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THE USE OF MOLECULAR BIOLOGY TECHNIQUES FOR THE DIAGNOSIS AND EPIDEMIOLOGICAL STUDY OF FOOT-AND-MOUTH DISEASE VIRUS IN THAILAND

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Abstract

THE USE OF MOLECULAR BIOLOGY TECHNIQUES FOR THE DIAGNOSIS AND EPIDEMIOLOGICAL STUDY OF FOOT-AND-MOUTH DISEASE VIRUS IN THAILAND

The detection of foot-and-mouth disease (FMD) virus from various kinds of field samples (tissue extract and cell culture isolate) was studied using the polymerase chain reaction (PCR) technique. The gene selected for diagnosis was the polymerase gene and an amplification target product of 454 bp in length was produced using AP5/AP6 primer sets. The PCR product was further examined by NcoI endonuclease digestion. The presence of the internal restriction site was confirmed by demonstration of two small fragments of 330 bp and 124 bp in length. Forty-nine samples that gave positive and negative results by ELISA typing and were positive by the PCR test were tested by NcoI digestion to confirm the results. About 10% of PCR products could not be confirmed by the method. Furthermore the FMD RNA polymerase gene could be detected by the PCR method in samples negative in both ELISA typing and the virus isolation test. A total of 23 samples were examined and compared after each stage of the testing process. At the end of the extraction for ELISA the amplification product band at 454 bp was detected in 74% of the negative tissue extract samples, and in 48% at the end of the virus isolation procedure. The PCR technique was shown to rapidly and sensitively detect FMD viral genome, when compared with virus titration by tissue culture infectious dose 50% ($TCID_{50}$) method. The PCR was about 10 times more sensitive than the virus titration technique in detection of virus. Therefore, the PCR technique can be used in conjunction with current procedures for FMD diagnosis, to support the routine standard ELISA typing and virus isolation test on clinical samples. The first step of the nucleotide sequencing technique was introduced with a view to study genomic differentiation of FMD outbreak viruses. The appropriate primer sets for each of the three endemic sero-types were optimized and used to detect the PCR products from field isolate viruses. The PCR products of FMDV type O, A and Asia I showed a clear band at 720 bp, 814 bp and 914 bp respectively. This work showed that the technique could be introduced to perform the nucleotide sequencing to support epidemiological investigations.

1. INTRODUCTION

The current diagnostic methods for foot-and-mouth disease virus (FMDV) such as antigen-capture enzyme immunosorbent assay (ELISA) [1], virus isolation, liquid phase blocking ELISA [2] and VIA-agar gel immunodiffusion (AGID) test [3] have been used for routine diagnosis at the FMD Center, Pakchong, Thailand for many years. The virus isolation test using primary cell culture of bovine thyroid gland [4] or primary lamb kidney [5] have been demonstrated to be the most sensitive for detection of virus in clinical samples and oesophageal-pharyngeal fluid or probang samples. Nevertheless, virus isolation procedure is slow, labor intensive and expensive. Recently, the molecular techniques polymerase chain reaction (PCR) and nucleotide sequencing have been published for detection of FMDV from animal tissue [6].

The oligonucleotide primers were selected for the conserved genomic sequences of the viral RNA polymerase (3D) gene [7] ideal for detection of all sero-types of FMDV. This technique may provide a rapid and sensitive laboratory diagnostic test. Rapid laboratory diagnosis and epidemiological investigation by molecular characterization of virus isolates from field are very important requirements in control and eradication of FMD in Thailand. The nucleotide sequencing at the VP1 FMDV genome, provides the most detailed information about an isolate. In this region sero-type O [8], sero-type A [9] and sero-type Asia I [10] are present. Nucleotide sequencing will be useful for tracing back to the origin of any virus causing outbreak in the country.

The aims of this study are:

(1) To established the PCR technology for detection of FMDV in various samples such as tissue samples and cell culture isolate samples in order to improve and develop the diagnostic technique.

(2) To establish the nucleotide sequencing of FMDV outbreak in Thailand to provide a basic molecular epidemiology information in order to trace back to the origin of virus causing outbreaks.

2. MATERIALS AND METHOD

2.1. Test samples

Routine diagnostic samples from cattle, buffaloes and pigs were submitted to the laboratory for FMD serotyping by ELISA test. The clinical samples collected from tongue or interdigital epithelial tissue were extracted by grinding with sand and prepared 10% suspension in phosphate buffer solution. The virus isolates were passaged three times in either primary lamb kidney cells or BHK-21 cells before use. Sample negative in initial typing test was inoculated onto tissue culture for attempted virus isolation and typing. The present study, the above samples were used to amplify viral RNA by PCR and nucleotide sequencing.

2.2. Viral RNA preparation

Viral RNA was extracted from test samples using TRIzol ™ (GIBCO, UK) according to the manufacturer's protocol.

2.3. Oligonucleotide primers

Oligonucleotide primers were synthesized as a single-stranded DNA using an automated synthesizer (Applied Biosytems, Foster City,USA) and the commercial random hexamer (Promega, USA) was also used for first strand synthesis in reverse transcription step and then two specific primer sets were used in the PCR amplification step, which based on sequences from highly conserved region within VP1,VP3 and 3D of FMD viral genome [7,11]. The primer sequences used in RT-PCR and sequencing are shown in Table I.

TABLE I. THE OLIGONUCLEOTIDE PRIMERS USED FOR RT-PCR AND SEQUENCING OF FOOT-AND-MOUTH DISEASE VIRUSES

Primer Designation	Primer sequence 5'→ 3'	Location	Product length (bp)
NK61	GACATGTCCTCCTGCATCTG	2B	Universal primer
O 1D - ROD1	TGTTGAAAACTACGGTGGTGA	1D	700 – 720
A 1C – 612	TAGCGCCGGCAAAGACTTTGA	1C	813 - 816
As1 1C- 505	TACACTGCTTCTGACGTGGC	1C	908 – 914
AP 5	AGGACAAAGCGCTGTTCCGC	3D	454
AP 6	TCAGGGTTGCAACCGACCGC	3D	Antisense primer

2.4. Reverse transcription

The viral RNA was reverse transcribed in 25 µl reaction mix cosisting of 5 µl of sample (extraction RNA suspension), 2.5 µl of 10 mM dNTP's, 5ul of 5X buffer, 1ul of MoMLV (200 U/ul, Promega, USA), 0.5 µl of 0.1M DTT, 1 µl of Random Hexamer or 25 pmol/1µl of NK61 primer. The reaction mixture was run in thermal cycler (Omnigene, Hybaid, UK) for 60 minutes at 42°C.

2.5. PCR amplification

The specific primer set of AP5/AP6, NK61/ID-ROD1, NK61/1C-612 and NK61/1C-505 were used for amplification of cDNA at each target gene of FMD polymerase (3D) segment and FMDV sero-type O, A and Asia I respectively.

The PCR mixture contained of 5 µl of cDNA, 5 µl of 10X Taq buffer, 1 µl of 1 mM dNTP's, 0.5 µl of Taq polymerase (Promega, USA), 3 µl of 25mM $MgCl_2$, 1 µl of 25 pmol Primer 1 and Primer 2 corresponding to the sero-type as described above and volume was made up to 50 µl with DEPC treated H_2O. The mixture was over lid with 20 µl of mineral oil. The PCR reaction was run in the thermal cycling block following the thermal profile: 94°C for 1 minute, 55°C for 1 minute, 72°C for 1.30 minutes for 30 cycles, final extension at 72°C for 5 minutes. After completion 5 µl of PCR products were analyzed on 1.5% agarose gel run at 100V for 30 minutes and stained with ethidium bromide (1 µl/ml) for 5 minutes then destained with distilled water for 10–15 minutes. The product was readily visible under the ultraviolet (UV) transilluminator.

2.6. Restriction enzyme analysis

A PCR product of 454 bp in length was presumed as positive for FMDV from the original sample. The PCR product was purified by using a Wizard Prep DNA purification kit (QIAGEN, Germany) according to the manufacturer's protocol. Confirmation of a specific PCR product was performed by using restriction enzyme digestion with NcoI endonuclease which digest the PCR product into 330 bp and 124 bp as described by Meyer et al [7].

3. RESULTS

The establishment of PCR technology for detection of FMD viral genome in infected tissue sample and cell culture isolate was successful. Field samples from cattle, buffaloes and pigs were used for amplification of viral RNA by PCR technique after those samples have been tested by ELISA typing and virus isolation.

The result in Fig. 1 shows the analysis of PCR product from field sample using appropriate primer sets of AP5/AP6 which complementary to the antigenomic sense at RNA polymerase gene position. The PCR product produced was the estimated length at 454 bp. The confirmation of the PCR product using restriction enzyme analysis of NcoI endonuclease was shown a clear digested fragment at 330 bp and 124 bp in length.

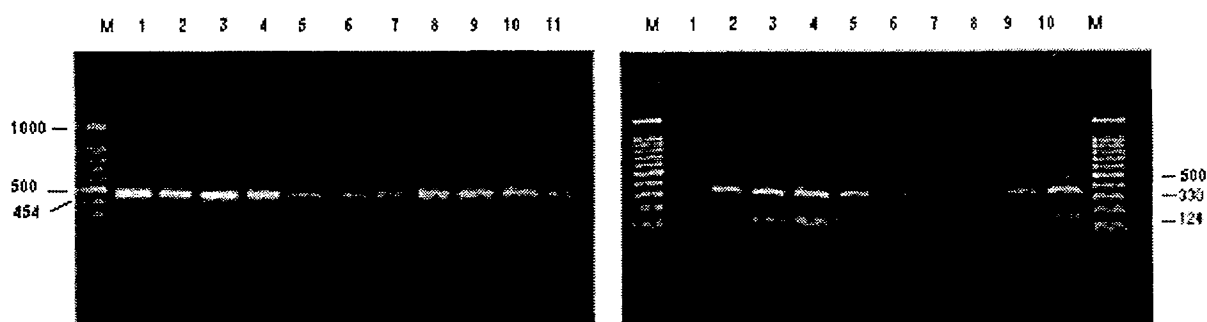

FIG. 1. Analysis of PCR product from field sample using 3D gene specific primer and confirmation of 454 bp band using NcoI enzyme digestion.

M = 100 bp Ladder DNA marker (Promega, USA),

Left gel: no. 1–10 = PCR product using AP5/AP6 primer set giving product band at 454 bp, No.11 = positive cont rol sample

Right gel: no. 1–10 = NcoI enzyme digest into 2 fragments of 330 bp and 124 bp.

By routine standard ELISA typing and virus isolation test of all field samples, twenty three samples, which were classified as negative following initial ELISA typing test and a subsequent virus isolation procedure, were tested by PCR to detect FMD RNA polymerase gene. The initial tissue sample extract was tested along with the matching passage 3 virus isolation tissue culture fluid. The result is shown in Table II.

In addition, a number of specimens were tested to examine the specificity of the PCR technique method. To confirm the PCR product, NcoI enzyme digestion was used. The samples selected were either original tissue sample extracts or virus isolation cell culture fluids. In Table III forty-nine samples regarded as ELISA positive and negative came from these sources. The result showed that 89.80% of the samples could be digested into small fragments of 330 bp and 124 bp by NcoI enzyme reaction. This demonstrates the consisting of the restriction site of NcoI enzyme in most of positive samples and remaining only 10.20% was not digested by NcoI enzyme reaction. The sensitivity of detection of FMD antigen by virus titration using tissue culture infectious dose 50% ($TCID_{50}$) method and PCR technique was studied and compared. The result in Table IV shows that the PCR technique was $1\log_{10}$ more sensitive than the tissue culture method.

TABLE II. PCR RESULT BY DETECTING 3D GENE OF FMDV FROM BOTH TISSUE EXTRACT AND VIRUS ISOLATION SAMPLES WHICH GIVING ALL NEGATIVE RESULT BY ELISA TYPING TEST.

Total sample	Source of sample	PCR test using primer sets of AP5/AP6	
		Positive	Negative
23	Tissue extract fluid	17 (73.91%)	6 (26.09%)
23	Cell culture isolate fluid	11 (47.82%)	12 (52.18%)

Positive = PCR positive result and the 454 bp of PCR product band was detected.
Negative = PCR negative result and the 454 bp of PCR product band was not detected.

TABLE III. COMPARISON OF ENZYME DIGESTION ANALYSIS OF PCR PRODUCT BAND AT 454 BP FROM VARIOUS TISSUE EXTRACTS AND CELL CULTURE ISOLATION FLUIDS WHICH GAVE POSITIVE AND NEGATIVE RESULT BY STANDARD ELISA TYPING.

Type of Sample	Total Samples	Positive PCR giving product Band at 454 bp	NcoI Digestion giving product band at 330bp and 124bp	
			Positive	Negative
Positive by ELISA typing	31	31	30	1
Negative by ELISA typing	18	18	14	4
Total sample	49	49	44 (89.80%)	5 (10.20%)

TABLE IV. COMPARISON OF PCR BAND AND VIRUS TITRATION FOR DETECTION OF FMDV TYPE A.

FMDV type A $10^{7.5}$ $TCID_{50}$ / 0.1ml	Cytopathic effect (CPE) from virus titration	PCR result
Undiluted	+	+ (Strong band)
10^{-1}	+	+ (Strong band)
10^{-2}	+	+ (Strong band)
10^{-3}	+	+ (Strong band)
10^{-4}	+	+ (Strong band)
10^{-5}	+	+ (Strong band)
10^{-6}	+	+ (Weak band)
10^{-7}	–	+ (Weak band)
10^{-8}	–	–

+ = Positive result, CPE occurred in virus titration method or PCR product band detected in PCR method
– = Negative result, No CPE occurred in virus titration method or no PCR product detected in PCR method

The use of PCR technology for epidemiological studies of FMD field isolate viruses type O, A and Asia I was introduced. This initial work was to establish the basis for nucleotide sequencing in order to study the genomic differentiation of FMD field outbreak viruses in Thailand. The appropriate primer set of NK61/1D-ROD1, NK61/1C-612 and NK61/1C-505 were used to amplify the VP1 and VP3 target gene of FMDV type O, A and Asia I, which giving product length at 720 bp, 814 bp and 914 bp respectively. The result is shown in Fig. 2.

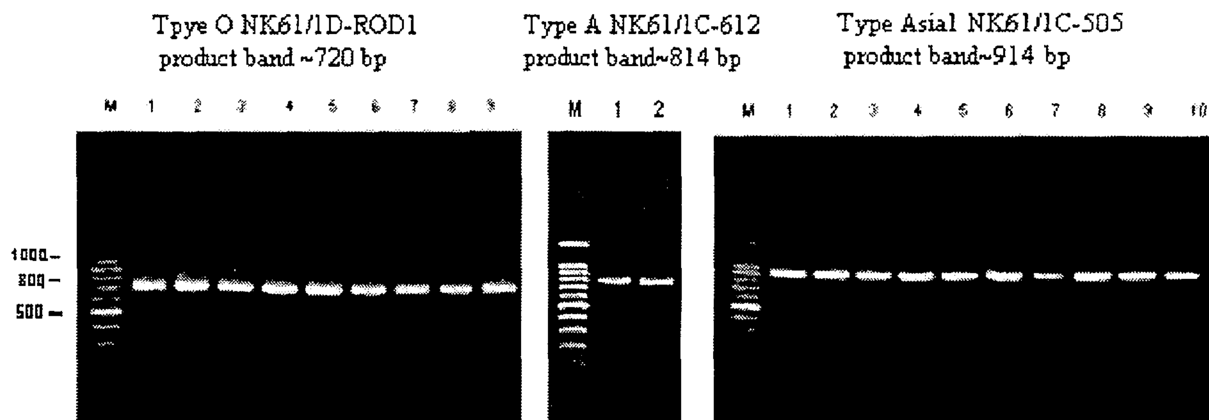

FIG. 2. Analysis of FMD field isolate virus type O, A and Asia I giving PCR product band at 720 bp, 814 bp and 914 bp respectively which were used for nucleotide sequencing.

M = 100 bp ladder DNA marker (Promega, USA)

4. DISCUSSION

PCR technology was successful in amplification of a portion of the FMDV RNA polymerase gene in various types of samples, tissue extracts and cell culture isolation supernatant fluids. The PCR primer set of AP5/AP6 was designed from a highly conserved region of FMDV sequence at 3D region [12] and allowed the production of a PCR product of 454 bp for all sero-types. The enzymatic RNA digestion of the polymerase gene product using NcoI was demonstrated to be a highly specific and sensitive method to confirm the identity of the PCR of 454 bp product band into 330 bp and 124 bp [7]. This sequence of the RNA polymerase gene contained a unique restriction site for NcoI endonuclease producing 330 bp and 124 bp products.

An interesting result observed was that virus was detected by PCR amplification of cDNA in 74% of the original 'negative' tissue extracts, and was still detected in 48% of the same group of samples after 3 serial passages in tissue culture (Table II). This suggests that either viral RNA persisted during the tissue culture isolation procedure or that there was a low level of virus replication for some specimens without observable sample cytopathic effects.

The PCR technique when compared with the tissue culture $TCID_{50}$ method for virus infectivity was shown to be a highly sensitive detecting method [13]. In this study a comparison of the two methods was made using a type A vaccine strain. The PCR method detected virus about 10-fold more dilute than the cell culture titration, i.e. the difference in sensitivity is about 10 TCID50 in 0.1 ml.

The current procedure of rapid and accurate diagnosis of FMDV outbreak is very important for the control and eradication of FMD programmes in the field. In addition, it is important to monitor outbreak viruses to ensure the continued suitability of vaccine viruses. The PCR technique can be used in conjunction with current procedures for FMD diagnosis to support the routine standard ELISA typing and virus isolation test in clinical specimens that may have a low level of virus. Poor quality samples often create a problem in the routine standard ELISA typing test because of undetectable levels of virus. If the PCR could be incorporated into routine diagnosis this would increase the overall sensitivity of virus detection procedures. However there would also be a need to introduce sero-type specific primer sets for sero-type identification [14,15]. Nested PCR would be needed to increase the specificity and sensitivity of the PCR, but the rink of cross contamination would be increased.

The first steps of the nucleotide sequencing technique were introduced at the FMD laboratory with a view to future study of the molecular epidemiology of FMDV field isolates in Thailand and the region. The primer sets of NK61/1D-ROD1, NK61/1C-612, and NK61/1C-505 were optimized and used to produce the PCR products from a collection of field isolate viruses. The PCR products of FMDV type O, A and Asia I were shown clearly as bands at 720 bp, 814 bp and 914 bp respectively. In the future we hope to apply nucleotide sequencing to investigate the epidemiology of FMD to assist the control and eradication efforts in Thailand and our neighbouing countries [11].

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