EVALUATION OF PRIMERS FOR PCR AMPLIFICATION OF RNA POLYMERASE GENE SEQUENCES OF FOOT-AND-MOUTH DISEASE VIRUS

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Summary. – Eight oligonucleotide primers in 7 different combinations were used to amplify 3D gene sequences of foot-and-mouth disease virus (FMDV) by reverse transcription-polymerase chain reaction (RT-PCR). Six of the primers were designed at this laboratory. All the primer combinations could specifically amplify 3D gene sequences of FMDV serotypes O, A, and C. The largest fragment amplified was of 1,393 bp and the smallest was of 208 bp in size. The 1,393 bp fragment included sequences from the preceeding P18 region of FMDV genome. The second largest fragment of 734 bp included sequences from the 3'-extracistronic region of viral genome. The remaining fragments were amplified either from the 3'- or 5'-half of the 3D gene. Specific amplification of the entire 3D gene in fragments of different size showed sequence conservation in the 3D genomic region of FMDV and usefulness of the primers reported in detecting inapparent or persistent FMDV infection in susceptible animals by RT-PCR.

Key words: foot-and-mouth disease virus; 3D gene; reverse transcription-polymerase chain reaction; persistent infection

FMD is a highly contagious viral disease of cattle, sheep, goat, pig and several species of wild animals. It is one of the most economically important viral diseases of domestic animals (Pereira, 1981). The causative virus, FMDV, is a member of the family *Picornaviridae* and exists in 7 antigenically and genetically distinct serotypes, viz. O, A, C, Asia 1, and SAT1-3. The virus genome consists of a single-stranded positive-sense RNA molecule of approximately 8 kb (Sanger, 1979) which is replicated by a virus-coded RNA polymerase (Polatnick and Arlinghaus, 1967). Establishment of persistent/inapparent infection (carrier status without clinical signs) in unvaccinated as well as vaccinated animals is well documented in ruminants, mainly cattle (Burrows, 1966; Salt *et al.,* 1996). In such animals, the oropharyngeal area is the main site of virus multiplication (Van Bekkum *et al.,* 1960; Burrows, 1966). Animals that recover from the disease can remain virus carriers for several months or years after infection (Sutmoller and Gaggero, 1965; McVicar and Sutmoller, 1976; Salt *et al.,* 1996) and may intermittently shed the virus and cause FMD outbreaks (Lubroth and Brown, 1995). Therefore, accurate identification of animals free from inapparent FMDV infection needed for maintenance of disease-free herds in endemic countries and ensuring FMD-free status of the animals before their introduction into the countries free from the disease (Hofner *et al.,* 1993) requires sensitive and fool-proof laboratory techniques. To meet this necessity, the non-structural polypeptides of the virus have been exploited in differentiating healthy animals from those infected. The viral RNA polymerase, also known as the virus-infection associated (VIA) antigen (Newman *et al.,* 1979), is one of the non-structural proteins extensively used for this purpose, as the presence of serum antibodies to this antigen in susceptible animals indicates FMDV infection (Cowan and Graves, 1966; McVicar and Sutmoller, 1970; Pinto and Hedger, 1978; Neitzer *et al.,* 1991). However, the specificity of the

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Abbreviations: AMV = avian myeloblastosis virus; FMD = foot-and-mouth disease; FMDV = FMD virus; nt = nucleotide; PCR = polymerase chain reaction; RT = reverse transcription; VIA = virus-infection associated

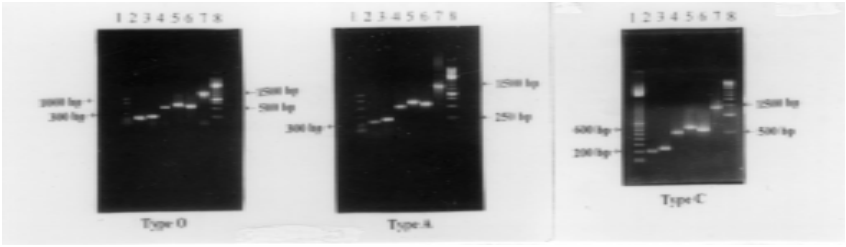

Fig. 1
Regions of FMDV 3D gene and its flanking sequences amplified by RT-PCR using different oligonucleotide primers
The 3D gene region comprises 1410 nt followed by a termination codon for the whole genome (Robertson *et al.,* 1983). The V14/12 primer combination includes sequences from the 3'-extracistronic region of the viral genome. The P18 genomic region is upstream of the 3D gene.

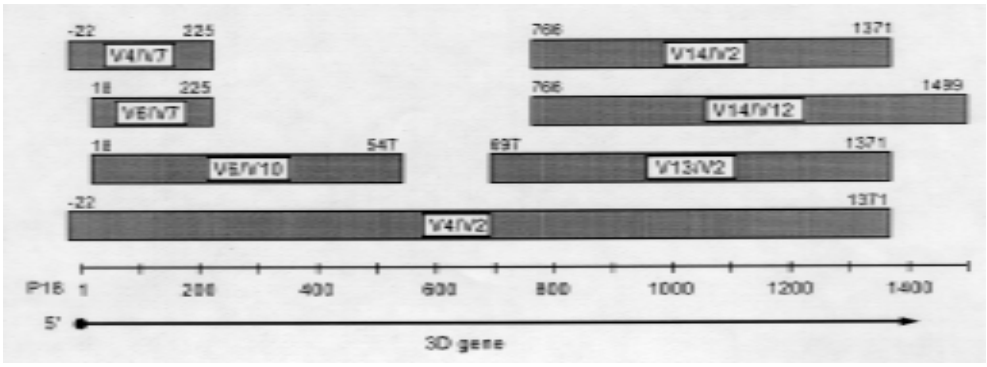

Fig. 2
Agarose gel electrophoresis of the RT-PCR products amplified from FMDV RNA of serotypes O, A, and C using different primer combinations
DNA ladders (lanes 1 and 8); 208 bp fragment amplified with primers V6/V7 (lanes 2); 254 bp fragment amplified with primers V4/V7 (lanes 3); 530 bp fragment amplified with primers V6/V10 (lanes 4); 675 bp fragment amplified with primers V13/V2 (lanes 5); 606 bp fragment amplified with primers V14/V2 (lanes 6); 1,393 bp fragment amplified with primers V4/V2 (lanes 7).

3D antibody response in distinguishing infected animals from those vaccinated was found doubtful (Lubroth and Brown, 1995). In countries like India, where the disease is endemic and the vaccination is mostly restricted to organised herds, the 3D antibody response can distinguish healthy animals from those infected. It is established that the RNA polymerase gene of FMDV has minimum homology with that of other picornaviruses (Rodriguez *et al.,* 1992). This polymerase is coded by the 3D region of FMDV genome located close to its 3'-end and consists of 1410 nucleotides (nt) (Robertson *et al.,* 1983). Though the 7 serotypes of FMDV are divided into two groups (O, A, C, and Asia 1 and SAT1-3) according to their genome homology (Robson *et al.,* 1977), the nucleotide sequence reported for the 3D gene region of different European strains of serotypes O, A, and C (Robertson *et al.,* 1983; Carroll *et al.,* 1984; Forss *et al.,* 1984; Martinez-Salas *et al.,* 1985) shows a high degree of sequence conservation. There are several regions in 3D gene which are highly conserved among FMD viruses but not among other picornaviruses (Martinez-Salas *et al.,* 1985; Rodriguez *et al.,* 1992). Due to a similarity (86.5% homology) in 3D genomic region across the major serotypes of FMDV (Martinez-Salas *et al.,* 1985), the RT-PCR amplification of segments within this gene region has been very fast, sensitive, and reliable in detecting the presence of the virus in persistently infected cattle and other biological materials (Meyer *et al.,* 1991; Rodriguez *et al.,* 1992, 1994; Prato Murphy *et al.,* 1994). In these studies, sequences at the 5'-end of 3D gene were amplified as an indicator of the presence of the virus. However, the potential of this PCR-based approach may be further extended as several other regions, mainly the 3'-end of 3D gene, remain to be tested (Rodriguez *et al.,* 1992).

In the present study, we used 8 oligonucleotide primers (Table 1) covering the entire length of 3D gene and flanking its different regions (Fig. 1), and evaluated their suita-

Table 1. Location and polarity of the primers used for PCR amplification of 3D gene sequences of FMDV

Primer designation	Primer sequence[a] (5' to 3')	Location (nt) and polarity	Reference
V6	CAGAGATGTGGAAGAGCGCG	18-37, +	Rodriguez *et al.,*1992
V7	GCGGAACAGCGCTTTGTCC	225-207, –	Rodriguez *et al.,*1992
V10	CATCGACAATGCGAGTCTTGCC	547-526, –	This study
V14	ATGTTTGAGGAGGTGTTCCGC	766-786, +	This study
V13	AGAAATGTGTGGGACGTGGAC	700-720, +	This study
V2	TGATCTGTAGCTTGGTATCT	1371-1352, –	This study
V12[b]	GGAAGCGGGAAAAGCTCTTT	1499-1480, –	This study
V4[c]	TGACCCTGAACCACAACACG	5565-5584, +	This study

[a]Primer sequences (except V4) were derived from the 3D gene sequence of FMDV A12 (Robertson *et al.,* 1983).
[b]Located in the 3'-extracistronic region of FMDV genome.
[c]Located at the 3'-end of the P18 region (Robertson *et al.,* 1985).

bility in amplifying by RT-PCR similar regions in FMDV serotypes O, A, and C. In this way, a suitable region in 3D gene was identified which can be unambiguously amplified from samples/materials containing the virus, and which represents a highly sensitive tool for declaring animals/materials free from the virus.

Indian reference strains of serotypes O (IND R2/75), A (IND 17/77), and C (Bombay/64) in the form of virus-infected BHK-21 cell culture fluid, were used in the study. The oligonucleotide primers were derived from the nucleotide sequence of 3D gene reported earlier for FMDV A12 (Robertson *et al.,* 1983, 1985) and were synthesized commercially (Genset). Total RNA was extracted from the infected cell culture fluids by the guanidine thiocyanate method (RNAgents, Promega) and reverse transcribed using avian myeloblastosis virus (AMV) reverse transcriptase and oligo$(dT)_{15}$-primer (Reverse Transcription System, Promega). The first strand cDNAs of all the three virus serotypes were subjected to PCR using 7 different primer combinations (Fig. 1) to amplify 3D gene sequences. The PCR was carried out in a 50 µl volume which contained 4 µl each of RT product (cDNA) and 10 × reaction buffer, 2.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, 25 pmoles each of direct and reverse primers, and 2.5 U of Taq DNA polymerase (Promega) in a reaction buffer. PCR mixtures were kept at 95°C for 4 mins and then the amplification was carried out for 40 cycles at 95°C for 1 min, at 55°C for 1 min, and at 72°C for 1.5 min, followed by a final extension step at 72°C for 5 mins. PCR products (5 µl) were electrophoresed in 1% agarose gel containing 0.5 µg/ml ethidium bromide.

The results of RT-PCR are shown in Fig. 2. With all the 7 primer combinations, there was no non-specific priming and PCR products of the expected size only were obtained. The largest amplified fragment of 1,393 bp included 22 nt from the 3'-end of P18 region (a neighbour sequence to the 5'-end of 3D gene). The smallest fragment of 208 bp was amplified from the 5'-end of 3D gene region. The second largest fragment of 734 bp obtained with the primer pair V14/V12 included nucleotides from the 3'-untranslated (extracistronic) region of viral genome. Other primer combinations amplified sequences either from the 3'- or 5'-half of 3D gene region. The primers did not recognize cDNA derived from RNA extracted from uninfected cells.

The results of this study showed that all the 7 primer combinations used could specifically amplify regions of 3D gene of FMDV serotypes O, A, and C, and that the 3D gene sequences were conserved among the FMDV serotypes tested. Compared to the RNA polymerase gene, higher variability was observed in the 3'-extracistronic region of the genome among FMDV serotypes O, A, and C (Martinez-Salas *et al.,* 1985). The oligonucleotide primers proved to be specific for 3D gene of FMDV as they failed to amplify non-viral RNAs extracted from non-infected BHK-21 and bovine kidney cells (data not shown). The 3D gene-specific primers reported in the present study can be very useful in detecting inapparent FMDV infection in animals by RT-PCR. To achieve this aim, any particular region of 3D gene can be targeted using suitable primers.

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