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Technical Report 11

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INDONESIA

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Report prepared for
the Government of the Republic of Indonesia

by

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ISOLATION OF ARBOVIRUSES, THEIR IDENTIFICATION AND THE IDENTIFICATION OF THEIR CULICOIDES VECTORS IN INDONESIA

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Project code:	INS/5/021 (INS/88/013)
Project title:	Agricultural Production - Phase 11
Expert mission No.:	11-67
Mission title:	Animal Diseases, Arbovirus studies.
Counterpart Inst.:	Research Institute for Animal Diseases (BALIVET), Bogor, Indonesia.

TERMS OF REFERENCE

In February-March 1991 a two week mission was undertaken to Bogor, Indonesia. The terms of reference as quoted by IAEA are as follows:

1) Assist counterparts in on-going studies to determine the incidence and importance of arbovirus infection in ruminants in Indonesia.

2) Specifically, assist with identification of the vectors which transmit bluetongue and related arbovirus infections.

3) Develop work plans for future studies and advise IAEA on inputs required.

In addition but compatible with those terms of reference, the counterparts also requested more specific advise on:

(i) Handling systems for Culicoides spp. collected for virus isolation.

(ii) Identification of Culicoides to be used for virus isolation.

(iii) Processing method for use with Culicoides collected for virus isolation.

(iv) Improved strategies for identifying virus isolates.

(v) Technology for starting Culicoides colonies.

(vi) Practical aspects of carrying out Culicoides ecology studies.

(i) Staff

The arbovirus group at the Research Institute for Animal Diseases, Bogor, consists of 3 senior personnel (Dr I Sendow - Project Leader & Veterinarian, Sukarsih - Entomologist, Eha - Scientist) and one technician. All Senior personnel have completed periods of overseas training.

In addition to full time staff, the arbovirus group also benefits from various support personnel, including a tissue culture section where BHK-21 cells, VERO cells C6/36 cells etc. are maintained and supplied regularly to the group.

The working relationship within the arbovirus group and with Dr P Daniels, the resident 'Supervisor' seems to be good. The members of the group are enthusiastic about their work and keen for it to succeed.

(ii) Status of work

Epidemiological studies on arboviruses and in particular orbiviruses have been carried out at Bogor since 1987 under the financial auspices of SIDA/IAEA. The project is therefore at an advanced level of implementation and at least 7 papers based mainly upon orbivirus serology and isolation of orbiviruses from ruminants have been published. As part of this work, sentinel herd/flocks of cattle and sheep/goats have been established at various times at; Cikidang (65 Km S. of Bogor), Cisarua (20 Km S.W. of Bogor) and Depok (30 Km N. of Bogor). No serum conversions to any of the viruses under study were detected by AGID

tests in over 16 months monitoring of the sheep/goat flask at Cikidang while low levels of sero-conversion were detected in Friesian cattle at Cisarua. These conversions occurred mainly in April, May & June. At Depok high levels of sero conversion occurred. usually during February-March so that by May virtually every animal was sero positive.

To date bluetongue virus (BTV) types 7 & 9 plus an untyped epizootic haemorrhagic disease virus (EHD) have been isolated from cattle at Depok. Numerous other, so far unidentified isolates, have also been made from ruminants but none have yet been made from vector species of insects. Further the composition, seasonal prevalence and distribution of potential arthropod vector populations (Culicoides spp.) in the areas under study is poorly understood.

(iii) Equipment and facilities

a) Buildings & laboratories - The quality of these is good and of the standard required for arbovirus studies.

b) Equipment & reagents - In general these were appropriate for the work being carried out. Dissection and high powered microscopes were available and are suitable for the entomological aspects of the work. A selection of vertebrate and invertebrate cell lines are also usually available for virus isolation and propagation. Provision of cold storage space (Liq. N_2 , -20°C, -70°C) is adequate, though in view of the large number of sera being collected additional space at -20°C may soon be necessary.

The BTV competitive ELISA was not being used while the expert was 'on site'. This was apparently due both to a lack of the mouse

monoclonal antibody (3-17-A3) and the standardised BTV ELISA antigen. Ten mls of 3-17-A3 were supplied by the expert on arrival and appropriate amounts of the standardised antigen will be despatched shortly.

Embryonating hen eggs (ECE) are available as a primary screen for virus isolation but their high cost is a major constraint upon this part of the group's work.

Four ground glass insect homogenisers are in use. However, if serious attempts are to be made to isolate viruses from insects at least 20 are necessary. Thought should be given to acquiring these without delay.

A number of CDC and NAMRU insect collecting light traps are available for monitoring vector insect populations and for collecting insects for virus isolation. These traps are of low power with a feeble light source. They are by no means ideal for the projected work. Thought should be given to 'upgrading' these items of apparatus.

A Pirbright-type miniature light trap had been supplied to the project and delivered according to IAEA instructions to Jakarta, 12 days before the arrival of the expert. This apparatus was still unavailable at the time of departure of the expert 27 days after its arrival in Indonesia. The project staff at Bogor were unaware of its arrival until enlightened by the expert. Communication between the UNDP office in Jakarta and BALIVET Bogor requires improvement. Fortunately a duplicate light trap carried by the expert on his arrival was available during the mission.

(iv) Supplies - On arrival at Bogor supplies of various laboratory reagents not locally available were delivered to the arbovirus group.

(1) VERO cells

(2) PDAM (12.5 litres)

(3) L15 media (20 litres)

(4) BTV Mab. (10 mls)

(5) Egg drill & bit.

WORK PROGRAMME

After protocol visits a tour of inspection was arranged of the various laboratory facilities in the Departments of Virology and Parasitology (Entomology) at BALIVET.

Subsequently, preliminary discussions were held with the 3 senior members of the arbovirus group and with Dr P Daniels to formulate an appropriate work programme and for familiarisation purposes. It was confirmed that the major requirement of the arbovirus group was to develop and implement an effective programme to identify the main BTV and EHDV vectors in Indonesia and to link the distribution and seasonal prevalence of these vectors to sero conversation in the sentinel herds/flocks. The subsequent 14 days were spent attempting to fulfil these requirements. In brief the following activities were undertaken.

1. The Sentinel Herds - The sentinel herds were visited at Depok (farms of Mr Madio & Mr Ali Udin), Cisarua & Cikidang (sheep & goats). The cattle herds at Depok are in an area of secondary rain forest at an altitude of about 95 m above sea level and with an annual rainfall of 3000 mm. The cattle are zero grazed and are kept in open sided sheds. Manure is habitually dumped over an area of some 20 - 50 sq metres

adjoining the animal housing and provides an ideal breeding site for Culicoides, particularly Avaritia species.

At Cisarua the sentinel herd of cattle was at an altitude of 1070 m in an area with an average annual rainfall of over 3500 mm. The area was more open than Depok and was surrounded by grassland and dwellings. Manure was not stored adjoining the cattle sheds but was removed several times a week. The overall impression was that this is a less favourable breeding site for Culicoides.

At Cikidang (alt. 450 m, ann. rainfall 3500 mm) the sentinel flock of sheep and goats was kept in a wooden 'pen' mounted 1 m above the ground. Although without walls the low overhanging roof and barred sides gave a more enclosed 'feel' than with the cattle sheds. The sheep and goats were allowed out for brief periods during the day but were housed during the evening and night. No other large animals were seen in the immediate area. The general impression was that the climate in this region is suitable for Culicoides but the absence of breeding sites and alternative hosts would mitigate against large populations of vector species.

Light traps were operated on several nights at the Depok and Cisarua sentinel herds to collect Culicoides both for identification and for virus isolation. Initially 8 assorted CDC & NAMRU traps and one Pirbright trap were operated each night. However, as the CDC light traps caught on the average fewer than 100 Culicoides per night while the Pirbright trap caught between 5 and 10,000 Culicoides per night, after the first night the Pirbright trap was used alone.

2. Culicoides Identification - For identification purposes it was recommended that Culicoides species be collected into PBS (or isotonic saline) containing approx. 0.1% detergent as a wetting agent. It was further recommended that collections should be retrieved on the following morning and the insect catch transferred into 2 - 5% formalin without delay or at most within 8 hours of retrieval. Formalin is preferable to alcohol since: it does not harden the insects (which would make for difficult slide mounting), it does not evaporate as readily and it is less liable to cause fading of wing markings. Should it be anticipated that retrieval of insect collections may take longer than 8 hours from capture, then the insects should be collected directly into 2 - 5% formalin plus a wetting agent.

On return to the laboratory, Culicoides species should be sorted from the remainder of the insect catch and should be stored in a cool (<20°C) dark area.

Some Culicoides can be identified to species under the dissecting microscope by the use of wing markings alone. However, many species can only be identified with certainty after dissection, slide mounting and examination under a high powered light microscope. It was recommended that the 'Pirbright-method' of slide mounting Culicoides be adopted (Annex 1).

A number of slide mounted Culicoides plus several thousand intact Culicoides were taken to the UK by the expert for confirmation of identification. A full list of the species of Culicoides identified from Indonesia, including slide mounted specimens and photograph will be sent to the project entomologist in due course.

3. Virus Isolation from Culicoides - Culicoides had previously been collected in Indonesia for virus isolation, in cages suspended from NAMRU/CDC light traps. This method catches small numbers of insects and is only suitable if the insects are returned to the laboratory without delay. Otherwise the specimens desiccate, which makes identification difficult and is likely to inactivate any virus which is present. Furthermore, desiccation makes division of the Culicoides into the various categories used during virus isolation procedures virtually impossible [(i) males, (ii) blood engorged females, (iii) non-blood engorged, nulliparous females, (iv) non-blood engorged parous females]. This is a major drawback since meaningful data on vector competence for viruses can only be obtained via isolations from the Culicoides in category (iv). The reasons for this fact and a recommended protocol for the collection and handling of Culicoides for virus isolation is shown in Annex 2. In more detail and in order to facilitate sorting of Culicoides to optimise the chances of isolating viruses from them, it is further recommended that:

(i) Insect collections should be made into PBS (or isotonic saline) including antibiotics (broad spectrum, 200 iu/ml) and 0.1% wetting agent.

(ii) Wherever possible insect collections should be returned to the laboratory on the same night as collection, being transported in a cool box and being stored at 4°C.

(iii) Insect collections should be sorted to species or species groups on the day following capture (see Annex 2).

(iv) Culicoides species or species groups should be separated into pools, each pool consisting of 1 - 200 Culicoides.

(v) Each pool of Culicoides should be rinsed twice in fresh PBS plus antibiotics.

(vi) If necessary, pools of Culicoides may be stored in 2 mls of PDAM plus antibiotics at 4°C for periods of 1 - 2 weeks.

(vii) Each pool of Culicoides should be ground up in 2 mls of fresh PBS plus antibiotics in a Griffiths tube or similar. Separate grinders should be used for each pool.

(viii) Spin the ground (homogenised) Culicoides at 2000 rpm for 5 minutes.

(ix) Filter the supernatants (45 μm disposable filter) into sterile containers.

(x) Mix 1 ml of each supernatant with an equal volume of PDAM and keep at -70°C for reference. Keep the second ml of supernatant at 4°C until required.

(xi) Inoculate 0.1 mls of a 1 in 10 dilution of each supernatant, iv. into each of 3 - 4, 11 day old embryonating hens eggs. If the solution proves toxic use a 1 in 100 dilution.

(xii) Observe for 5 - 6 days.

(xiii) Grind up the hearts of the embryos dying between 2 - 6 days post infection in 2 mls of sterile PBS plus antibiotics.

(xiv) Spin at 2000 rpm for 5 minutes.

(xv) Filter the supernatant as previously.

(xvi) Inoculate onto BHK-21 cells and if available C6/36 Aedes albopictus cells.

(xvii) After 7 days sonicate the Ae albopictus cells and inoculate onto fresh BHK-21 cells.

(xviii) Observe the BHK-21 cells for cytopathic effects.

(xix) Apply virus identification procedures (AGID, ELISA, SN_{50}) should isolates be detected.

When attempting to isolate viruses from insects speed is of the essence. Therefore, since many Culicoides are either impossible or exceedingly difficult to identify to species without slide mounting it is recommended that Culicoides collections should be divided into the following easily differentiated species or species groups:-

a) Sub group Avaritia, excluding C.actoni.

b) C.actoni.

c) C.oxystoma.

d) C.peregrinus (Sub genus Hoffmania)

e) C.sumatrae (sub genus Hoffmania)

f) Sub genus Trithecoides.

g) Other species of Culicoides.

With the exception of N. America virtually all BTV and EHDV vectors have been identified within the subgroup Avaritia and/or C.schultzei group (oxystoma), therefore these divisions are in descending order of their likely importance.

4. Further Discussions - Towards the end of the consultancy further discussions were held with project staff on a number of topics including the 1991-92 projected work plan.

a) In general the projected work plan is appropriate to the resources available. However, in the expert's opinion, it would not be possible to carry out a meaningful pathogenesis study or a gene mapping programme in the present phase of the project although this work should form part of any future phase.

b) Suggestions were made concerning the unreliable results of the immunofluorescent antibody test (IFAT) which is being used as a group specific test to identify orbiviruses, rhabdoviruses, bunyaviruses and flaviviruses. It is essential with this test to include the appropriate positive and negative controls every time that the test is carried out.

c) Culicoides colonisation - No Indonesian species of Culicoides have been or are being maintained as self-sustaining colonies. Nevertheless the expert does maintain two other species of Culicoides in the laboratories of the IAH Pirbright. The technology for maintaining these species could be transferred to Bogor (import requirements permitting) where appropriate facilities have been identified. However, colonisation of

Culicoides is invariably time consuming and labour intensive and the advantages to the project at this stage in its implementation are not immediately clear to the expert. It should be considered very carefully why such colonies may be necessary and what use is to be made of them before valuable and scarce resources are committed.

d) The expert agreed to take several Indonesian cattle virus isolates to the UK for typing at the IAH, Pirbright.

CONCLUSIONS AND RECOMMENDATIONS

In general, the work being carried out by the project staff in the context of an arbovirological-epidemiological study is of high standard and much valuable information has already been gathered.

The major impact of the present assignment has been to refine and redirect that part of the programme dealing with the isolation of viruses from insect vectors. There is now a realistic chance that isolations will be made and the major vectors identified.

However, as in any epidemiological study involving arboviruses, it is important that virus activity in both the vector insect and vertebrate populations should be studied in concert to gain a proper understanding of the disease picture. It is also vital not only to collect potential vectors for virus isolation, in the places of and at the times of sero conversion in the vertebrates but vector population studies should continue throughout the year in an attempt to link seasonal virus activity in vertebrates with vector prevalence and distribution.

It is recommended that:

1. Culicoides identification should be based upon slide mounted specimens using the Pirbright method (Annex 1). Confirmation of identification should be sought from the relevant experts in the field as and when necessary.

2. A reference collection of Indonesian Culicoides should be built up under the care of the project entomologist at Bogor.

3. Culicoides population studies (prevalence) should continue throughout the year and for the duration of the project based in the vicinity of the sentinel herds at both Depok and Cirarua.

4. Virus isolation from Culicoides should be attempted using the methods outlined in this report. Isolation should only be attempted at those times of the year and in those areas where sero-conversion in vertebrates indicates that transmission is taking place.

5. The quality of the insect collecting traps should be upgraded to take maximum advantage of the times when virus isolation is likely.

6. The use of the BTV ELISA in conjunction with the AGID test for preliminary virus identification should recommence as soon as possible. The IFAT at present in use at Bogor should be standardised and only used with the appropriate controls.

7. Data on sero conversion and virus activity in sentinel herds should be analysed in the light of Culicoides activity in the same areas.

8. Initiation of Culicoides colonies is time consuming and labour intensive. This is not recommended at the present time and at the present staff complement.

9. Gene mapping and pathogenesis are important parts of any investigation on BTV. However, with the time constraints of the present phase of this project and at the present level of resources it is not possible to carry out meaningful studies. This area of work is therefore not recommended at the moment but it would form an important part of any future study.

Annex 1

Slide Mounting Culicoides for identification

1. Specimens preserved in 2 - 5% formalin or 70% ethyl alcohol may be used but formalin is best since it does not harden the insects.

2. Soak the specimens overnight in a saturated solution of alcoholic phenol to clear and soften.

3. Take from alcoholic phenol and blot to remove excess liquid.

4. Mount in 4 'spots' of Canada balsam (diluted in alcoholic phenol) in the following way:

   (i) Top right-hand spot, 1 wing

   (ii) Top left-hand spot, head (both antennae and mouthparts showing)

   (iii) Bottom left-hand spot, abdomen (ventral side up showing spermathecae or male genitalia).

   (iv) Bottom right-hand spot, rest of insect (cut off mesonotum from thorax and mount dorsal side up).

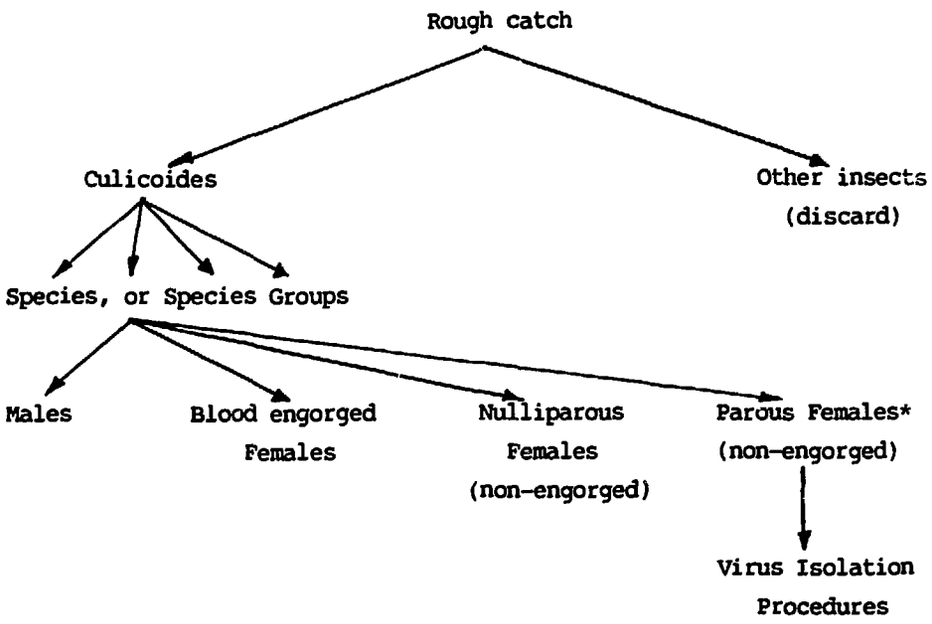
5. Cover each of the 4 'spots' with an 8 mm. square cover slip.

6. Label with; species name, male or female, Country and area of collection, date of collection, name of collector.

7. The mounting medium will take at least 48 hours to dry.

Annex 2

Sorting Culicoides collected by light trap for Virus Isolation



Male Culicoides	- Don't bite, no evidence for transoverial transmission. Won't contain virus.
Female Culicoides, non-engorged, nulliparous	- Not had a blood meal,
Female Culicoides, blood engorged	- May contain virus, but this may be in the meal, no information on vector potential.
Female Culicoides, non-engorged, parous	- Have had blood meal and digested it. May contain virus, may have significance in terms of vector potential.

NOTE - Most species of Culicoides develop a burgundy red pigment in the abdomen when parous. The abdomen of nulliparous Culicoides is without this pigment.