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Development of multiplex RT-PCR for detection and differentiation of foot-and-mouth disease virus O and A serotypes in Turkey

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Abstract: In this study, a two-step multiplex reverse transcription-polymerase chain reaction (mRT-PCR) was developed and evaluated using designed primers for the simultaneous detection and differentiation of Turkish foot-and-mouth disease virus (FMDV) serotypes A and O from clinical samples. In the method, a mix of one universal reverse primer designed from the 2B gene and two serotype-specific forward primers designed from the hypervariable regions of the capsid VP1-1D coding gene of FMDV were used. Totally, 272 FMDV-infected samples collected between 2006 and 2008 constituted the material of the study. mRT-PCR was compared to ELISA in terms of positivity percentage (%) and the difference between the two tests was found to be statistically significant ($P < 0.05$). In the study, 148 (54.4%) of 272 samples were undetermined by ELISA. Ninety-nine (66.8%) of these 148 undetermined samples were found FMDV RNA-positive with mRT-PCR. Consequently, the diagnostic sensitivity and specificity of mRT-PCR was determined as 95% and 84%, respectively. The study showed that mRT-PCR was more efficient than ELISA and concluded that the technique can be used as an alternative to ELISA for molecular typing of FMDV samples. This is the first report providing the differentiation of FMDV serotypes A and O in Turkey by mRT-PCR.

Key words: Foot-and-mouth disease virus, diagnosis, mRT-PCR

1. Introduction

Foot-and-mouth disease (FMD) is a global, highly contagious, and economically devastating disease of both wild and domestic cloven-hooved animals. The FMD virus (FMDV) belongs to the genus *Aphthovirus* of the family *Picornaviridae*. It is 28–30 nm in size and contains a positive-stranded RNA genome of about 8500 nucleotides. FMDV has 7 serotypes (A, O, Asia-1, SAT 1, SAT 2, SAT 3, C) and many subtypes. Although cross-immunity has not been observed among the serotypes, partial immunity has been reported among the subtypes (1–3). High mutation rates and quasispecies population structure in FMDV triggers many antigenically and genetically divergent strains within each serotype of the virus, all of which cause difficulties in control of the disease (4–6).

In Turkey, FMD is generally endemic with different subtypes of O, A, and Asia-1 serotypes since 1952. FMDV A serotype-A Iran 2005 (ASIA topotype) and O serotype-O Panasia II (ME-SA topotype) strains were prevalent during the study period (2006–2008). FMDV strain A Iran 2005 caused many outbreaks starting at the end of 2005 and occurred through 2006. An FMDV serotype Asia-1 outbreak occurred in 2011, 9 years after the first Asia-1 epidemic in 2002. There was no Asia-1 outbreak during this study period.

FMDV diagnosis is based on clinical signs, followed by confirmation by laboratory tests. Virus isolation in cell culture is considered to be the gold standard for FMDV detection. However, it takes 1–4 days to obtain a definitive result, thus delaying the initiation of outbreak control procedures in the field. Antigen-detection ELISA is considered by the World Organisation for Animal Health (OIE) as the preferred method for FMDV antigen detection and serotyping (7,8). Although ELISA is much faster than virus isolation, it has lower sensitivity (9,10). In general, samples are first tested by ELISA, and consequently ELISA-negative samples were inoculated into cell culture followed by the confirmation of the virus serotype by ELISA in the case of virus propagation (8). This process is time-consuming. Therefore, it is essential to have a reliable molecular technique for the rapid typing of FMDV. In particular, RT-PCR studies for typing of FMDV (9,11–15) and its derivatives such as Ag-RT-PCR ELISA (16), mRT-PCR (12,13,17–20), and real-time RT-PCR

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(21,22) have provided new approaches for rapid detection and typing of FMDV. For example, mRT-PCR can detect different genes simultaneously in the same PCR reaction mix, providing a useful technique for FMDV typing. Nevertheless, there are very limited articles on FMDV typing with mRT-PCR (12,13,17,18,20). Multiplex primer design for FMDV typing is quite a challenging step. The major obstacle is the high variability in the FMDV genome due to high mutation rates and lack of sequences that are conserved within but restricted to a particular serotype (21). Another problem originates from the nature of the mRT-PCR technique, as a number of primers have to be used in same PCR reaction and these may interact with each other and finally may limit the sensitivity of the test (23,24). The aim of the current study was to develop a two-step mRT-PCR for the differentiation of FMDV A and O serotypes circulating in Turkey.

2. Materials and methods

2.1. Cells and virus samples

Vaccine strains O1/Manisa/TUR/69, O/JOR5/2006, A22/Mahmatlı/TUR/64, and A/TUR1/2006 maintained in a BHK-21 (baby hamster kidney) cell line were used as the reference strains for serotypes O and A during the test optimization. For evaluation of mRT-PCR, samples including tongue and heart epithelia were collected from 272 cattle suspected of being infected with FMD from different provinces of Turkey. The numbers of samples collected per year were as follows: 50 of 272 samples were collected in 2006, 119 in 2007, and 103 in 2008.

First, all 272 samples were subjected to indirect ELISA. Sixty-five of 272 ELISA-tested samples were inoculated into cell culture to evaluate mRT-PCR efficiency with cultured samples. The number of samples used in the study according to sample type is summarized in Table 1.

2.2. Virus isolation

Epithelium samples (1 g) were taken from phosphate-buffered saline (PBS)/glycerol and a suspension was prepared by grinding the sample with sterile sand in a sterile pestle and mortar with 9 mL of PBS. This homogenate was clarified on a bench centrifuge at 3000 rpm for 30 min. The supernatant was filtered with 0.45-µm and 0.20-µm filters and such suspensions of field samples suspected to contain FMDV were inoculated onto established BHK-21 cell lines (1 mL/25-cm^2 cell culture flask). Cell cultures stayed in an incubator for adsorption at 37 °C for 1 h and 5 mL of G-MEM (Glasgow MEM BHK 21 1X (GIBCO, Cat. No. 21710) virus medium was added and cultures were examined for cytopathic effect (CPE) for 48 h. If 75%–80% CPE was detected, the cells were frozen and thawed, then centrifuged at 4 °C and 3000 rpm for 30 min. Finally, the supernatant was taken, labeled, and stored at –70 °C until use.

Table 1. Sample numbers according to sample type.

Sample numbers	
Sample type	
Clinical*	Cell-cultured
207	65
272	

*Tongue and heart epithelia samples were collected as clinical specimens.

2.3. Oligonucleotide primers

The universal reverse primer (BES-VP1R) was designed from the conserved 2B region based on the alignment of VP1 genomic sequences of serotype O and A selected from the GenBank nucleotide database. Two serotype-specific forward primers (O1F and A1F) were designed from the hypervariable regions of the capsid coding gene (VP1/1D) following the alignments of VP1 (1D) gene sequences of serotypes O and A selected from the GenBank nucleotide database and conserved sequences unique to each serotype were identified and used for primer design. Details of the designed oligonucleotide primers are shown in Table 2.

All steps of primer design were performed with a commercial licensed software package (MacVector, USA) and free web-based computer programs (Primer 3.0, mfold). Each set of primers was confirmed for specificity by BLAST searches.

2.4. RNA extraction and reverse transcription (RT)

RNA was isolated with TRIzol (GIBCO Life Technologies, UK) according to the manufacturer's protocol. The method of choice was the one developed by Chomczynski and Sacchi using guanidine thiocyanate (25). Briefly, virus suspensions were taken from deepfreeze, then thawed at room temperature, and 200 µL of chloroform was added to the suspensions. The aqueous layer containing the RNA was removed, and the RNA was recovered by precipitation with isopropyl alcohol and washed with 70% ethanol. The RNA was resuspended in RNase-free water. RNA purity was also measured spectrophotometrically (260/280). RNA concentrations and purities were 55–172 ng/µL and 1.50–1.90 in the samples, respectively.

RT reaction was modified and performed according to the recommendations of Sambrook et al. (26). First, 5 µL of template RNA and 2 µL of 10 µM primer (novel designed primer BES) were heated to 65 °C for 5 min and cooled to 4 °C. The total RT reaction volume of 20 µL was obtained by adding 4 µL of 5X AMV RT buffer, 0.5 µL of 10 mM dNTPs, 0.5 µL of 20 U RNasin, 0.5 µL of DTT, 0.5 µL of 10 U AMV reverse transcriptase, and 7 µL of RNase-

Table 2. Primers designed for FMDV typing in the study.

Primer	Sequence	Sense	Gene	Serotype	Tm	PCR product (bp)
A1F	ACTCTACTGCCCYAGRCCACTG	+	VP1	A	60 °C	218
O1F	CGGGTGACTGAACTGCTTTA	+	VP1	O	61 °C	253
BES	TTGACATGTCCTCCTGCATCT	-	2AB	Genus-specific	60 °C	

IUB nucleotide symbols (Y: C or T; R: A or G).

free water. RT was carried out at 50 °C for 60 min. The reaction was terminated by incubation at 85 °C for 5 min and obtained cDNA was stored at –20 °C until use.

2.5. Polymerase chain reactions (uniplex and multiplex) and mRT-PCR optimization

Uniplex and multiplex RT-PCR reactions were modified and performed according to the recommendations of Sambrook et al. (26) and Henagariu (24). mRT-PCR was carried out using a HotStar Taq DNA Polymerase Kit (QIAGEN, Germany). Uniplex RT-PCR was carried out to determine whether the designed primers amplified their respective target sequences. The uniplex RT-PCR reaction mix and cycling conditions were the same as those for mRT-PCR, except that 1.5 µL of of 25 mM $MgCl_2$ was used. The PCR reaction mix contained 2 µL of cDNA, 2.5 µL of 10X PCR buffer, 3.0 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mix, 2 µL of 20 µM genome-specific reverse primer, 1 µL of 10 µM of each serotype-specific forward primer, 0.25 µl of 1.25 U HotStar Taq DNA polymerase, and 12.75 µL of RNase-free water. All RT-PCRs were performed in a final reaction volume of 25 µL for one cycle at 94 °C for 15 min; 30 cycles each of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 2 min; and one finally cycle of 72 °C for 5 min. The PCR products were analyzed on 1.5% agarose gel and visualized by ethidium bromide staining. Based on the primer performances in uniplex RT-PCR, a universal reverse primer and a serotype-specific forward primer specific to each serotype were chosen. The specificity of these primers when combined to amplify homologous cDNA templates was examined using the HotStar Taq Polymerase Kit (QIAGEN). The rest of the reaction mix and cycle conditions were the same as in the uniplex RT-PCR. Optimization of mRT-PCR was performed with varying reaction components and cycle conditions for each parameter at a time.

2.6. Analytical sensitivity and specificity of mRT-PCR

To determine analytical sensitivity (detection limit), 7.5 $TCID_{50}$/mL O and A serotype viruses were prepared and viruses using a series of 10-fold dilution were tested by ELISA and mRT-PCR simultaneously. For testing the specificity of the method, bovine herpes virus-1 (BHV-1: strain Germany, strain 2204, and strain Schönböken), bovine viral diarrhea virus (BVD: genotype 1, NADL, and genotype 2 CS8644), vesicular stomatitis virus (strains Indiana and New Jersey), and blue tongue virus (BTV: serotypes 1 to 24) were used.

2.7. Statistical methods

The degree of agreement between ELISA and uniplex and mRT-PCR assays in detecting and serotyping FMDV was calculated by Cohen's kappa values (kappa statistics). The mean positivity of ELISA and mRT-PCR were compared by Student's t-test. For the description of the agreement of the results and determination of the diagnostic sensitivity of mRT-PCR, Spearman correlation coefficients were calculated (SPSS 11.0, SPSS Inc., USA).

2.8. Sensitivity of mRT-PCR

As the first step, the degree of agreement between ELISA, uniplex assays, and mRT-PCR assays in detecting and typing FMDV was calculated. For this purpose, 45 out of 272 samples were tested with uniplex RT-PCR. Since the degree of accuracy between PCR and ELISA was rather low (kappa = 0.2), mRT-PCR was compared to ELISA in terms of the % positivity. Degree of accuracy between uniplex RT-PCR and mRT-PCR was high (kappa = 0.73). Therefore, uniplex RT-PCR was used for determination of mRT-PCR diagnostic sensitivity.

3. Results

3.1. mRT-PCR

The primers were cocktailed together in one multiplex reaction regarding their suitability to simultaneously amplify their respective targets determined previously in uniplex RT-PCRs. Amplicons of the desired sizes of 253 bp and 218 bp in length were obtained respectively for serotypes O and A (Figure).

3.2. mRT-PCR optimization

mRT-PCR was performed with previously characterized A and O serotypes at annealing temperatures of 55–61 °C. Other reaction conditions were kept constant. At temperatures of 59 °C and 61 °C specific amplification of the target sequence was obtained with both of the two serotypes without important variation in band intensity.

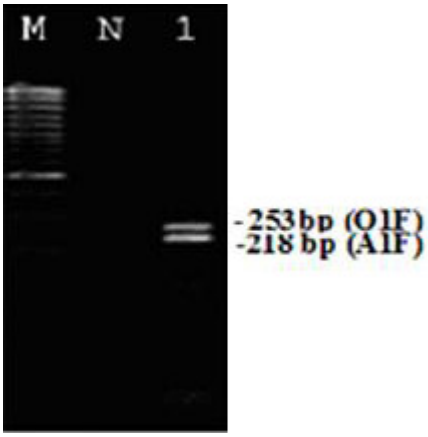

Figure. mRT-PCR results with FMDV primers.
M: 100-bp DNA ladder (Fermentas, Vilnius, Lithuania). Lane N: Negative control, lane 1: mRT-PCR result of primer pairs (A1F, O1F, BES) and FMDV serotypes A and O cDNA.

The best amplifications were obtained with final PCR concentrations of 1.5 mM $MgCl_2$ and 200 µM dNTPs. Taq DNA polymerase (1.25 U) was used in mRT-PCR for both of the serotypes. Primers were used in 10 µM concentrations for serotype-specific primers and 20 µM for the reverse primer since the latter was used for both serotype-specific primers in the reaction. Changing numbers of cycles (30 and 40 cycles) were studied for optimal performance. The lower cycle number was preferred since no difference was found in PCR results due to the cycle numbers.

3.3. Comparison of mRT-PCR and ELISA

mRT-PCR was found positive in the fifth dilution step ($10^{3.5}$ $TCID_{50}$/mL) for the FMDV O serotype and in the fourth dilution step ($10^{4.5}$ $TCID_{50}$/mL) for the FMDV A serotype. ELISA was found positive just until the second dilution step for both serotypes of FMDV ($10^{5.5}$ $TCID_{50}$/mL). Hence, mRT-PCR revealed a better analytic sensitivity than ELISA.

mRT-PCR clearly detected and differentiated almost all FMDV samples, yielding the expected 218-bp and 253-bp PCR products for serotypes A and O, respectively. Only 12 samples gave negative results with mRT-PCR. Nonspecific reactions or cross-amplifications were not observed in the assay. The difference between ELISA and mRT-PCR results was found to be statistically significant with both clinical and cell-cultured samples ($P < 0.05$). In the study, 148 (54.4%) of 272 samples were undetermined by ELISA, and 99 (66.8%) of these were found positive with mRT-PCR. The detailed results of mRT-PCR and ELISA assays are shown in Tables 3 and 4.

4. Discussion

Rapid identification of serotypes is a crucial step to understand the epidemiology of FMDV in a geographic location. Furthermore, it is of utmost importance to decide the appropriate vaccine strains for the control of a novel virus outbreak. The gold standard "virus isolation" and antigen detection sandwich ELISA methods have been used for FMDV typing for many years. However, low sensitivities and longer analysis time make these two methods inadequate. RT-PCR-based FMDV typing methods (RT-PCR, mRT-PCR, real-time PCR) provide the required advantages to researchers since they are faster and more sensitive than ELISA and virus isolation (13,18,21). In one of these techniques, mRT-PCR, more

Table 3. Typing results of samples with mRT-PCR and ELISA assays.

Serotype	ELISA-positive samples				ELISA-negative samples			
	mRT-PCR positive		mRT-PCR negative		mRT-PCR positive		mRT-PCR negative	
	Field	Cell-cultured	Field	Cell-cultured	Field	Cell-cultured	Field	Cell-cultured
O	32	14	5	3	38	9	28	2
A	37	29	3	1	46	6	18	1
Total	69	43	8	4	84	15	46	3

Table 4. Comparison (% positivity and statistical importance) of mRT-PCR and ELISA results.

Sample	mRT-PCR (positive/total)	ELISA (positive/total)	Statistical importance (P)
Total	77.5% (211/272)	45.5 % (124/272)	$P < 0.05$
Clinical	73.9% (153/207)	37.1% (77/207)	$P < 0.05$
Cell-cultured	89.2% (58/65)	72.3% (47/65)	$P < 0.05$

than one target sequence could be amplified in the same PCR reaction since it helps to investigate different genes in the same PCR reaction simultaneously (23,24). Therefore, in this study, mRT-PCR was used to differentiate FMDV serotypes.

Reliable and efficient mRT-PCR amplification depends mostly on the quality of the designed primers (24,27). A poorly designed primer pair may amplify a PCR product in low quantity or no product at all and thus be insensitive. This may be due to nonspecific amplification, primer-dimer formation, or amplicon secondary structure, leading to the failure of the reaction (27,28). Hence, all of the multiplex primers should be designed according to the above mentioned parameters, which is crucial for successful mRT-PCR (24,27). VP1-1D is a most immunodominant region defining viral antigenicity and is responsible for serotype and subtype differences (2,29). Similar to previous studies (12,13,17), in this research, the VP1-1D gene region was preferred for serotype-specific primer design. It is known that there are 30%–50% nucleotide differences between the FMDV VP1 genes (3). This seems to facilitate serotype-specific primer design. However, it is a challenging task, depending on the high variability of the FMDV genome, and lack of enough sequences that are conserved within but restricted to a particular serotype causes the cross-reactions in mRT-PCR assays (21). We thus selected optimal primer pairs providing minimum cross-reactions. Nevertheless, during FMDV replication, due to its error-prone nature and quasispecies structure, designed primers will not always succeed in detecting some virus strains (14). For this reason, regarding probable FMDV serotype variability in a particular geographic location, revisions of the primer sequences should be performed periodically. For a diagnosis laboratory, tailoring primers for FMDV should be a continuous dynamic action evolving in parallel to FMDV population dynamics in the field. Expecting high mutation rates, especially in FMDV A serotypes, degenerate primers might be a rational design approach to overcome this disadvantage. For this reason, degenerate primers were designed and used for identification of FMDV serotype A in this study. In some of the previous studies, degenerate primers were also used (12,18,20). However, Reid et al. (14) mentioned that degenerate primers could cause a decrease in serotype specificity.

In some studies on mRT-PCR for FMDV typing (12,13,17), it was indicated that RT-PCR did not successfully work with clinical specimens. Conversely, another study (18) showed that mRT-PCR got satisfactory results with clinical samples; the researchers reported that the method has better efficiency than ELISA and mRT-PCR might be used as a second-round test to define ELISA-negative samples. In other research (20), one-step mRT-PCR was studied for FMDV typing and the results were quite satisfactory with clinical samples.

In the current study, a statistically significant difference ($P < 0.05$) was obtained regarding % positivity results between ELISA and mRT-PCR in clinical specimens. This result is similar to the results reported by Grindharan et al. (18) since ELISA and PCR detect different viral components: capsid 146S antigen and viral RNA, respectively. FMDV has no viral envelope. For this reason, it can be easily degenerated in field conditions by high temperature, acidic pH, etc., and RNA can also be degraded. Nevertheless, such samples may still have adequate quantities of intact RNA for RT-PCR (30).

The most essential disadvantage of PCR diagnosis is false negative results due to PCR inhibitors inherent to the sample or manipulation errors in the laboratory. Therefore, clinical sample quality has the same degree of importance as primer optimization conditions in the laboratory. Although PCR specificity is affected mainly by primer design, PCR sensitivity is affected by many factors such as RNA extraction methods, RNA quality in the field samples, or primer design (24,26,27). Hence, well-designed and optimized PCR conditions, minimization of manipulation errors in the laboratory, and follow-up of current circulating strains as soon as possible will provide important implications for FMDV molecular diagnosis and FMD control.

In conclusion, this was the first study to be conducted to evaluate mRT-PCR efficiency for FMDV diagnosis in Turkey and the study showed that the present method is more sensitive and specific than ELISA. Diagnosis techniques based on PCR are crucial for rapid identification of an epidemic serotype and thus the vaccine strain. Development of real-time mRT-PCR is urgently needed to improve the diagnostic sensitivity and the specificity of conventional gel-based mRT-PCR and a loop-mediated isothermal amplification method for rapid identification of FMDV serotypes in the field.

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