. RESULTS

All clinical samples tested were positive by virus isolation as well as by RT-PCR assays. The universal pestivirus 324/326 primers selected from the 5'-NC region provided 280 bp amplicons. To confirm their specificity for CSFV, the 284 bp DNA fragments were cleaved by Bg/II. The amplicons were cut into two bands (243 bp and 41 bp) indicating the CSFV origin. The S1/S2 primers selected from the NS5B region provided a 449 bp band. Further primers from the E2 (gp55L/gp55U) and the NS2 regions (gp54L/gp54U) resulted in the 307 bp and 467 bp fragments, respectively.

4. CONCLUSIONS

The four RT-PCR assays were able to detect CSFV in all 20 clinical samples which had been collected from dead swine and wild boars during the outbreaks of CSF in Slovakia in 1993 and 1994. The quality of the selected RT-PCR primers was determined as follows: gp55L/gp55U (E2), 324/326 (5'-NC), S1/S2 (NS5B) and gp54L/gp54U (NS2 genomic region). We conclude that gp55L/gp55U primers are the most suitable for direct detection of CSFV by RT-PCR in tissue homogenates of diseased animals.

IAEA-SM-348/25P

THE SERVICE LABORATORY — A GTZ-BgVV PROJECT:
HEALTH PROTECTION
THROUGH ADAPTED VETERINARY
DIAGNOSTIC TECHNIQUES

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The customary diagnostic methods of today have been developed in industrialized countries. High costs for personnel resulted in a trend towards automation and prefabricated test kits. Consequently, these techniques are not sufficiently adapted to local conditions in developing countries, where, as a rule, skilled and ancillary staff is available whereas foreign currency reserves for purchasing laboratory equipment and material from abroad are rather limited. Furthermore, the training of personnel
from developing countries has usually been oriented towards the non-transferable standards and methods of industrialized countries. This leads to a long term dependence of the diagnostic services on external funding. A diagnostic technology adapted to the specific local conditions of developing countries is needed to overcome this situation.

The project activities concentrate on serological diagnostic work. Here, basic knowledge of the common diagnostic techniques and their set-up for specific diseases, methods for the production of related reagents (antigens, antibodies, conjugates, complement, etc.) and cleaning procedures for the reuse of 'one way' plastic material is spread by training programmes, specific publications and information leaflets. For two of the more complex test procedures, the most frequently quoted prescribed test for international trade, CFT, and the increasingly important ELISA (OIE, Manual of Standards for Diagnostic Techniques, Paris, 1992), we have calculated the cost reduction potential of adaptation through self-production of reagents and reuse of plastic materials. Material costs per microtitre test plate for the diagnosis of brucellosis can be reduced from US $3.79 to 0.82 for CFT and from US $3.88 to 1.13 for ELISA. In comparison, commercial ELISA kits cost about US $80 to 90 per plate (e.g. Bommeli, IDEXX, Boehringer).

1. EXAMPLES OF SPECIFIC ACTIVITIES

1.1. IgY technology

Antibodies of good specificity can be produced very efficiently and economically in eggs of immunized hens. The housing and keeping of hens is simple, and antibodies are harvested by extraction from the eggs in amounts of 250–1000 mg per egg. Subsequently, antibody purification makes specific pathogen free conditions dispensable. Except for the ordinary CFT, these antibodies can be applied in the same way as mammalian antibodies, including production of conjugates.

1.2. Affinity chromatography

The possibility to selectively extract specific antibodies through affinity chromatography makes the great financial and technical input for the production of monoclonal antibodies dispensable for the vast majority of diagnostic applications. This method enables diagnostic institutions in developing countries to build up a sustainable production of high quality diagnostic antibodies. Now, the important advantages of purified polyclonal antibodies over monoclonal antibodies could be even more enhanced by a significant cost reduction in affinity chromatography. By replacing the common bead formed agaroses by pulverized polystyrene, the costs for
the chromatographic matrix can be reduced from US $6/g (Sepharose 4B) to US $2/kg (PS 158k).

1.3. Cleaning device for microtitre tips

With regard to material, one of the main cost factors related to the application of microtitre methods in serological diagnosis is the consumption of one way plastic material, i.e. microtitre plates and pipette tips. These consumables can be reused if they have been thoroughly cleaned from remains of biological reagents. For the cleaning of pipette tips, a simple cleaning device was designed in order to assure proper cleaning, facilitate the cleaning process and reduce the time required.

2. TRAINING

Training courses and programmes cover serological diagnosis of the major zoonoses and livestock diseases, production of related bioreagents, food hygiene and handling, and maintenance and repair of common laboratory equipment. Since 1988, 289 veterinarians, technologists and senior technicians have been trained in 15 national and 6 regional training courses in 18 countries in Africa and Asia. 111 trainees from Africa, Asia and Latin America have undergone specific mid- or long term training programmes at the BgVV in Berlin.

IAEA-SM-348/26P

PREPARATION OF RADIOIMMUNOASSAY (RIA) FOR THE DETERMINATION OF TYPES A AND B STAPHYLOCOCCAL ENTEROTOXINS

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1. AIMS

Methods with minimal detection limit are required for the determination of staphylococcal enterotoxins (SEs). These methods include a radioimmunoassay
(RIA). The paper describes the preparation procedures of the high affinity specific antiserum against staphylococcal A and B enterotoxins (SEA and SEB), the preparation of the tracers of $^{125}$I-SEA, $^{125}$I-SEB and the parameters of the radioimmunoassay test.

2. METHODS

The strain of FRI 722 (Bergdoll, USA) was used for the production of SEA and that of CCM 5757 (CCM Brno, Czech Republic) for the production of SEB. The purified enterotoxins A and B were used for preparation of antisera by immunization of the New Zealand white rabbits by the method of Vaitukaitis et al. (1971). The equilibrium constant, binding capacity, Gibb's standard free energy change, changes in standard enthalpy and entropy were determined in prepared antisera. Tracers of $^{125}$I-SEA and $^{125}$I-SEB were prepared by the chloramine T method of Greenwood and Hunter (1963). Na$^{125}$I (FY Amersham, IMS 30) was used for iodination. Non-specific bound (NSB), specific bound ($B_0$), 50% intercept, recovery, sensitivity and precision were determined by means of the standard curve.

3. RESULTS

The obtained titres of antisera against SEA were 1:20 000 and those against SEB 1:80 000. The equilibrium constant of antisera was approximately $10^{10}$ L·mol$^{-1}$, the binding capacity was $10^{-12}$ mol·L$^{-1}$. The change of Gibb's standard free energy, $\Delta^0 G$, ranged from $-55$ to $-60$ kJ·mL$^{-1}$. The standard enthalpy change was $\Delta^0 H = -14.02$ kJ·mol$^{-1}$. The entropics term of the reaction was $\Delta^0 S = 145.21$ J·mol$^{-1}$·K$^{-1}$. The tracer had the following parameters: the specific activity of $^{125}$I-SEA and $^{125}$I-SEB ranged from 1.0 to 1.25 TBq·g$^{-1}$; the immunoreactivity with an excess of antiserum was from 80 to 84%, the radiochemical purity (content per cent of $^{125}$I$^{-}$) was 2–4%. The developed RIA method had the following characteristics: NSB was from 1 to 3%; $B_0$ was 35–45%; 50% intercept from 8–12 ng·mL$^{-1}$; recovery 88–115%; sensitivity of determination 0.5 ng·mL$^{-1}$; intraassay $-CV$ 8.3% at 10 ng·mL$^{-1}$; interassay $-CV$ 9.2% at 10 ng·mL$^{-1}$.

4. CONCLUSIONS

Antiserum against SEA and SEB and tracers of $^{125}$I-SEA and $^{125}$I-SEB were prepared and their basic characteristics established. The prepared substances are satisfactory in their quantitative and qualitative parameters for immunochemical
systems such as RIA, ELISA and fluorescent immunoassay (FIA), in which high sensitivity of determination is required. A sensible and precise radioimmunoassay method for the determination of SEA and SEB was developed.

IAEA-SM-348/27P

FIELD TRAIL OF BRUCELLOSIS COMPETITIVE ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

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1. AIMS

The purpose of this study was to compare the diagnostic performance characteristics of the competitive (C) ELISA for the diagnosis of bovine brucellosis and the differentiation of \textit{Brucella} infected from \textit{Brucella} vaccinated cattle to the indirect (I) ELISA and the conventional serological techniques used in Argentina.

2. METHODS

*Experimental design:* Three test groups were analysed.
Group (a): 500 sera from non-vaccinated brucellosis free cattle herds.
Group (b): 1000 sera from S19 calfhood vaccinated cattle from brucellosis free herds.
Group (c): 1000 sera from vaccinated cattle from \textit{Brucella} infected herds where field strains of \textit{B. abortus} were isolated. For this experiment, samples were selected from four different farms in each group (12 farms studied).

*Serological tests:* The following conventional tests were used: buffered plate agglutination test (BPAT), Rose Bengal test (RBT), 2-mercaptoethanol (2ME), complement fixation test (CFT).
The following ELISA tests were used: indirect ELISA using smooth lipopolysaccharide (SLPS) antigen and M23 conjugated (a modification of I-ELISA using sera pretreated with EDTA/EGTA was also performed), C-ELISA using o-polysaccharide of \textit{B. abortus} 1119-3 antigen and YST9Mab conjugated, and C-ELISA using SLPS antigen and the mouse monoclonal antibody (M84 MAb) conjugated. The cut-off value was set at 40% P for I-ELISA and 40% I for C-ELISA. The results were recorded as the mean optical density (OD) value of the duplicates. The BPA and RBT antigen and all ELISA reagents and plates were provided by the IAEA. Antigens for 2 ME and CFT were elaborated at INTA, following standard procedures.

3. RESULTS

In group (a) (negatives), the diagnostic specificity was 100% in CFT, 100% in 2ME, 98.6% in I-ELISA, 99.8% in C-ELISA, 99.8% in BPAT and 97.7% in RBT. In group (b) (vaccinated), the CFT diagnostic specificity was 96.4%, in 2ME 93.6%, in I-ELISA 95.8%, in C-ELISA 97.8%, in BPAT 37.6% and in RBT 35.4%. In group (c) (infected), the relative sensibility to CFT (100% reactors 1/10 or more) was 99.8% in 2ME, in I-ELISA 98.2%, in C-ELISA 97.3%, in BPAT 99.6% and in RBT 96%.

4. CONCLUSIONS

The diagnostic specificity and relative sensibility of ELISA was comparable to CFT and 2ME, which are the official complementary tests in Argentina. Besides, C-ELISA showed the best performance in analysing vaccinated animals. The application of the ELISA test is largely feasible because of its reproducibility and easy standardization. In addition, although CFT has a great performance, its application is cumbersome; the 2ME test lasted for 48 h and is toxic. Both the C-ELISA, using MAb M84, and the I-ELISA, using MAb M23, have been demonstrated to have the best performance. Owing to our field conditions (brucellosis prevalence and mandatory vaccination of female calves) the competitive ELISA should be applied as a complement of the official screening tests such as BPA. These results suggest that the ELISA test would be very useful in contributing to the control and eradication programme of bovine brucellosis in Argentina.
1. AIMS

In Chile, as in several other Latin American countries, brucellosis is an enzootic disease. As there is a government voluntary control programme for brucellosis, some farms are free from this disease. Currently, however, the government has initiated an eradication programme. This programme requires more accurate diagnostic techniques.

The objective of this research was to compare an indirect enzyme immunoassay (I-ELISA) with the milk ring test (MRT) for detection of antibodies in milk. A second objective was to compare the I-ELISA, the Rose Bengal test (RBT) and the complement fixation test (CFT) for the detection of serum antibody.

2. METHODS

The I-ELISA was used on both milk and serum samples. This assay used Brucella lipopolysaccharide (LPS) as the antigen, and a mouse monoclonal antibody against bovine IgG1 conjugated with horseradish peroxidase, for the detection of bound antibody. The substrate was hydrogen peroxide, the chromogen was ABTS and sodium dodecyl sulphate was used as a stopping agent.

The MRT and the RBT were performed according to the techniques described by Alton et al. in 1990. The C'EPANZO procedure was used for the CFT. Antigens were prepared at the Instituto de Microbiología, Universidad Austral de Chile. Test samples were divided into three groups: 510 from brucellosis free farms where all calves were vaccinated with B. abortus strain 19; 503 from cattle calves from a farm with B. abortus infection, which were vaccinated with strain 19; and 19 from non-vaccinated infected cattle. Serum and milk samples were collected from all the animals.

The I-ELISA cut-off values obtained by ROC analysis gave 15% positivity (% P) for milk and 26% P for serum. If the CFT was used as the reference test, the
I-ELISAs gave the best overall sensitivity and specificity. The I-ELISA sensitivity for milk was 97.6% and 98.3% for serum. The I-ELISA specificity was 99.2% for milk and 100% for serum.

4. CONCLUSIONS

The I-ELISAs detect more infected cattle than the RBT and MRT in infected herds because of their higher analytical sensitivity. Sensitivity and specificity of the I-ELISAs were higher for both milk and serum than for the conventional tests. The sensitivity of the I-ELISA for milk, using a cut-off of 15%, was 97.6%, and the specificity was 99.2%. The sensitivity of the I-ELISA for antibody detection in serum, using a cut-off value of 26%, was 98.3%, and the specificity was 100%.

IAEA-SM-348/30P

USE OF LIQUID PHASE ELISA KIT FOR DETECTION OF ANTIBODIES AGAINST FOOT-AND-MOUTH DISEASE VIRUS (FMDV) IN COLOMBIA

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Colombia has FMDV type O and type A in some regions with endemic characteristics. At present, the country is involved in the hemispheric plan for eradication of FMDV. To attain this objective, it is necessary to use modern techniques with high sensitivity and specificity as opposed to traditional serological tests.

The use of liquid phase ELISA is of great benefit in places where prevention, control and eradication programmes for FMD are running. This technique gives more security because it is very sensitive and specific and the results are obtained on the same day. In addition, it does not need any cell culture nor a CO₂ environment.

The test is based on specific blocking of the liquid phase of FMDV antigen by antibodies in the test sample. After the test serum has been allowed to react with the specific FMDV antigen, the test serum/antigen mixture is transferred to an ELISA plate coated with FMDV serotype specific trapping antibodies. The presence of antibodies to FMDV in the serum sample will result in the formation of immune complexes and, consequently, reduce the amount of free antigen trapped by the
immobilized rabbit antisera. In turn, fewer guinea pig anti-FMDV detecting antibodies will react in the next incubation step. After the addition of enzyme labelled horseradish peroxidase, anti-guinea-pig Ig and substrate/chromogen solution, a reduction in colour development will be observed when compared to controls containing free antigens only.

To develop the initial experience in Colombia, we tested 360 bovine sera divided into the three animal categories:

(a) 120 bovine sera came from areas free of FMDV and were sent by the CPFA.
(b) 120 bovine sera came from vaccinated animals with trivalent vaccine, also sent from the CPFA.
(c) 120 bovine sera came from animals infected with the FMDV obtained from outbreaks which had naturally occurred in different regions of Colombia.

The samples were tested in a microneutralization plate assay against FMDV type O and type A as well as in the liquid phase blocking ELISA.

The results of the liquid phase ELISA are still being processed since we are still adjusting the titres of some of the reagents. The reason for this is that after following the procedures in the bench protocol, the EDI program did not accept the given date and all the plates were rejected.

The use of new techniques, such as the liquid phase ELISA, is of great use in places where prevention, control and eradication programmes for FMDV already exist.

We are likely to use it as a routine tool in places where the vaccines are systematically applied every six months.

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EVIDENCE OF MYCOTOXINS (AFLATOXIN B1 AND OCHRATOXIN A) USING THE RADIOIMMUNOASSAY (RIA) IN NATURALLY CONTAMINATED CEREALS

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1. AIMS

The aim of our study was to gain starting information on the aflatoxin B1 and ochratoxin A levels in cereals and feed mixtures which are in use in poultry breeding.
2. METHODS

Kits for RIA of aflatoxin B1 and ochratoxin A were obtained from Putz Co, Lúcky, Slovakia. This assay has the advantage of high specificity and sensitivity. RIA of aflatoxin B1 was equally specific for aflatoxins B1, G1, B2, M1 and G2, with cross-reactions of 100, 73, 10.2, 4.7 and 1.1%, respectively. The sensitivity of the assay ranges from 0.4 to 0.5 µg·kg⁻¹. These kits contain standards of aflatoxin B1 0.05–3.2 ng·mL⁻¹ radioligand, rabbit polyclonal antiserum. T-2 toxin was determined by the ELISA method in co-operation with the Biotechnological Centre, Gödöllö, Hungary.

3. RESULTS

The degree of aflatoxin B1 contamination in wheat ranged from 0.028 to 0.125 µg·kg⁻¹ in different regions of eastern Slovakia. Aflatoxin B1 levels in maize ranged from 0.166 to 0.707 µg·kg⁻¹. The levels of ochratoxin A and T-2 toxin were 0.015 µg·kg⁻¹ and 0.5 ng·mL⁻¹, respectively. The examination of feed mixtures BR 1 and BR 2 revealed the following degrees of contamination by mycotoxins: aflatoxin B1: 0.99–2.77 µg·kg⁻¹; ochratoxin A: 0.00–0.31 µg·kg⁻¹; T-2 toxin: 0.5 ng·mL⁻¹.

4. CONCLUSIONS

To ascertain the presence of mycotoxins, we examined the cultivars of cereals (maize and wheat) and the feed mixtures. The cereals came from different regions of eastern Slovakia (Družstevná, Čečejovce, Čaňa). In all cereals examined, the low mycotoxin levels did not exceed the tolerance limit set by hygienic standard (Sv. 61, 1986, No. 69). In wheat, the contamination by aflatoxin B1 ranged from 0.028 to 0.125 µg·kg⁻¹. In maize, the contamination by aflatoxin B1 ranged from 0.166 to 0.707 µg·kg⁻¹. The results enhance our knowledge of feedstuff and feed mixture contamination in poultry breeding.
VALIDATION OF AN FAO/IAEA/PANAFTOSA ELISA KIT FOR THE DETECTION OF ANTIBODIES AGAINST FOOT-AND-MOUTH DISEASE VIRUS IN VENEZUELA

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1. AIMS

The aim was to validate an immunoenzymatic competition test in liquid phase (ELISA/CFL) developed for the qualitative and quantitative detection of antibodies to foot-and-mouth disease virus (FMDV) serotypes O and A.

Specificity and sensitivity of the ELISA/CFL make it a valuable instrument for evaluating the immune conditions of herds as well as the immune response and immunity duration in vaccinated animals.

2. METHODS

240 samples of bovine sera were studied by the ELISA/CFL technique used as a screening or titration assay.

Trapping antibodies of rabbit antisera (whole serum), specific for either FMDV serotype O or A, were used for absorption into polystyrene microplates; this constituted the solid phase of the test.

The antigen–antibody reaction (liquid phase) was carried out and incubated in auxiliar polypropylene microplates. After the test, the sera were allowed to react with the specific FMDV antigen, then the test sera/antigen mixture was transferred to a microplate coated with FMDV serotype specific trapping antibodies and subsequently incubated.

The presence of antibodies to FMDV in the serum samples resulted in the formation of immune complexes and, consequently, reduced the amount of free antigen trapped by the rabbit antibodies absorbed to the microplates.

Next, after an incubation period, guinea pig anti-FMDV specific detecting antibodies were added and then bound to the trapped antigen in the reaction. After a second incubation period, horseradish peroxidase conjugated rabbit anti-guinea-pig immunoglobulin was added, and, after a third period of incubation, a substrate/chromogen solution was added; the reaction was observed as a colour development whose optical density was compared with the control values of the test.
3. RESULTS

A screening test with the application of ELISA/CFL showed that 120 sera were positive and belonged to cattle vaccinated with oil trivalent inactivated vaccine; they were sampled 30 days after vaccination, the remaining 120 sera showing a negative result.

The positive sera were titred to serotypes O and A, by using fivefold dilution. Sera were regarded as positive when they gave percentage of inhibition (PI) values equal to or higher than 50. The range of titre for serotype O was between 1:50 and >1:1250 and for serotype A between 1:22 and >1:1250. It was observed that the majority of sera with high titres were serotype O.

4. CONCLUSIONS

(1) The ELISA/CFL technique demonstrated its sensitivity and specificity; it is capable of discriminating positive from negative sera and is very useful for titration of antibodies.

(2) The process of using a previously frozen solid phase did not allow us to reach acceptance levels; therefore, a solid phase prepared the day before was used in our assay.

(3) As to the first level of acceptance by the optic density of the antigen control, 23 of the 38 processed microplates were accepted for serotype O; for serotype A, 16 of the 29 processed microplates were accepted, some of them by approximation to the values of the acceptance range.

(4) As to the second level of acceptance based on the PI of the controls — CA, strongly positive (C++), moderately positive (C+) and negative (C-) — it was observed that the high variability of serotypes, microplates and working sessions did not allow a correct test validation. It is considered necessary to review the PI ranges in order to achieve the replication needed.
IMMUNOASSAYS FOR THE DIAGNOSIS OF Brucella suis INFECTION IN PIGS — PRELIMINARY STUDY

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1. INTRODUCTION

Serological procedures to detect antibody against Brucella were developed for diagnosing bovine brucellosis and have been adapted for testing swine sera. The original tube and plate agglutination test and additional, more specific tests such as the buffered plate antigen (BPAT) or 2-mercaptoethanol (ME) utilize Brucella abortus whole cells as antigen. Regardless of the serological test used for swine diagnosis, detection of 80–90% of individual infected swine must be regarded as the best result that can be achieved at present.

A primary binding assay such as the enzyme linked immunosorbent assay (ELISA) improved sensitivity and specificity of the measurement of bovine antibody to B. abortus. Limited investigation has been reported about the use of immunoassays for the diagnosis of swine brucellosis. The purpose of this preliminary study is to evaluate the immunoassays developed by K. Nielsen and collaborators for the serological diagnosis of bovine brucellosis using swine sera by comparing test results of BPAT and ME assays now available in diagnostic laboratories. Sera from brucellosis free herds (from Canada) and from swine infected groups (from Argentina) where Brucella suis was isolated were tested.

2. MATERIALS AND METHODS

The following swine serum samples were used for testing: 952 pig sera sent from ADRI (Canada) and collected from uninfected herds. 1169 serum samples collected from groups from swine abattoirs where Brucella suis was isolated from lymph nodes at least once in each group. The BPAT and ME tests were performed according to the Manual de procedimientos (Res. SENASA 1269/93). The I-ELISA and C-ELISA assays both use B. abortus lipopolysaccharide immobilized on a polystyrene matrix as antigen.
The I-ELISA uses a monoclonal antibody to swine IgGI (M-167) conjugated with horseradish peroxidase as detection reagent. Optical density readings are taken after 10 min of incubation with the substrate/chromogen, and data are presented as a percentage of positive control serum included in each plate (% positivity). The C-ELISA utilizes a monoclonal antibody (M-84) with specificity for an epitope of the o-polysaccharide obtained from *B. abortus* and a goat anti-mouse IgG antibody enzyme conjugate. Optical density readings are taken after 10 min of incubation with the substrate/chromogen, and the data are presented as a percentage of inhibition relative to the buffer (uninhibited control).

3. RESULTS

The 952 pig sera collected from uninfected herds gave a mean (% positivity) value of 6.86 and a standard deviation (SD) of 5.65 in the I-ELISA test. The I-ELISA cut-off value was calculated as the mean +3SD (25%). This cut-off was confirmed using both positive and negative serum samples by receiver operating characteristic (ROC) analysis. The I-ELISA specificity was 99.8%. Two serological false positives were evident when the I-ELISA cut-off value was 25%. Therefore, a cut-off value of 30% was selected to achieve a specificity value of 100% with the negative sera.

The C-ELISA test performed on the same samples gave a mean (% inhibition) value of 8.4 and an SD of 11.3. The C-ELISA initial cut-off value was calculated as the mean +3 SD (42%). The specificity value was 100%. The same data analysed by ROC analysis using both positive and negative serum samples gave a cut-off of 30%. This resulted in a specificity of 99.6%, while the sensitivity increased. Therefore, a value of 30% inhibition, or greater, of the reactivity relative to the buffer control was considered a positive reaction.

The BPAT, ME, I-ELISA and C-ELISA assays were performed on 1169 serum samples collected from infected groups. 668 sera gave BPAT and ME negative reactions. Among these 668 sera, one serum gave I-ELISA and C-ELISA positive reactions and another serum gave I-ELISA negative and C-ELISA positive reactions. 501 sera gave BPAT positive reactions. The I-ELISA and C-ELISA assays performed on these 501 sera gave 443 and 439 positive reactions, respectively. The ME test performed on the same 501 sera gave 424 positive reactions. Two of these 424 sera gave I-ELISA and C-ELISA negative reactions. This resulted in a relative sensitivity of I-ELISA to the ME test of 99.5% and to the ME/I-ELISA test of 95%. The kappa index was 95.8%. The C-ELISA relative sensitivity to the ME test was 99.5%, and to the ME/C-ELISA test it was 96%. The kappa index was 96.5%. The relatively high rate of sera yielding positive ELISA assays compared to the ME positive reactions is not due to ELISA false positive reactions (I-ELISA specificity was 100% and C-ELISA specificity was 99.6%), but may be due to ME false negative reactions.
EVALUATION OF A MILK-ELISA TEST FOR BRUCELLOSIS THROUGH REPEATED SAMPLING ON AN INFECTED HERD

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1. AIMS

The aim was to evaluate the ability of a milk-ELISA test (M-EIA) to detect infected animals, in comparison with the Rose Bengal plate test (RBPT), the complement fixation test (CFT) and the milk ring test (MRT), and to estimate the probability of M-EIA and MRT detecting infected animals in three subsequent samplings and testings in relation to directives 64/432/EEC and 91/499/EEC regarding the application of MRT and M-EIA, respectively, and stating that in countries free from Brucella during the last four years serological tests may be substituted by three M-EIAs or three MRTs.

2. METHODS

Tests: RBPT, CFT, MRT and M-EIA using an LPS antigen of Brucella abortus 99W. The M-EIA was standardized to have a specificity of 99.5%.

Case definition: any animal from an infected herd that proved positive in at least one of the four tests performed or any culture in at least one of the four samplings performed. Herd: a herd of 77 cows in which three abortions were reported and B. melitensis biotype 2 was isolated from aborted foetuses. Sampling: all animals were blood and milk sampled at the start of the study. The remaining cows, after culling CFT positive reactors, were tested every 45 days for a total period of 135 days.

3. RESULTS

The initial prevalence of infection was 57.1% (44 infected animals, 30 of which, i.e. 39%, were CFT positive) on the basis of the adopted case definition. As a whole, 71 cows fulfilled the case definition during the whole study period. The
sensitivities recorded for each test were: RBPT = 59.0%, CFT = 36.0%, MRT on individual samples = 47.5%, M-EIA on individual samples = 92.3%. Regarding the use of the tests on bulk milk, MRT was able to detect infected milk diluted up to 1:80 in negative milk, and M-EIA was able to detect infected milk diluted 1:1600 in negative milk.

Test sensitivities established after repeated samplings: RBPT = 70.4% (four samplings), CFT = 50.7% (four samplings), MRT = 68.1% (three samplings), and M-EIA = 96.9% (three samplings).

4. CONCLUSIONS

The sensitivity of RBPT and CFT in the case study was lower than the values normally reported in the literature for both cattle and sheep infection. Likewise, the sensitivity of MRT was lower than the values normally observed in infected herds. The M-EIA was the most sensitive test when performed on individual milk. On the basis of the test sensitivities estimated in this study and of tests specificities reported in the literature, the expected predictive value for positive results of the adopted case definition would have been 94.6% (equivalent to two false positive cases) after the first sampling of animals, and the predictive value for negative results would have been 99.2% (equivalent to zero false negatives). The use of CFT as a confirmatory test implies a predictive value for positive results of 98.4% (equivalent to one false positive case) and a predictive value for negative results of 60.11% (equivalent to 13 false negative cases). In fact, six more animals positive to CFT have been detected in the following samplings.

The higher sensitivity of M-EIA in three subsequent samplings (96.9%) and its ability to detect positive milk diluted up to 1:1600 in negative milk indicates that the testing of bulk milk as stated by directive 91/499/EEC is a valid and inexpensive method of detecting the infection. Conversely, the lower sensitivity of MRT and its ability to detect infected milk diluted 1:5–1:80 in negative milk indicates that the use of MRT according to directive 64/432/EEC may be useful only in the case of small sized herds. Repeated milk testing by M-EIA would certainly increase the chances of an identification of positive animals.
IMPROVED DIAGNOSIS
OF CONTAGIOUS BOVINE PLEUROPNEUMONIA (CBPP)
BY STANDARD SEROLOGICAL TECHNIQUES
AND DNA AMPLIFICATION

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1. AIMS

The aim was to improve the diagnosis of contagious bovine pleuropneumonia (CBPP) in Botswana by the implementation and establishment of the OIE standard complement fixation test (CFT) method, and to demonstrate a diagnostic system in a 96 well format coupled with a colorimetric detection of the PCR amplified product suitable for moderate to high sample numbers for the detection of Mycoplasma mycoides mycoides SC (MmmSC) from nasal swabs, lung tissue and cultures.

2. METHODS

The OIE standard method for complement fixation testing for CBPP has been described; it is an adaptation for microplates of the procedure by Campbell and Turner. A 6% suspension of sensitized red blood cells, a crude, phenolized antigen and complement were titrated to achieve the optimal sensitivity. Standard sera approved by the OIE were used as controls. DNA amplification by PCR on nasal swabs was performed as described by Bashiruddin and others. The PCR product was digested with the restriction enzyme Asn1 for the identification of MmmSC. Amplification from clinical material and isolates collected in Italy was performed with primers modified as described by Kemp and others. PCR products were captured in coated ELISA plates and detected immunologically. Positive samples were confirmed as MmmSC by typical patterns obtained after the digestion of PCR products with Asn1.
3. RESULTS

Sera from five animals in Xaudum valley of Botswana from one of the first outbreaks ranged between ++/1:40 to +++/1:320. Another set of 20 sera collected from a clinically sick herd in Mohembo, northwestern Botswana, was tested for CF antibodies against *MmmSC* and titres ranged from non-reactive to +++/1:1280. There was 65% positivity by the CFT in this herd. Of the 37 animals studied from one of the last outbreaks in northern Italy, 51% were positive by the CFT. They were asymptomatic. PCR from nasal swabs taken from these animals was positive in 45% of the cases, cultures from lung tissue were positive in 19% of cases. Together, CFT and nasal swab PCR were able to diagnose CBPP in 60% of the cases. All strains of *MmmSC* from Europe and Africa tested so far gave amplification products which produce identical bands after *Asn1* digestion. Colorimetric detection of PCR products yielded clear distinctions between positive and negative results as judged by agarose gel electrophoresis and could be performed within 2 h after the completion of PCR. Restriction enzyme digestion for 1 h was sufficient for the discrimination between *MmmSC* and other *M. mycoides* ssp.

4. CONCLUSIONS

The standard method of the complement fixation test for CBPP is the official test for the diagnosis of CBPP throughout Europe and Africa, and the establishment of this technique, through reciprocal visits and the provision of training, was achieved in Botswana. Sera separated in regional laboratories were sent to the National Veterinary Laboratory for CFT, where a capacity for processing about 7000 sera per week was developed. The shedding of organisms from apparently healthy animals could be demonstrated by PCR from nasal swabs. From a single sampling, PCR from nasal swabs was not able to detect all serologically positive animals. However, *MmmSC* was detected in nasal secretions in four serologically negative cases, indicating the possibility of earlier detection with PCR than with CFT. The microplate format of the PCR product detection system was a more rapid method for increased sample numbers and took advantage of standard equipment used for ELISA, including microplate washers, readers and automation. In practice, nasal swabs and blood could be taken at the same time from each animal for aerological and molecular analyses. An increase in the accuracy of overall diagnosis could be gained by considering the cumulative results from both tests, but validation of these results requires the testing of a larger sample number.
A POLYMERASE CHAIN REACTION (PCR) SYSTEM TO DETECT Babesia equi IN BLOOD OF ITALIAN HORSES

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1. AIMS

The aim was to develop a new and more sensitive diagnostic alternative to detect Babesia equi infection in horses. A PCR system, which amplified a 664bp region of the 16S-like rRNA coding region, was designed and validated by comparing its diagnostic performance with those of the complement fixation test (CFT) and the microscopic examination.

2. METHODS

Blood specimens from 107 Italian horses were collected in EDTA and plain vacutainer tubes. To assess B. equi infection, sera in plain vacutainers were examined by CFT whereas thin film smears were stained with Giemsa, and DNA was extracted from EDTA blood specimens for PCR. The PCR primers, which were chosen from DNA sequence coding for the 16S-like rRNA amplified a 664 bp product. PCR products were detected by electrophoresis in 1% agarose gel and visualized by UV illumination of ethidium bromide stained DNA. The reaction was optimized by using B. equi isolates, and its specificity was tested against different species of Babesia as well as other closely related protozoan strains and control DNA from normal horse blood. The PCR results were compared with those obtained with CFT and microscopy. The kappa statistic was used to assess the agreement between PCR and microscopy.

3. RESULTS

The optimized reaction was specific for B. equi when tested against several protozoa and DNA from normal horse blood. A significantly higher proportion (P < 0.05) of infected animals (24.3%) was detected by PCR than by either CFT or microscopy.
The same proportion of infected animals was detected by the latter two methods (12.1% and 8.4%, respectively). The observed agreement proportion between the three tests was 72.0%, whereas it was 82.2% between PCR and CFT, 83.2% between microscopy and CFT and 78.5% between PCR and microscopy. The kappa value for agreement beyond chance between the two direct methods (PCR and microscopy) was calculated to be 25.1%.

4. CONCLUSIONS

The fact that rDNA, because of structural constraints in rRNA, is a stable gene with very little intraspecific variation makes this area of the genome an attractive target for PCR. The chosen primers were designed to amplify the 16S-like rDNA target of *B. equi*, exclusively. The optimized PCR was experimentally shown to be specific for the target, and, importantly, there were no amplification products with the host species DNA. Samples from cultured, stored and freshly obtained blood were amplified. In this study, the PCR was the test which detected the greatest number of positive samples. Interestingly, the kappa value measuring agreement beyond chance between PCR and microscopic examination was low, which, in turn, might suggest an underestimation of the prevalence of infection when assessed by this method only.

In conclusion, the PCR which amplifies the 16S-like rDNA provides an accurate and reliable means for detecting and identifying infection with *B. equi*, and its relative simplicity and adaptability to the handling of large sample numbers make it particularly suited for rapid, routine screening as an adjunct to standard microscopy and serology, which could result in a general diagnostic performance of equine babesiosis. It might be especially useful where there is an urgency in obtaining results as is the case in the import/export testing of horses and for monitoring the progress of therapy. Its higher sensitivity could also be useful in better defining the carrier state in equine babesiosis.
USE OF BIOTECHNOLOGY IN DIAGNOSIS IN PERU

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1. AIMS

Molecular biology techniques were used for isolation, identification and assessment of immunoreactive proteins of some bacteria and viruses affecting domestic animals and birds. Protein profiles of mycoplasmas, adenoviruses, reovirus, bronchitis virus, gumboro virus and Newcastle disease virus have been identified. RNA extraction from faeces of dairy calves, piglets and birds allowed detection of RNA viruses such as rotavirus and reovirus. PCR improved mycoplasma diagnosis for its sensitivity and specificity and rapid results.

2. METHODS

SDS-PAGE electrophoresis was used for protein profile studies of mycoplasmas of goats, birds, dairy cattle and avian viruses.

Isolation of mycoplasma was carried out in WJ broth and protein collected after treatment with buffer extraction and boiling. Running gel of 10–12% and stacking gel of 4.5% were used. Gels were stained with Coomassie blue.

Faeces from pigs and calves affected with diarrhoea and birds affected with maladsorption syndrome were treated with sodium acetate buffer and phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 1200g for 20 min. The upper layer was collected and mixed with 50% sucrose. RNA electrophoresis was conducted with 6% running gel and stained with silver.

Protein profiles and immunogenic proteins of avian viruses were obtained with SDS-PAGE and western blot analysis. Protein transfer was carried out under semi-dry conditions, and reactions were developed with a TMB kit.

PCR for avian mycoplasma was conducted by using GAG CTA ATC TGT AAA GTT GGT C and GCT TCC TTC CGG TTA CGA AC primers and 1% agarose. DNA was extracted form tracheal swab samples and amplified in the thermal cycler. PCR
amplification was electrophoresed at 100 V for 30 min and stained with ethidium bromide and detected by UV photography.

3. RESULTS

SDS-PAGE allowed differentiation of goat and avian mycoplasmas. The molecular weights of avian viruses and their immunogenic proteins were identified by western blot analysis. The prevalence of viral diarrhoea caused by rotavirus in dairy calves and piglets was assessed. The genomic patterns of viral RNA corresponded to group A, with 11 genomic bands. Also chicken affected with maladsorption syndrome showed the presence of electropherotype with 10 genomic bands of reovirus, within 7 and 15 days of age. PCR amplification of chicken mycoplasma was about 200 bp in size.

4. CONCLUSIONS

Molecular biology techniques developed in our laboratory have enabled us to identify the main pathogenic bacteria and viruses affecting domestic animals and birds, the immune response to them and RNA viruses causing diarrhoea. In addition, PCR has allowed us to reach a rapid and accurate diagnosis of chicken mycoplasma within 24 h.

IAEA-SM-348/40P

THE INTERNATIONAL FOUNDATION FOR SCIENCE (IFS)

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The International Foundation for Science (IFS), founded in 1972, is a non-governmental organization with a membership of 94 scientific academies and research councils in 80 countries, out of which three quarters are in developing countries and one quarter in industrial countries. The Foundation is governed by an international Board of Trustees, which is elected every three years. The Secretariat, with a staff of 22, is located in Stockholm, Sweden.

The Foundation supports young scientists of merit from developing countries. The focus is on the individual more than on the project, with the aim of building up local research capacities. The research must fall within one of the following areas:
aquatic resources, animal production, crop science, forestry/agroforestry, food science and natural products. Besides being from a developing country, the researcher must also carry out the research in a developing country. His/her institution is expected to provide salaries and basic research facilities.

Research in the animal production area includes breeding, genetics and reproduction; health and diseases; nutrition and the development, production and conservation of feeds; animal traction and animal production systems. The project should be relevant to developing countries and research oriented, not only transfer of technology. All research proposals should aim at contributing to ecologically, socially and economically sustainable development.

To date, IFS has supported some 2500 researchers in 93 countries, out of whom animal production grantees make up about 23%. Some 250 projects are ongoing at the moment on highly diverse subjects such as reproduction of timber elephants in Myanmar, feeding systems for pigs based on sugar cane juice in Colombia, development of a vaccine against a skin disease in camels in Kenya, etc.

In addition to the research grants, IFS provides supporting services such as help to purchase equipment, travel grants to attend international meetings, organization of workshops and help with literature and scientific contacts.

IAEA-SM-348/41P

IDENTIFICATION
OF BOVINE VIRAL DIARRHOEA VIRUS (BVDV) PERSISTENTLY INFECTED CALVES UNDER LOCAL MANAGEMENT PRACTICES IN URUGUAY

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1. AIMS

The aim was to identify BVDV persistently infected (PI) animals in dairy herds in Uruguay with the ultimate aim of controlling this disease.

2. METHODS

Bulk tank milk samples from six dairy farms were tested by the ELISA test for the presence of antibodies to BVDV. They were classified according to antibody
titles, in view of the optical density (OD) values obtained (Niskanen, 1993). Four farms were selected, and individual serum samples were obtained from all animals in each dairy farm, to be tested for the presence of antibodies by the ELISA test. All animals negative to antibodies were tested for the presence of circulating BVDV antigens, by the Ag-ELISA test.

3. RESULTS

The six dairy farms screened for BVDV antibodies in their bulk tank milk were classified as follows:

Two were classified as Class II, with OD values below 0.500 nm, and four as Class III, with OD values above 0.700 nm. Individual sera from all animals (350 sera) tested also for antibodies gave the following results: 337 positive and 13 negative. The 13 Ab-negative animals were also tested for the presence of circulating antigen; they were Ag-negative.

4. CONCLUSIONS

From the fact that no PI animals were identified after herd testing we may conclude that PI animals may have been culled or have died previously or that herd infection had resulted in abortions and reabsorptions rather than in the production of PI animals. Authors report that infection is self-limiting and that, if the incidence of infection becomes very high, more and more animals acquire immunity before breeding and fewer PI animals are born. A reduction in the number of PI animals then causes the incidence of infection to decrease again. However, it was discovered that the results were affected by a local management practice in Uruguay, i.e. the use of co-operative fields where several farmers with more animals than can be sustained by them alone share pastures. They keep in their farms only milking cows and those near confinement. Male calves are sold immediately after birth and heifers are sent to this co-operative field after weaning (eight to ten months old) and only return to the farm at seven months pregnancy. For this reason, it was not possible to finish the work as designed, because of the impossibility to sample all existing animals in each dairy. With this practice, there is a reduction by 50% of the possibility of PI animals, as males are eliminated from the herd immediately after birth. Female calves graze with milking cows and first pregnancy cows of seven to eight months pregnancy, with no possibility of producing a PI calf. The possibility of PI animals, being born at the farm, of infecting other animals exists at the co-operative field, where any female being served and pregnant might become infected by a PI and thus produce herself a PI animal. Perhaps, it might be interesting to test all animals in a co-operative field.
Further work is to be accomplished until the epidemiological behaviour of this disease is understood in Uruguay. This research work was performed with the assistance of IFS grant B-1134/F.

IAEA-SM-348/42P

CONTROL OF BRUCELLOSIS IN IRAQ

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1. AIMS

Brucellosis is the most widespread zoonotic disease and considered a great problem of major economic importance in most countries, particularly in those where no eradication programme is applied. In developing countries, ovine brucellosis is still a more frequent source of human infection, and attempts at eradication or campaigns for its control have often met with considerable difficulties. The Iraqi government has long been concerned with the danger brucellosis represents to public health and economy.

The Central Veterinary Diagnostic Laboratory at Baghdad has a long history of work on brucellosis since in 1977–1985 different biotypes of Brucella melitensis, in particular biotype III, were prevalent and isolated from aborted foetuses and from sheep and goat milk. In addition, two biotypes of Brucella abortus were isolated from aborted calves. As a result, goats, sheep and cattle have a very high risk of morbidity. Serious incidences of brucellosis have been observed in 12% of goats, 10% of sheep and 0.5% of cattle.

Recently, Iraq has faced an increasing incidence of the disease, because of inadequate observation of the rules and regulations concerning its control. Accordingly, Iraq has initiated action in co-operation with Jordan, Lebanon and the Syrian Arab Republic in order to develop a regional project in the framework of the elimination, prevention and eradication programme established in 1995.

2. METHODS

In the surveillance activity aimed at finding out the percentage of seropositive animals, random blood samples were collected from cattle, sheep and goats. A total of 45 100 samples were tested (cattle: 26 042 samples; sheep: 17 510 samples; goats:
TABLE I. PREVALENCE OF BRUCELLOSION AMONG CATTLE, SHEEP AND GOATS

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Number of serum samples</th>
<th>Number of RBT (+) samples</th>
<th>Number of SAT (+) samples</th>
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<tr>
<td>Cattle</td>
<td>26,042</td>
<td>350 (1.34%)</td>
<td>217 (0.83%)</td>
</tr>
<tr>
<td>Sheep</td>
<td>17,518</td>
<td>2600 (14.84%)</td>
<td>2201 (12.56%)</td>
</tr>
<tr>
<td>Goats</td>
<td>1,540</td>
<td>156 (10.71%)</td>
<td>130 (8.44%)</td>
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1540 samples. The sera were screened for the presence of Brucella antibodies by the Rose Bengal test (RBT) and with the serum agglutination test (SAT). Table I summarizes the results of RBT and SAT.

4. CONCLUSIONS

Sensitivity, specificity and limitation of serological tests play an important role in the diagnosis and control of brucellosis. The results of RBT and SAT have detected higher percentages of positive reactors and a higher incidence of infection recorded in sheep, goats and cattle. These results demonstrate a higher prevalence of ovine brucellosis; it is concluded that B. melitensis represents a hazard to animal populations and public health in Iraq.

To control the infections, it is very important to start a long term strategy for three consecutive years, a campaign to vaccinate three to eight months old females of sheeps and goats with B. melitensis Rev. 1 vaccine to create a new generation of sheep and goats free from brucellosis.
THE LITHUANIAN IBR/IPV CONTROL PROGRAMME

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1. AIMS

In Lithuania, preliminary serological investigations of infectious bovine rhinotracheitis (IBR) in 1992 showed a high seroprevalence in animals at artificial insemination (AI) and bull growing centres. On the basis of these investigations, the Lithuanian IBR/IPV control programme was developed and approved on 7 October 1993. The main features of this programme are routine testing of all animals in AI and bull growing centres twice a year, virological examination of semen, removal of seropositive animals, testing of blood serum samples from high production dairy herds, and transport restriction and quarantine measures.

2. METHODS

Blood sera of bulls from AI centres and from calves and cows from dairy herds were examined by an enzyme linked immunosorbent assay (ELISA, Bioveta, Czech Republic). Primary bovine testicle cells were used for virus isolation. All samples were passaged at least three times, and samples showing cytopathic effects (CPEs) were examined by the direct immunofluorescence (IF) method (Gamakon/IBR, Mevak, Slovak Republic).

3. RESULTS

Serological investigations in bull rearing stations revealed that IBR is rather widespread: 52.5% of the tested calves were seropositive. On the basis of these results and those obtained in 1993, the programme for IBR eradication in breeding cattle was approved. Seropositive animals were eliminated from the stock, and
subsequently only 5.7–5.9% of the animals there were diagnosed seropositive in 1994 and 1995. Investigations in breeding cows revealed that the prevalence of BHV-1 was higher than in other cattle. Although after the application of the IBR eradication programme the number of seropositive animals decreased from 78.9% in 1993 to 41.1% in 1994, the investigations performed in 1996 showed that 66.1% of the highly productive cows were seropositive. Blood serum analysis in the three largest and some of the smaller Lithuanian AI centres revealed that before the implementation of the IBR control programme (in 1993) 49.8% of bulls were infected with BHV-1. Therefore, the IBR eradication programme was most strictly implemented on those farms, and as a result only 3.5% of the bulls tested appeared seropositive in 1996.

At the same time, bull semen was also examined for BHV-1. In 1993, semen samples from 65 seropositive bulls from AI centres were examined, and in 16.92% of the cases BHV-1 was isolated and identified in the cell cultures after three to four passages. It must be pointed out that the examined bulls had recently suffered from balanopostitis. Later on, the semen stored in liquid nitrogen was examined. In 1994 and 1995, 5.3% and 2.8% of the samples, respectively, were diagnosed positive for BHV-1. BHV-1 contaminated semen lots were eliminated.

4. CONCLUSIONS

Serological tests carried out in 1994 and 1995 on bulls from AI centres revealed that the initial implementation of the IBR eradication programme produced good results. In 1993, 49.8% of bulls were diagnosed seropositive, whereas in 1996 the number of seropositive bulls had significantly decreased to 3.5%. However, many cows on different farms have been in contact with BH and may be considered BHV carriers. The results of our survey could be associated with the occurrence of reproductive disorders in Lithuanian cows. However, the results of our investigations show that AI bulls are shedding BHV-1, and only after the implementation of the IBR eradication programme the number of BHV-1 positive semen samples decreased from 16.9% (1993) to 2.8% (1995). Our investigations show that IBR eradication in Lithuania is problematic. Despite some progress in AI and bull growing stations, other methods of IBR control such as vaccination with recently developed marker vaccines must be attempted.
ELISA — THE METHOD OF CHOICE FOR ASSAY OF ANTIBODIES IN SERA OF LIVESTOCK

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1. INTRODUCTION

Q fever, a coxiellosis, in animals is a worldwide zoonosis caused by *Coxiella burnetii*. Domestic ruminants — sheep, goats and cattle — are the main reservoirs of *C. burnetii* responsible for both human and livestock infections. Serology remains the major means of confirmation of clinically suspect *C. burnetii* infection as well as for seroepidemiological studies. A sandwich enzyme linked immunosorbent assay (ELISA) has been used to detect antibodies in different animal sera and was compared with three commonly used serological methods. Six antigenic preparations obtained by different methods from *C. burnetii* strain Nine Mile in phases I and II have also been compared.

2. METHODS

ELISA was used to detect whole serum antibodies in goat, sheep and aborted cow sera, and the results were related to those from complement fixation (CF), microagglutination (MA) and immunofluorescence (MIF) tests. The cut-off values were as follows: serum dilutions of 1/8 for CF, 1/16 for MA and 1/32 for the MIF. In ELISA, positive sera were considered to be those that yielded serum dilutions equal to, or greater than, the mean plus three standard deviations of the mean of 24 goat and sheep sera considered negative which had been collected from different parts of Slovakia. Sera from 91 aborted cows were collected in farms located near Bratislava.

*Antigens* I and II: purified whole cell phase I (EP₃) and phase II (EP₁₆₄) cells, respectively, grown in chicken embryo yolk sacs; III: the so called phase II artificial antigens obtained from phase I cells in EP₆ by potassium periodate treatment; IV: a complex phase I and phase II antigen prepared by the whole cell phase I in EP₆.
subjected to mild acid hydrolysis; V: phase I antigen treated with a chloroform–methanol mixture; VI: residuum left after treatment of phase I whole cells with trichloracetic acid. All antigens were adjusted to the concentration of 1 mg/mL.

3. RESULTS

Testing of sera of aborted cows with ELISA, MA and MIF tests for the presence of antibodies to *C. burnetii* resulted in the highest proportion of positive sera in ELISA (41%) with antigen phase II and 16% with antigen phase I, respectively. In the MA test, only 25% of the sera were positive, and in the CF test 34% of the sera were positive, both with phase II antigen. Examination of goat and sheep sera revealed virtually equal reactivity with all antigens used in ELISA, but not in other tests. In the MA and MIF tests, the most sensitive was antigen II, followed by antigens III and IV detecting predominantly phase II antibodies. In the C test, which was found to be least sensitive (although our results could be affected by the anticomplementarity of some sera), the best reactivity was observed with antigen II detecting only phase II antibodies.

4. CONCLUSIONS

Our results indicate that the detection of antibodies to *C. burnetii* can be influenced not only by different antigenic preparations, but also by different serological methods. The advantageous sandwich ELISA with its simplicity, automation and objective reading represents the method of choice for screening great numbers of samples and for evaluating epizootiological situations.
Monoclonal antibodies (MAbs) directed against intracytoplasmic antigens from *Trypanosoma vivax*, *T. congolense* and *T. brucei* bloodstream forms released following cell destruction were developed [1] and incorporated into a sandwich enzyme linked immunosorbent assay (ELISA) [2]. As supported by the Joint FAO/IAEA Animal Production and Health Subprogramme, the ELISA technology was transferred to National Agricultural Research Systems (NARS) in Africa and used in combination with standard parasitological techniques for monitoring tsetse and bovine trypanosomosis control programmes. Referring to the results of the assay field evaluation from 15 African countries [3, 4], the FAO/IAEA direct antigen sandwich ELISA had a diagnostic specificity in the range of 94–100% using a cut-off value of 10% positivity when applied to negative cattle populations in tsetse free areas. In comparison with other diagnostic techniques, the results from positive cattle populations in tsetse infested areas demonstrated that the ELISA had greater diagnostic sensitivity than parasitological techniques in the detection of *T. brucei*, but had less in the detection of *T. congolense* or *T. vivax*. However, the diagnostic sensitivity of the ELISA varied enormously between animals. Consequently, in addition to routine...
activities in the trypanosomosis programme, the FAO/IAEA Agriculture and Biotechnology Laboratory became involved in the provision of assistance for the reassessment of the diagnostic sensitivity of the immunoassay in collaboration with international institutes. The aim of the present study was to investigate the capability of the IgG\textsubscript{1} isotype MAb Tv27 to detect antigens in experimentally \textit{T. vivax} infected goats. Moreover, two IgM isotype MAbs (Tc39, Tbrl), stated to be species specific for \textit{T. congolense} and \textit{T. brucei} antigens, respectively, were assessed for potential cross-reactivity to \textit{T. vivax} released antigens.

Before infection of two female goats from Austria, EDTA blood samples were tested negative for the presence of haemoparasites using giemsa stained blood smears and dark ground/phase contrast buffy coat techniques (BCTs). The packed cell volume (PCV) and the body temperature were found to be at physiologically normal levels. Serum samples were tested negative for antigens of \textit{T. vivax}, \textit{T. brucei} and \textit{T. congolense} by ELISA. For the experiment, a cloned population IL2160 of West African \textit{T. vivax} was propagated in mice and separated from blood by anion exchange chromatography. The eluted trypanosomes were washed 5 times in 0.01 M phosphate buffered saline plus glucose (1%), pH8.0, to remove all mouse components attached to the trypanosomal surface. This was done to prevent interference by mouse protein induced antibodies with mouse monoclonal antibodies used in the ELISA. The goats were infected by intramuscular injection with 3 mL of \(5 \times 10^7\)/mL of \textit{T. vivax} bloodstream forms. The course of infection was assessed by determining the PCV, rectal body temperature, other clinical signs and the day when parasites and antigens first appeared in the systemic circulation. Trypanosomes first appeared in both goats on day 5 post-infection (pi), followed by antigen on day 5 pi and day 7 pi, respectively. At the same time, a decrease in PCV values was observed which returned to physiologically normal level upon treatment with Berenil® on day 103 and day 125 pi, respectively, after which no parasites were observed. During the same period, the increase of body temperature above 40°C was observed, and the development of clinical signs, such as anaemia, dyspnoea, somnolence and temporary clouding in both eyes, correlated with the appearance of trypanosomes in circulation but not with the presence of trypanosomal antigens. Shortly upon infection, a correlation was observed between positive ELISA and positive BCT results following the onset of the first parasitaemic wave. Thereafter, numerous parasitaemic waves were recorded before \textit{T. vivax} antigens could be detected by ELISA. For the period up to day 103 pi and 125 pi, respectively, \textit{T. vivax} trypanosomes were found on 31/75 sampling days (41.3%) and 33/82 sampling days (40.2%), respectively. During the same period, the BCT failed to detect trypanosomes on 8/75 sampling days and 19/82 sampling days, respectively, when the goats were tested positive for \textit{T. vivax} by ELISA. Although the results demonstrated that the combination of ELISA and BCT may improve diagnosis of trypanosomosis in both goats, the \textit{T. vivax} antigen ELISA alone was considered unsatisfactory; the diagnostic sensitivity of the ELISA was found to be 30.7% and
45.1%, respectively. This could be caused by capture and detecting antibodies used in the ELISA competing for the same specific \textit{T. vivax} epitope, and/or by released trypanosomal antigens which were masked by goat produced antibodies forming immune complexes during the course of the infection. Using MAb \textit{Tc39}, the ELISA had less diagnostic sensitivity than the BCT detecting \textit{T. vivax} trypanosomes. In contrast, for MAb \textit{Tbr7} the diagnostic ELISA sensitivity was found superior to that of BCT. The latter observation might be the reason why the ELISA sensitivity would appear to have been improved, judging from an analysis of ELISA data based on MAbs \textit{Tv27}, \textit{Tb7} and \textit{Tc39} (50.7% and 74.4%, respectively). Taking into account that a cloned \textit{T. vivax} stabilate was used for experimental infection and no MAb reactivity to Berenil® dissolved in negative serum was found in previous studies, these findings provide evidence of potential cross-reactivity of the two IgMs against either \textit{T. vivax} released antigens or non-specific components in goat serum giving false positive results. Interestingly, one goat remained positive by ELISA until the end of the experiments (day 416 pi); using MAbs \textit{Tbr7} and \textit{Tv27}, the optical density values were similar to those previously observed at parasite confirmed stages. Therefore, false positive results possibly caused by circulating anti-idiotypic antibodies in chronic infections were considered as well.

The results of this study, seen in the context of those obtained from various studies conducted under experimental and field conditions, emphasize the urgent need for the development and validation of a second generation of improved, sensitive, specific and reliable immunoassays which are suitable for use under the conditions prevailing in tsetse infested regions.

**REFERENCES**


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