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Full Article

Rapid and accurate diagnosis of Foot-and-mouth disease virus by Real-time PCR in field samples

Jeirani[1*], F., Ahmadi[2], A., Azimi[1], M., Mahravani[1], H.

1. Department of FMD, Razi Vaccine and Serum Research Institute, Karaj, Iran
2. Jahrom University of Medical Sciences, Jahrom, Iran

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ABSTRACT

During 2010-2011, Real-time PCR procedure was used to detecting FMDV RNA on 147 epithelium samples from the field. In this survey, for Real-time PCR from 3D gene segment as conserve region selected for tracking all of seven serotypes FMDV. The assay detected the viral RNA in all serotypes of FMDV. The rRT-PCR specifically detected FMD virus in sample with greater sensitivity than our conventional RT-PCR procedure and our routine diagnostic procedures. Sensitivity of this technique was estimated by 10-log serials dilution Asia virus RNA that defined $10^{1.2}$ TCID50/ml. Also, to assess the specificity of primer pairs related to 3D segment and specific probe was used Swine vesicular disease virus (SVDV) and bluetongue virus (BTV) isolates RNA. The results showed that rRT-PCR is seen as a powerful and valuable tool concomitant with routine diagnostic methods for FMD virus diagnosis.

Keywords: Real-time RT-PCR, conventional RT-PCR, Foot-and-mouth disease virus

INTRODUCTION

Foot-and-mouth disease virus (FMDV), as a filterable agent, is a highly contagious in domestic cloven-hoofed animals and economically the most important viral disease of livestock. FMD virus belongs to Picornaviridae family and Aphthovirus genus, have seven serotypes (A, C, O, Asia, SAT 1, 2, 3) and multiple subtype within any serotype. Organization of RNA virus genome is a single-stranded, plus-sense strand and size of approximately 8/5 kb(Alexandersen et al 2003, Grubman & Baxt 2004). The regions of FMD virus genome as 3D and 2B segments are nearly conserved, was used for detection of all of seven serotypes in clinical samples as epithelium suspension (ES), milk, oesophageal–pharngeal (probang) (Vangrysperre & De Clercq 1996, Amaral-Doel *et al* 1993, Callahan *et al* 2002).

More than twenty years, At the OIE/FAO World Reference Laboratory for Foot-and-Mouth Disease (WRL for FMD), has ordered to the use of traditional diagnosis such as indirect sandwich enzyme-link immunosorbent assay (ELISA) for detection of FMD virus antigen (Pan-serotypic detection) and virus isolation in cell culture(Ferris & Dawson 1988). But Serological methods as the ELISA, is not 100% sensitive that simultaneously inoculated onto cell cultures and the specificity of isolated viruses confirmed by the ELISA(Paixão *et al* 2008). Generally,

*Author for correspondence.Email: f.jeirani@rvsri.ir

these tests only used to confirm the clinical diagnosis and identify the FMDV serotypes; however have not the ability to determine the possible source of infection in outbreak.

In recent years, various molecular methods as conventional RT-PCR be used to the universal detection of FMDV serotypes and serotype- specific results as a source of material for the early detection of FMD virus(Marquardt *et al* 1995, Reid *et al* 1998). Conventional RT-PCR can be combined with sequence analysis to study genetic correlations between field isolates and help to molecular epidemiology (Reid *et al* 1999, Knowles & Samuel 1994, Knowles & Samuel 2003). With this condition, none have sufficient sensitivity and conjunction with other techniques as ELISA and VI. Other formats used as PCR-ELISA that increase sensitivity and diagnostic rate, however it has disadvantage as labor-intensive(Alexandersen *et al* 2000). Fluorogenic RT-PCR has been used successfully for a quantitative time course study to investigate early pathogenesis of FMD in pigs infected by contact challenge with FMD virus serotype O1 Lausanne(Oleksiewicz *et al* 2001). This method Indicate that could be a reliable method for the diagnosis of FMD virus to provide quantitative results within 24 h of sample receipt without the obstacles associated with conventional RT-PCR. Fluorogenic Real-time RT-PCR method has a number of benefits than conventional RT-PCR. This method removed the processing of the post-PCR and can be increased output of samples with fewer the work steps. The purpose of the present study is to applying of portable rRT-PCR procedure as assistant additional tools for detection of all seven serotypes of FMDV in biological samples submitted to IRL for FMDV during the year and comparison of sensitivity of this rRT-PCR with other diagnostic methods.

MATERIALS AND METHODS

Clinical samples. one hundred and forty-seven samples of Epithelium suspension (ES) were received from different provinces in Iran during the period of the study (2009-2010 years). These samples were selected from all of FMDV carrier animals. Moreover, seven samples of the inoculated cell culture from seven FMDV serotypes (that has confirmed by WRL for FMDV) and a sample of epithelium from non-infected calf were selected as positive and negative control respectively. For analysis of the specificity of primers and probe rRT-PCR method applies to Blue-tongue virus (BTV) and Swine vesicular disease virus (SVDV) isolates.

Virus isolation. Porcine kidney cells line (IB-RS-2) was used for virus isolation on submitted epithelial suspension. 0.5 ml of epithelial suspension (ES) was inoculated to cell culture and then the plates were incubated for 72 h at 37 °C and 5% of CO2. The infected cultures were daily examined to check the appearance of cytopathic effects. In the case of lack of the effects, the cell cultures were frozen and then thawed out and used to inoculate fresh cultures.

Viral RNA extraction. total RNA was extracted from every unknown and control samples by High Pure Viral RNA Kit (Roche) according to manufactures instructions. Finally extracted RNA stored at -20 **ºC** and then was used in Real-time RT-PCR and conventional RT-PCR.

Real-time RT-PCR probe and primers. in our study, a set of primer used for detecting FMDV fragment 3D (polymerase RNA gene), as indicated in table1**.** These primers and probe was previously described by Callahan el al. 2002. The probe in Real-time RT-PCR reaction can detect the sequence of 3D gene region in all of the FMDV serotypes. Also the probe labeled with 6-carboxyfluorescein (FAM) at the 5' end, and the quencher tetramethyl rhodamine (TAMRA) at the 3' end.

Real-time RT-PCR reaction. rRT-PCR reaction optimized based on one-step procedure combined with reverse transcription and Real-time assay. So, Real-time reaction carried out by Superscript III/Platinum Taq one-step rRT-PCR kit (Invitrogen).The 20µl reaction mix (total volume) in Real-time including in

following: 0.4 µl MgSo4, 10µl 2x-reactions buffer, 2.6µl DEPC water, 0.4 µl Enzyme Superscript III/Platinum, 0.25 µl each of the primers, 0.1 µl of probe, 6µl extracted RNA then the tube was placed into a Corrbett Rotor-Gene ™ 6 PCR device. The amplification was done at the following temperature cycle: Reverse transcription (one cycle), 50 ºC for 30 minute, the initial denaturing (one cycle), 95 ºC for 2 minute, 95 ºC for 30 second and 60 ºC for 30 second, 40 cycle (PCR and rRT-PCR). The fluorescence rate acquired at the annealing/extension steps in 60 ºC. Ct value for every sample was designed from the point at which fluorescence crossed a threshold line. Standard curve was calculated by Rotor-Gene ™ software by including six standard dilutions of FMD Asia RNA ($10^{6.2}$- $10^{1.2}$ TCID50/ml) in each reaction.

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

Name	Sequence (5-3)	Target gene
Forward primer (P32)	**(5´ACTGGGTTTTACAAACCTGTGA-3´)**	3D
Reverse primer (P33)	**(5´-GCGAGTCCTGCCACGGA-3´)**	3D
Probe	**(5´-FAM-TCCTTTGCACGCCGTGGGAC-TAMRA-3´)**	3D

Conventional RT-PCR. This reaction was performed in 147 suspected epithelium samples and negative/positive controls. These oligonucleotide primers (P32, P33) related to 2B gene region for amplification 131bp segment as a nearly conserved gene in all FMDV serotypes. Sequence of primer was designed as previous study described by Vangrysperre al 1996. The one-step reaction optimized as Real-time reaction. The 50 µl reaction mix (total volume) including: 1.5 µl of each primer,1µl enzyme, 2µl $MgCl_2$, 1µl DNTPs, 5µl Buffer 10x, 6µl extracted RNA and 32 µl DEPC water. PCR amplification was performed by Eppendorf thermocycler machine. The PCR reaction was done at the following temperature cycle: RT reaction,42 ºC for 40 minute (1 cycle), initial denaturation, 94 ºC for 4 minute (1 cycle), 40 cycles for denaturation, at 94 ºC for 45 seconds, annealing, at 55^0C for 45 seconds, extension, at 72 ºC for 45 seconds and final extension at 72 ºC for 10minute. Finally, PCR product run in1.5% agarose gels and staining with Ethidium bromide (1 µg/ml).

Analytical sensitivity and specificity. The analytical sensitivity of the rRT-PCR established by a serial dilution (10-fold) of FMDV serotype Asia RNA with $10^{6.2}$TCID50/ml value. RNA was extracted from of each dilution for rRT-PCR analysis by using High pure viral RNA kit (Roche). Also for the sensitivity of conventional RT-PCR checked by a serial dilution (10-fold) FMD serotype Asia RNA. The analysis of Specificity of these methods was performed by of two BTV and SVDV isolates RNAs due to undistinguished of clinical signs of FMDV.

RESULTS

The results of the isolation of the FMDV in cell cultures, the detection of FMDV RNA by the RT-PCR and rRT-PCR assay are shown in Table 2. The presence of infectious FMDV was found 79.5% of suspected samples that produced recognizable cythopatic effects (CPE) but the remaining samples were negative (no CPE). As regards, the results of the rRT-PCR assay were assessed by the CT value. The negative CT value for any test and control sample considered CT of ≥40.0 and was selected as the positive/negative cut-off CT values. After evaluating 147 submitted samples, 97.2% and 89.1% of cases were detected by rRT-PCR assay and gel-based PCR procedures respectively. In general, VI positive samples was compatible with positive results in rRT–PCR assay so that all of this samples had CT value <40, therefore there were no false negative results in rRT–PCR assay. Sensitivity of rRT–PCR and conventional RT-PCR was assigned by a serial dilution (10-fold) FMD serotype Asia RNA. The result showed that detection limit of FMDV Asia RNA in rRT–PCR and conventional RT-PCR was $10^{1.2}$ and $10^{3.2}$ TCID50/ml respectively (figure 1, 2). The minimum traceable

concentration of RNA extracted from a viral stock by rRT–PCR was $10^{1.2}$ TCID50/ml that was CT-value equal to 37 (data not shown). Also for analyzing the specificity of both methods, BTV and SVDV RNAs was evaluated by FMDV primers and probe and no amplification was detected.

Table 2. Comparison of virus isolation,conventional RT–PCR and Real-time PCR for FMD virus diagnosis on clinical samples

Diagnostic Methods on ES	Negative results	Positive results
Virus isolation	30	117
conventional RT-PCR	16	131
Real-time PCR	4	143

DISCUSSION

In our country, FMD virus is endemic, therefore rapid and accurate diagnosis can play an important role for the implementation of effective measures to control the spread of disease. rRT–PCR or flourogenic methods has been become important for virus detection of animal diseases as CMV and blue-tongue virus (BTV) where accurate and rapid results is essential for screening large numbers of samples without cross-contamination(Azimi *et al* 2011, Kearns *et al* 2001). Also, this procedure can also be applied for diagnosis of FMD virus in submitted samples, especially in rapid and prolonged outbreaks that the number of samples is high. In this study, the diagnosis of FMD virus in suspected samples submitted to the IRL(Iran reference laboratory for FMD virus) was performed with the Taqman probe/primer along with reference samples of all of seven serotypes of FMD virus because confirmatory evaluation of flourogenic method. Indeed, further development of diagnostic methods will increase tests capability for detection of FMD virus in biological samples. Validation of Real-time PCR assays in reference laboratories, such as IHA Pirbright confirmed that this technique has sensitivity greater or equal to VI. So OIE has introduced Real-time PCR as a standard golden test for FMDV detection(OIE 2008).

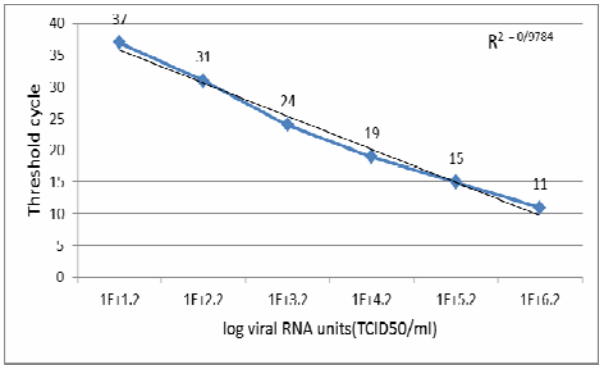

Figure1. In this figure, sensitivity of rRT-PCR by a 10-fold serial dilution of Asia RNA and linearity rRT-PCR for FMDV detection was depicted. As shown there is reverse correlation between viral RNA dilutions (equivalent to TCID50/ml on logarithmic scale) and cycle threshold values.

The advantages of Real-time PCR as diagnostic novel approach against other procedures are including: high sensitivity and processing of large number of field samples in each run, more rapid than the conventional RT-PCR, no need to analyze PCR products by relatively insensitive gel electrophoresis and staining with Ethidium bromide, as carcinogens dyes (Niedblski *et al* 2010, Jiménez-Clavero *et al* 2006).

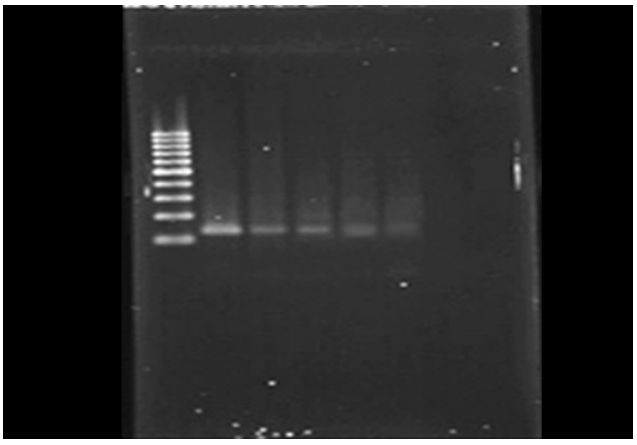

Figure2. In this figure, is seen electrophoresis results for the evaluating of sensitivity of conventional RT-PCR. Left to right: lane 1, 100bp ladder. Lanes 2, FMDV Asia serotype as positive control. From lane 3 – 8 a 10-fold serial dilutions of FMDV Asia serotype RNA ($10^{6.2}$ -$10^{1.2}$) respectively.

Other usefulness is the measurement of PCR products in the exponential phase of the reaction; therefore eliminate the variability related to gel-based reaction as conventional RT-PCR(Arya *et al* 2005).

Furthermore, final results will be faster than gel-based RT-PCR. The length of the Real-time PCR Amplicons in short (less than 150bp), furthermore annealing/extension steps merged, therefore the possibility of the target degradation during the reaction reduced. In addition to highly genetic mutation in FMD virus genome and the circulating of the virus serotypes in different host therefore detection of virus genetic materials in clinical samples is extraordinary important. The regions of FMD virus genome such as 2B, 3D, 5UTR (IRES) are approximately conserved in all of the FMD virus serotypes, so are less affected by mutation that is based on previous studies(King *et al* 2006, Vangrysperre & De Clercq 1996). Furthermore use of this target segments for detection of FMDV RNA is a benefit. In this survey, we used 3D and 2B region as target and also one-step method in both molecular procedures that improved the assays by quicker reaction to set-up, cheaper to use, less processing of samples and decreasing pipetting errors(Wacker & Godard 2005). The results indicated that only four samples by rRT–PCR as well as virus isolation in cell culture not detected, therefore there were no false negative results obtained with rRT–PCR that found in previous studies(Shaw *et al* 2004). In certain instances the samples which were diagnosed as FMD virus positive differed between the conventional RT–PCR assays and rRT–PCR, e.g. rRT–PCR detected FMD viral RNA in 12 of 16 samples which had been evaluated as negative by the conventional RT–PCR assays. Furthermore, these results showed highly sensitivity of Real-time PCR methodology than conventional RT-PCR that considered to other studies(Azimi *et al* 2011). The CT of 40 chosen as a positive cut-off was adopted as CT value by others workers (Reid *et al* 2009, Shaw *et al* 2004). In rRT–PCR assay Ct value more than 40 taken to account as negative result, because the Ct value of $10^{1.2}$ TCID50/ml (as detection limit viral RNA by Real-time PCR) was equal to 37. The next dilution did not show any detectable Ct value therefore it was considered as a negative sample. Real-time PCR technique was detected FMD virus genetic materials in most epithelium samples than the conventional RT-PCR, On the other hand, Ct value of eleven of the negative samples by conventional RT-PCR was lower than 40, Thereby demonstrating the slightly higher level of sensitivity achievable with this system (Taqman probe/primer) compared to the single primer used in the conventional RT-PCR. Sensitivity of Real-time PCR was $10^{1.2}$ and also similar to previous study that greater than conventional RT-PCR (the sensitivity was $10^{3.2}$), means the delectability of FMD virus RNA in fluorogenic RT-PCR is a range of one hundred rates than conventional RT-PCR, therefore was increase diagnostic capacity. This data showed that the negative samples by VI have Ct value more than 38 that confirm the greater sensitivity of Real-time PCR than VI. Since, the weak storage condition on the samples causes FMD virus loss infectivity, however the viral RNA tracing was performed by Real-time PCR, therefore can used this method as rapid and initial screening test in outbreaks (Fosgate *et al* 2008).

As FMD cannot be clinically distinguished from other vesicular diseases such as SVD and BTV, each undistinguishable disease should be considered as FMD and the precise differential diagnosis must be performed. We found that these pathogens can be differentiated in rRT-PCR assay with FMDV specific primers. These results were in agreement with the previous observations (Fernández *et al* 2008, Shaw *et al* 2007, Hindson *et al* 2008).

Moreover, the isolation of the virus from the disease outbreak enables obtaining the material for a further characteristic of isolates and the disease immunoprophylaxis. Finally, the finding of this survey indicated that applying of Real-time PCR because sufficient output and rapid processing as valuable tool along with traditional procedures is useful and necessary. Also, we suggested other studies on large and different number of field samples performed, using of this technique for viral quantitation in suspected samples such as probang and serotyping accomplished by rRT–PCR which uses specific-serotype probe-primer sets.

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