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Original Article

Diagnosis of Foot-and-Mouth Disease Virus by Real Time Reverse Transcription Polymerase Chain Reaction Assay in Iran

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Abstract

Background and Aims: Accurate and rapid diagnosis is necessary for effective control and prevention of foot-and-mouth disease (FMD). In present study, was evaluated real time reverse transcription-polymerase chain reaction (rRT-PCR) assay along with diagnostic routine methods for the detection of all seven serotypes of FMD virus (FMDV), namely O, C, A, SAT1, 2, 3 and Asia 1 in biological samples at the reference laboratory for FMD, Iran.

Materials and Methods: Two different RT-PCR assays targeting two different regions 5´ untranslated region (5´-UTR) and RNA polymerase (3D) of the FMDV genome were used to confirm the presence of FMDV in epithelial suspensions.

Results: In the two methods the viral RNA in all tested archival serotypes of FMDV were detected. Specificity of this reaction was confirmed by the use of swine vesicular disease virus and blue-tongue. The amount of cycle threshold (C_T) value of all seven serotypes was different and the lowest and highest of C_T value achieved for SAT3, A, O types and SAT2, C types, respectively.

Conclusion: The results showed that RT-PCR was more sensitive and effective than routine diagnostic methods. Furthermore, RT-PCR as a strong and valuable tool concomitant with diagnostic routine methods facilitate monitoring the fields FMDV strains and suggested that the use of the multiple diagnostic targets could enhance the sensitivity of the molecular methods for the detection of FMDV.

Keywords: Foot-and-Mouth Disease Virus; ELISA; real-time PCR; conventional RT-PCR

Introduction

Foot-and-mouth disease virus (FMDV) belongs to the genus aphthovirus of the Picornaviridae. FMD is a highly contagious vesicular disease that affects cloven-hoofed domestic and wild animals including goat, pig and sheep (1). Generally, FMD consists of seven serotypes (A, C, O, Asia 1, SAT1, 2, 3) and some subtypes in each serotype (2). The disease is contagious that imposes economic losses to the live-stock and their products due to reduction in productivity, mortality of young animals and affects international trade of live-stock industry thus control of this condition is dependent upon early tracing and monitoring. Iran is an endemic region of FMD and due to neighboring Afghanistan, Saudi Arabia and Pakistan, monitoring the emergence of new

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strains of FMDV in the these regions is important (3). The clinical symptoms is different in various hosts and are not discernable from some diseases such as swine vesicular disease (SVD) and vesicular stomatitis diseases (VSV). Therefore, definitive diagnosis of the disease was performed by laboratory methods (4).

Definitive diagnosis of FMD requires detection of the virus, viral antigen or genetic materials in clinical specimens. Usually sample of choice should be vesicular epithelium from clinically affected animals since, during the acute stage of the disease, it is rich in virus. For almost ten years, virus isolation (VI) and enzyme-linked immunosorbent assay (ELISA) methods have been considered as diagnostic tests for FMD (5). The VI is a sensitive and valid diagnostic method but it is hard, time-consuming. The ELISA is a rapid method to detection infected or non-infectious virus antigen and the specificity of isolated viruses confirmed by the ELISA is not 100% and needs cell suspensions to confirm the diagnosis of virus type (6-8). Both of methods are recommend by World reference Laboratory for FMD (7). If the result of the ELISA in epithelium sample is negative, it cannot be definitely said that the negative result is true because it may be due to the low and unsuitable concentration of antigen therefore detection of FMDV will be postponed at least for 4 days which increase the diagnostic capability of FMDV in the epithelium. In recent years, with the progress made in molecular biology, various reverse transcription polymerase chain reaction (RT-PCR) methods are used to detect and identity FMDV isolations (9-11). Although RT-PCR is used to determine genetics conditions of existing strains in the field and determining the origin of virus infection in epidemics regions (12), its limitation is that only a few number of samples in each reaction series can not be used (13). Reverse transcription polymerase chain method (RT-PCR) is also useful for typing FMDV isolates based on sequencing (14) but this method does not have optimal results in terms of specificity and sensitivity (1).

Recently, real time reverse transcription-polymerase chain reaction (RT-PCR) assays have been developed with the capability of detecting FMDV in a variety of sample matrices (15, 16). These assays characteristically possess both high analytical sensitivity and high specificity. The ability to detect diverse FMDV strains (pan-reactivity) is an additional requirement for any routine FMD diagnostic assay. Although many of the published RT-PCR assays are reported to be able to recognize members of all 7 FMDV serotypes, detailed evaluation of diagnostic sensitivity to comparison methods such as VI and Ag-ELISA has been published for only RT-PCR assay (17). The previous study concluded that rRT-PCR method had superior sensitivity than established diagnostic methods, for FMDV was detected in 18% more in the samples compared with VI and Ag-ELISA combined. The aim of this study was to compare the methods recommended by the OIE for FMDV detection and to evaluate their usefulness.

Methods

Clinical samples

Epithelial suspensions of samples from sick animals were obtained from different provinces during the years of 2009-2010 and submitted to the reference laboratory for FMD, Iran. A 10% suspension was prepared in 0.04 M phosphate buffer from each vesicular epithelium. All FMDV isolates were obtained from the Institute for Animal Health (IAH, Pirbright). Negative control viruses were vesicular stomatitis virus (VSV), swine vesicular disease virus (SVDV) and an epithelium sample of uninfected calf epithelium. Also A blue-tongue virus (BTV) and swine vesicular disease virus (SVDV) isolates were used to check the specificity of RT-PCR reaction.

Virus isolation and ELISA

0.2 mL Filtered epithelium samples were inoculated to sensitive cell such as porcine kidney line cell (IB-RS-2) and to investigate the cytopathic effects (CPE) that were monitored for 24-72 hours.

Cultures that did not CPE than were frozen at -70°C and because monitoring of CPE was re-inoculated onto IB-RS-2 cells. At the order of

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reference laboratory for FMD, ELISA was used to detect FMDV antigen (7). This ELISA was pan-serotypic and the results were read in 492-630 nm. Of course the negative ELISA samples after inoculation to cell culture and using cell culture suspension were analyzed in ELISA for typing.

Viral RNA extraction

Viral RNA was extracted using high pure viral RNA extraction kit (Roche) according to the manufacturer's instructions. The extracted RNA was stored at -70°C and was used in conventional RT-PCR and RT-PCR reactions.

Conventional RT-PCR

This reaction was performed using OneStep RT-PCR (AccuPower® RT-PCR PreMix from Bioneer) Kit. P32 and P33 primers of 2B region of FMDV genome to amplify the fragment of 131 bp length because universal detection of all seven serotypes of this virus (11). The reaction mixture with a volume of 40 µl comprised: 10 µl of 5 x buffer RT-PCR, 2 µl of dNTPs, 0.5 µl of each primer: 2 µl of enzyme mix, and 25 µl of RNase free water. The mixture was supplemented with 10 µl of RNA (total volume of 50 µl). The reaction was performed in an Eppendorf Thermocycler. Amplification was performed at the following temperature cycle: reverse transcription reaction, 42°C for 40 minute (1 cycle), initial denaturation, 94°C for 4 minute (1 cycle), 40 cycles for denaturation, at 94°C for 45 seconds, annealing, at 55°C for 45 seconds, extension, at 72°C for 45 seconds and final extension at 72°C for 10 minute respectively. Finally, the amplification products were separated electrophoretically in a 1.5% agarose gel

Oligonucleotides primers and TaqMan probe

Two primer/probe series of 5´-UTR and 3D gene regions in previous studies were used (15, 18). Primer and probe sequences for detection of FMD virus are given in Table 1.

Conditions of Real-time RT-PCR

The reaction was performed according to the Rotor–Gene ™ 6000 machine by Superscript III/Platinum Taq one-step rRT-PCR kit (Invitrogen). The reaction mixture at a volume of 20 µl comprised: 0.4 µl MgSo4, 10 µl 2x-reaction buffer, 2.6 µl RNase free water, 0.4 µl Enzyme Superscript III/Platinum , 0.25 µl of each primers, 0.1 µl probe and 6 µl RNA. The tube was placed into Rotor-Gene ™ 6000 PCR machine and the amplification was done at the following temperature cycle: RT reaction (1 cycle), at 50°C for 30 minute, initial denaturation (one cycle), at 95°C for 2 minute, 40 cycles at 95 °C for 15 seconds and at 60°C for 30 seconds. The fluorescence acquired at the end of the 60ºC annealing/extension step. Cycle threshold (Ct) value for each sample was

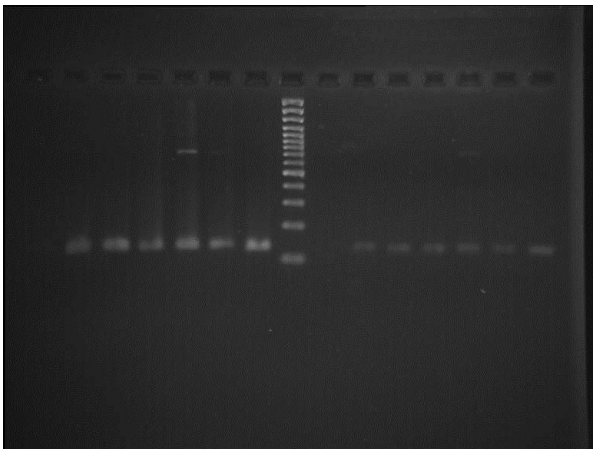

Fig. 1. Electrophoretic separation of amplification products. Of left: Lines: 1-6 FMD type A, O, Asia 1, SAT1, 2, 3, 7-DNA marker (100 bp DNA ladder), 8 – negative control, 9 – positive control ,10-14 FMD type A, O, SAT1, 2, 3, respectively.

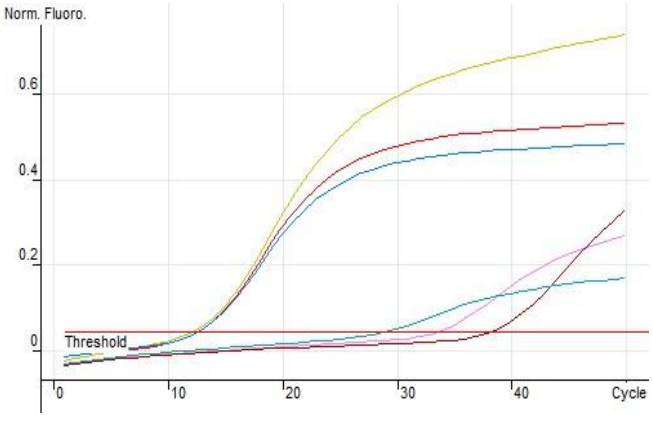

Fig. 2. Logarithmic fluorescence plots versuscycle number resulting from the determination of FMDV RNA in tested samples.

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Table 1. Primer and probe sequences for detection of FMD virus serotypes in rRT-PCR reaction.

Target Gene	Primer/probe name	Sequences
3D	Forward primer	5´ACTGGGTTTTACAAACCTGTGA-3´
3D	Reverse primer	5´-GCGAGTCCTGCCACGGA-3´
3D	probe	5´-FAM-TCCTTTGCACGCCGTGGGAC-TAMRA-3´
IRES	Forward primer	5´-CACYTYAAGRTGACAYTGRTACTGGTAC-3´
IRES	Forward primer	5´-CAGATYCCRAGTGWCICITGTTA-3´
IRES	probe	5´-FAM-CCTCGGGGTACCTGAAGGGCATCC-TAMRA-3´

determined from the point at which fluorescence breached a threshold fluorescence line.

Sensitivity and specificity of RT–PCR

The sensitivity of the RT –PCR was checked by a log-10 serial dilution of Asia 1 purified RNA. The RNA was extracted from a viral stock with $10^{4.8}$ $TCID_{50/ml}$ viral loads. Then RNA of each dilution of virus was extracted and analyze for the sensitivity of RT–PCR. Specificity of the reaction was done by both of the BTV and SVDV isolated RNA because the clinical signs are undistinguished of FMD.

Results

ELISA and virus isolation

The results of virus inoculation (VI) in cell culture (IB-RS-2) and typing samples exists in IRL for FMD are summarized in Table 2. From the 161 epithelium samples (ES), recognizable by CPE were detected in 129 samples (80.1%), but the remaining samples didn't have any infectious virus (no CPE). It should be mentioned that CPE was appeared at different times (every 24 hr for 72 hr). Of 161 samples in ELISA method, only 72 samples were directly identified in terms of type (44.7%). Therefore, to complete the typing of the samples the negative samples were amplified in the cell culture (1-4 days) afterward they were used in ELISA which lead to that other 75 samples were positive. Therefore the detection rate of virus combined with ELISA/VI was 91.3%. In all of samples, only Asia 1, O, A serotypes were present, but most of them (52.7%) were type A.

Conventional RT-PCR

The length of PCR product was 131bp. FMDV detection by RT-PCR method was positive in 146 epithelium samples (90.7%) and the data are summarized in Table 2. There were no replication in negative control samples (BTV, SVDV isolates) but all positive samples of seven serotypes of FMDV were positive Fig. 1.

Real-time RT-PCR

Calculation of C_T -value in ES samples of FMDV reference viruses as positive control, BTV and SDVD viruses as negative control were performed by RT-PCR and the results of this method in comparison with other methods is shown in Table 2. Real-time PCR method detected all seven serotypes of FMDV. No C_T value was detected in FMDV negative samples. Also C_T of less than 20 as strong positive samples was considered. Among all ES, only 4 samples in RT-PCR were negative without C_T whereas also their outcomes in ELISA/VI and conventional RT-PCR methods were negative. Generally, an appropriate correlation was achieved between the results of RT-PCR and ELISA/VI (93.7%) among all samples. It should be mentioned that all positive samples based on VI were with C_T of less than 40, thus false negative results with RT-PCR were not achieved and on the other hand, all negative samples with RT-PCR were negative with ELISA/VI. Among samples, only 7 samples were with C_T=35-40 that considered as borderline. Of 15 negative RT-PCR samples, only 4 samples were not detected by RT-PCR but the other 11 samples

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Table 2: Comparison of diagnostic results for FMD virus by rRT-PCR, RT-PCR, ELISA and virus isolation on 161 field epithelial suspensions during 2009-2010, Iran

Diagnostic methods on ES[a]	Negative results	Positive results
Virus isolation	32 (19.9)	129 (80.1)
Real-time PCR	4 (2.5)	157 (97.5)
Conventional RT-PCR	15 (9.3)	146 (90.7)
ELISA	89 (55.28)	72 (44.72) [b]
ELISA/VI	14 (8.7)	147 (91.3)

[a] Epithelial suspensions ; [b] 72 Positive samples included 38, 33, and 1 cases for A, O, and Asia 1 serotypes, respectively.

Table 3: Results of control and reference FMD virus samples obtained by Real-time RT-PCR and RT-PCR assay in Iran.

Virus isolates Virus isolates	Result	
	RT-PCR	C_T- value
FMD A05	+	13.63
FMD C	+	18.64
FMD Asia	+	15.45
FMD SAT 1	+	14.56
FMD SAT 2	+	19.49
FMD SAT3	+	11.68
FMD O_{2010}	+	14.52
SVDV	-	NO C_T
BT	-	NO C_T
Negative Control (Uninfected calf)	-	NO C_T

Table 4. Sensitivity of Real-time RT-PCR assay by a 10-fold serial dilution of FMD serotype Asia 1 RNA.

Asia 1 dilutions ($TCID_{50}$)	C_T value
1000000	10.41
100000	14.53
10000	21.61
1000	27.82
100	31.77
10	32.95
1	37.54
0.1	NO C_T

have C_T=29-38 and also were positive ELISA/VI.

Sensitivity and specificity of Real-time PCR

A 10-fold serial dilution of the titrated virus FMDV/Asia 1 were prepared to determines sensitivity of RT-PCR. Viral RNA detection limit was achieved by RT-PCR 1 in $TCID_{50}$/ml. Seven subsequent dilutions up to detection limit of RT-PCR were positive considering C_T, which only no C_T Value detected 0.1 $TCID_{50/ml}$.

The amount of C_T in different dilutions is shown in Table 4. Also, no C_T to RNAs of SVD and BT virus isolated RNA of uninfected

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calf epithelium were detected and it shows that two series of primer/probe were specific for detection of FMDV genome (Table 3).

Discussion

Iran is an endemic region for FMDV and it is possible that every moment becomes epidemic which might lead to considerable economic losses. In emergencies, the speed and accuracy of diagnosis of FMD by clinical and laboratory methods are important in controlling and eradicating the disease. It is estimated that the interval between the collection of samples and their delivery to the laboratory was on average about one day therefore we should develop a rapid and reliable diagnosis methods beside the routine methods. There is the question of whether the tests employed were reliable that could have contributed to false negative test results. However this problem could affect the interpretation and significance of the results. The laboratory test procedures were all carried out as recommended at the time by the World Organization for Animal Health (OIE2000) and were recognized as highly sensitive. Therefore we as IRL for FMD should increase their laboratory diagnostic levels to rapid detection of FMD.

The antigen detection by ELISA is highly sensitive when applied to appropriate samples collected during the acute phase of the disease. Ideally, samples of animals are collected in acute stages of disease, when the concentration of virus is high. Unfortunately, suspected samples of FMD given to laboratories are collected in the period after acute stages of disease, in fact, when the virus is exist or it does not exist. However, its efficiency depends on the presence of a certain concentration of virus (or antigen), and it is improved when used in combination with VI in cell cultures of high sensitivity (particularly primary calf thyroid cells). Despite sensitivity of VI, is dependent upon the presence of infectious virus, but infectivity may be lost within samples of poor quality that have been inappropriately stored and dispatched to the laboratory. The results were available from antigen ELISA were ultimately scored as positive; the remainder were scored as positive after the amplification and isolation of virus in cell culture, which took up to four days. In Iran reference laboratories (IRL) for FMD Iran, Karaj, the diagnosis of FMD is done by VI and ELISA. Development of molecular methods as using RT-PCR (10, 19, 20), and RT-PCR (8, 16, 21) can offer advantages for the diagnosis of FMD on such material when it is used in addition to the conventional assays of antigen ELISA and VI.

The RT-PCR method used in the current study has proven to be highly sensitive and specific under laboratory and experimental conditions, which is supported by our results as all previously characterized field isolates resulted in positive reactions with low C_T values. In this study, an RT-PCR method with high sensitivity and specificity detecting RNA of FMDV in epithelium samples was described. Probe and primers of 5'UTR regions and 3D FMDV genome were used and they were specific for detection of each seven serotypes of FMDV as it was used already (15, 18). All of the samples that were positive by combined VI/ELISA were also positive by RT-PCR (Table 2). Furthermore, false negative results with RT-PCR were not achieved. Indeed, a recent study demonstrated that this method performed similarly to another RT-PCR protocol for amplification 5'UTR and 3D regions of the FMDV genome (22). However, samples from the area free of FMD, which were properly stored, were all negative by RT-PCR as well as by VI and ELISA, indicating a high specificity of this test as previously demonstrated (8, 15). A comprehensive recent study of specificity of this method confirmed a very high specificity, but even with the inconclusive reactions considered false-positives, the specificity was 99.9%, which may provide enough accuracy for a diagnosis during an outbreak, but may not be specific enough for surveillance in FMD-free areas (23).

The finding that 4 samples failed to yield a C_T value throws doubt on the provenance of the tested material, because stored cell culture-grown antigens to four of the isolates readily reacted in the rRT-PCR which had a nucleotide substitution in the probe site, enabling it to

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escape recognition by the routine diagnostic probe. The results suggest that it should be possible to classify samples definitively as either positive or negative for FMD by RT-PCR within a relatively short time. If the procedure can be transferred from the laboratory to the field and the time taken to complete a test can be markedly reduced, the RT-PCR could provide a rapid diagnosis on or near the farm and provide objective support to veterinary clinical judgment.

By two series of primer/probe diagnostic capability was increased in suspected samples of FMD. Calculated C_T in two series of primer/probe were in one range in epithelium samples (the results are not shown) that shows both regions are used to detect RNA of virus and these regions change less in types and FMDV subtypes. In the present study, the amount of C_T value of all seven serotypes was different and the least value of C_T achieved for SAT3, A, O, Asia 1 types and the most value of C_T obtained for SAT2, C types, respectively. It is due to the fact that there are more sequence information about Asia 1, O, A for design of primer and probe, and this observation is somewhat consistent with reports from several other studies (22, 24). Of course in addition to dominant serotype in Iran and this study, O and A serotype, is better probe and primers based on these types sequence due to possible detection in RT-PCR methods increased.

Real-time PCR detection limit was obtained 1 TCID50 and as C_T value >40 as positive/negative cut-off and to 7 dilutions up to detection limit have positive C_T and it was detected by Real-time PCR. Generally, RT-PCR had high sensitivity in comparison with diagnosis routine methods and conventional RT-PCR and the rate of FMDV detection in comparison with combined ELISA/VI and separated VI was increased and these evidences are similar to the previous studies (17). Although, foot-and-mouth disease is not clinically discernable of vesicular diseases of SVD and BT viruses, but in RT-PCR by specific primer/probe it is discernable (Table 3) and our results are similar to the previous studies (9). One of aims of this study detecting of RNA of this virus in epithelium sample with negative ELISA/VI result that it was done by RT-PCR. It is interesting that the results of ELISA/VI, conventional RT-PCR and RT-PCR are not compatible in some cases and it was seen in the results of other research (13).

Briefly, the current study indicates that one-step RT-PCR introduced recently in our laboratory is a high sensitivity, specificity and effectiveness of diagnosis of RT-PCR in comparison with conventional RT-PCR powerful technique for the reliable detection of FMDV in biological samples (25). Due to rapid prevalence of FMDV, many diagnostic methods were used and we shouldn't depend on only one method. Using RT-PCR method shows the results achieved at a short time and in comparison with diagnostic routine methods such as virus isolation, and ELISA-Ag are useful for testing many samples. Thus, it seems that RT-PCR can be used as a valuable tool to complete diagnostic routine methods and as a main method in initial diagnosis of FMDV. Finally, suggested other surveys focused on detection of specific-serotypes by RT-PCR that have need to specific-serotypes primer/probe at this methods.

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