ONE-TUBE AND ONE-BUFFER SYSTEM OF RT-PCR AMPLIFICATION OF 1D GENE OF FOOT-AND-MOUTH DISEASE VIRUS FIELD ISOLATES

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Summary. – A method of reverse transcription (RT) and polymerase chain reaction (PCR) amplification of 1D (VP1) gene of foot-and-mouth disease (FMD) virus using one reaction mixture containing both avian myeloblastosis virus (AMV) reverse transcriptase (RTase) and Tfl DNA polymerase is described. The procedure was time saving, made use of a single buffer for both RT and subsequent amplification and performed better than the two-step procedure usually conducted with Moloney murine leukemia virus (MMLV) RTase and Taq DNA polymerase for amplification of the VP1 gene of field isolates of FMD virus serotypes O, A, C and Asia 1. The failure to amplify the VP1 gene of many type O and Asia 1 viruses using MMLV RTase-Taq polymerase enzyme system could be overcome by performing RT of the viral genome at a higher temperature (48°C) with AMV RTase which is not possible with MMLV RTase.

Key words: foot-and-mouth disease virus; 1D gene; reverse transcription; polymerase chain reaction

The nucleotide sequencing of the VP1 gene of FMD virus isolates is essential in studying molecular epidemiology of the disease (Knowles and Samuel, 1994). Compared to a direct RNA sequencing, cycle sequencing has several advantages (Knowles and Samuel, 1994). Successful application of the cycle sequencing technique depends on efficient RT of the RNA template and susbsequent amplification of cDNA by PCR. The amplification of different genes of FMD virus by PCR is usually done in two steps using two separate reaction mixtures. In the first step, the RNA is reverse transcribed using MMLV/AMV RTase at 37°C – 42°C and then the cDNA is amplified using Taq DNA polymerase in the second step (Meyer *et al.*, 1991; Hofner *et al.*, 1993; Donn *et al.*, 1994; Rodriguez *et al.*, 1994). In the present communication, we report a method of RT-PCR which is much more sensitive and efficient than the commonly used technique mentioned above for amplification of the VP1 gene of FMD virus. The aim of this study was to amplify the VP1 gene of FMD virus field isolates of serotypes O, A(A22), C and Asia 1 by RT-PCR under different conditions so that the obtained product could be used in different downstream applications like cycle sequencing, cloning etc.

Ninety-nine field isolates (60 of type O, 4 of type A(A22), 5 of type C and 30 of type Asia 1) were used in the study. The viruses underwent 3-5 serial passages in BHK-21 cells. The infected cell culture fluids were clarified with equal volumes of chloroform. RNA was extracted from the cell culture fluids using RNeasy Total RNA Extraction Kit (Qiagen). The oligonucleotide primers were provided by Drs N.J. Knowles and A.R. Samuel of the World and Community Reference Laboratory for Foot-and-Mouth Disease, IAH, Pirbright, U.K. The synthesis of cDNA (first strand) and its

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Abbreviations: AMV = avian myeloblastosis virus; DEPC = diethyl pyrocarbonate; DTT = dithiothreitol; FMD = foot-and-mouth disease; MMLV = Moloney murine leukemia virus; PCR = polymerase chain reaction; RT = reverse transcription; RTase = reverse transcriptase

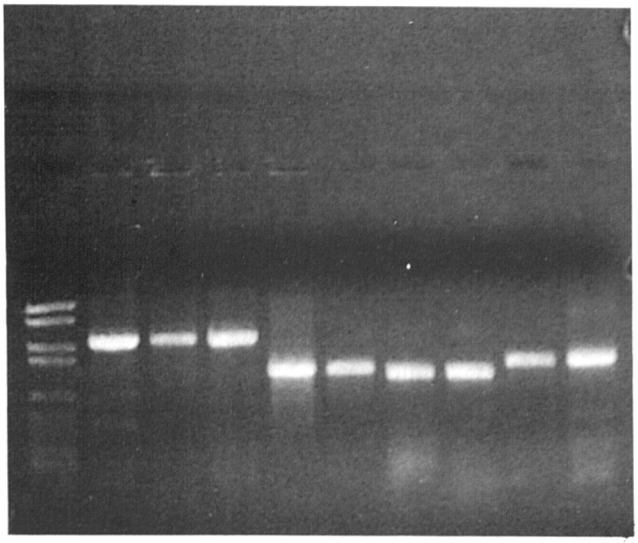

Fig. 1
Agarose gel electrophoresis of the RT-PCR amplified 1D gene of FMD virus field isolates using the one-tube and one-buffer system
DNA size markers (lane 1), virus type O isolates (lanes 2-4), virus type A(A22) isolates (lanes 5,6), virus type C isolates (lanes 7,8), and virus type Asia 1 isolates (lanes 9,10).

Table 1. Comparison of the positivities of amplification of FMD virus 1D gene by RT-PCR procedures I, II, and III

FMD virus type	Procedures		
	I	II	III
O	19/60[a]	19/60	60/60
A(A22)	4/4	4/4	4/4
C	5/5	5/5	5/5
Asia 1	16/30	6/30	30/30

[a]Ratio of positive to total RNA samples tested.
Procedure I: Two-step RT-PCR. MMLV RTase, Taq DNA polymerase. RT at 42°C. Procedure II: The same as I, except RT at 38°C. Procedure III: One-step RT-PCR. AMV RTase, Tfl DNA polymerase. RT at 48°C.

subsequent amplification was performed by three different procedures using universal (NK61, antisense) and serotype-specific (virus sense) primers (O-1C_{124}, A-1C_{562}, C-1C_{616}, Asia 1-1C_{505}).

Procedure I. RT was performed at 42°C for 60 mins with MMLV RTase (Promega) in an 1.5 ml microcentrifuge tube. The reaction mixture (25 µl) contained 14 µl of the RNA preparation, 100 U (0.5 µl) of MMLV RTase, 5 µl of 5x RT buffer (250 mmol/l Tris-HCl pH 8.3, 375 mmol/l KCl, 15 mmol/l $MgCl_2$ and 50 mmol/l dithiothreitol (DTT)), 1 µl (25 pmoles) of pNK61, 1 µl each of RNasin (3.3 U) and diethyl pyrocarbonate (DEPC)-treated water and 2.5 µl of 10 mmol/l dNTPs. The PCR amplification of reverse transcribed RNA was done in an 0.5 ml tube and the reaction mixture (50 µl) contained 5 µl of a template (cDNA), 1 µl of 10 mmol/l dNTPs, 3 µl of 25 mmol/l $MgCl_2$, 5 µl of 10x reaction buffer (500 mmol/l KCl, 100 mmol/l Tris-HCl pH 9.0, 1% Triton X-100), 1 µl (25 pmoles) each of pNK61 and type-specific primer, 0.5 µl (2.5 U) of Taq DNA polymerase (Promega) and 33.5 µl of DEPC-treated water. The mixture was overlaid with mineral oil and the amplification was carried out on a heating block (Hybaid) for 40 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 2 mins) followed by a final extension step at 72°C for 7 mins. The samples were then electrophoresed in 1% agarose gel containing ethidium bromide (0.5 µg/ml). DNA Molecular Weight Marker VI (Boehringer Mannheim) was used for reference.

Procedure II. RT with MMLV RTase was done at 38°C for 60 mins. The rest of the steps were similar to those of the procedure I.

Procedure III. RT as well as PCR amplification were performed in a single 0.5 ml tube with AMV RTase and Tfl DNA polymerase (Access RT-PCR Kit, Promega). The reaction mixture (50 µl) contained 4 µl of RNA sample, 10 µl of 5x reaction buffer (a component of the Access RT-PCR Kit), 1 µl of 10 mmol/l dNTPs, 2 µl of pNK61 (25 pmoles/µl), 1 µl (25 pmoles) of type-specific primer, 2 µl of 25 mmol/l $MgSO_4$, 1 µl (5 U) of AMV RTase, 0.5 µl (2.5 U) of Tfl DNA polymerase and 28.5 µl of DEPC-treated water. The mixture was overlaid with mineral oil and the reaction was carried out on the heating block for 1 cycle at 48°C for 45 mins (RT), 1 cycle at 94°C (AMV RTase inactivation and template denaturation) for 4 mins, 40 cycles (94°C for 30 secs, 60°C for 1 min, 68°C for 2 mins; the synthesis of the second strand and DNA amplification), followed by a final extension step at 68°C for 7 mins. The samples were then analyzed on agarose gel as mentioned before.

The results of RT-PCR amplification of RNA samples as assessed by agarose gel electrophoresis are presented in Table 1 which shows that the conditions of RT-PCR directly affected the results. The RNA of virus serotypes A and C could be amplified by all the three procedures. But RNA of most of the viruses of type O (41/60) and Asia 1 (14/30) could not be amplified by the procedures I and II. This failure in obtaining the RT-PCR products of RNAs of virus types O and Asia 1 was significant. However, RNA samples of the virus types O and Asia 1 which could not be amplified by the procedures I and II, could be successfully amplified using the one-tube AMV RTase-Tfl DNA polymerase reaction system (the procedure III, Fig. 1). This phenomenon may be attributed to the secondary structure of the RNAs extracted from these viruses which is destabilized only at a higher temperature (48°C) during the RT step facilitating the annealing of the primer and subsequent chain elongation. Once the RT was complete, further am-

plification could be done by the Tfl DNA polymerase. Besides, the use of this technique helped in saving valuable time and did not require change of the buffer system for PCR amplification of the cDNA. Recently, Vangrysperre and De Clercq (1996) have reported the use of the Tth enzyme for both RT and PCR amplification of FMD virus genome which required a change in buffering conditions following RT. But the technique employed in the present study makes use of a single buffer system for both RT and PCR so that the technique is more easy and handy and does not require manipulation of the reaction mixture following RT.

Thus the one-tube, one-buffer and two-enzyme RT-PCR amplification system using AMV RTase and Tfl DNA polymerase is a very sensitive and precise technique for successful amplification of the 1D gene of FMD virus isolates.

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