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Original Article

Comparison of Immune Assay and Molecular Methods For Diagnosis Of FMD Virus

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Abstract

Foot-and-mouth disease virus (FMDV) causes severe infection in livestock and makes considerable economic impacts. Therefore, a rapid, highly specific, and accurate method for the diagnosis of FMDV infections is required to ensure that appropriate treatment is administered to reduce economic losses. In this study, the diagnostic tests for FMDV detection by enzyme-linked immunosorbent (Ag-ELISA), reverse transcription–PCR (RT-PCR), and real-time RT-PCR (rRT-PCR) were carried out as the World Organization for Animal Health recommended and were based on the VP1 gene. Positive samples were detected by RT-PCR and rRT-PCR (73.16%) and by ELISA test (55.99%). According to the information obtained from the present study molecular methods provide much more reliable and definitive results than Immune assay methods.

Keywords: FMD virus, ELISA, RT-PCR, rRT-PCR

Introduction

Foot-and-mouth disease (FMD) is an extremely contagious disease affecting domestic and wild ruminants. The causative agent, FMD virus (FMDV) belongs to the family Picornaviridae in the genus Aphthovirus. FMDV particles comprise a single copy of the positive-sense RNA genome within a near-spherical protein capsid which contains 60 copies of 4 different structural proteins, 1A (VP4), 1B (VP2), 1C (VP3), and 1D (VP1) (1). The viral RNA is sufficient to initiate replication when introduced into the cytoplasm of cells (2). The disease has been observed in almost every part of the world where livestock is kept. More than 100 countries are still affected by FMD worldwide and the distribution of the disease roughly reflects economic development (3). Most of the developed countries are free from FMD, whereas the disease is present in many developing countries including Iran. The rapid and untraceable spread of the disease in affected animals generates significant economic losses locally and worldwide.

The Office International des Epizooties(OIE) recommended antigen enzyme-linked immunosorbent (Ag-ELISA), reverse transcription–PCR (RT-PCR), and real-time RT-PCR (rRT-PCR) assays for virus identification.

Conventional RT-PCR procedures using primers corresponding to the VP1 (1D) coding region for serotyping of FMDV have been reported (4). The coding sequence for the VP1 protein has been extensively used for molecular epidemiological studies (5-8). The VP1 protein is considered to be highly immunogenic but VP2 and VP3 also contribute to the antigenic properties of the virus (9).

MATERIALS AND METHODS

Sample Preparation

One hundred and eighteen epithelial samples from suspicious animals were obtained from 16 different provinces during 2014-2021 and submitted to the reference laboratory for FMD,

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Razi Vaccine and Serum Research Institute (Karaj, Iran). Epithelial Specimens were crushed in a pounder with the aid of the pestle to produce a 10% suspension (W/V) with Eagle's maintenance medium.

The suspension was clarified at centrifuge 2500 rpm/min (5810/5810 R, Eppendorf) for 15 min and the supernatant fluid was collected.

Typing by Enzyme-linked Immunosorbent (ELISA)

The supernatant fluid was typed by the sandwich capture ELISA method according to the Nijel recommendation. An indirect sandwich ELISA was employed (10). The FMDV antigen detection and serotyping ELISA (FMDV O, A, Asia1, C, SAT1-2) kit (Cat No: 79621, IZSLER, Italy) helps identify Foot-and-mouth disease viruses in samples. The sensitivity and specificity of the ELISA technique sufficiently recommend as a golden standard test to detect FMDV. The tests were performed according to the instructions of the manufacturer.

The OD values were measured by ELISA reader (BioTek 800 TS) at 450 nm and were recorded after correction for background reaction. OD values above 0.1 were positive and those under 0.1 were negative.

Conventional RT-PCR

RNA Extraction

Total RNA was extracted from the homogenized samples using RNA Extraction/purification kit (Bioer, Cat No: BSC77M1, Germany) according to the manufacturer's instructions.

Reverse Transcription

The single strand cDNA was synthesized using a Reverta-L RT reagents kit (Amplisens, Cat No: K3-4-100-CE, Russia).

The reaction mix-ture contained 10 µL extracted RNA template and 10 µL of ready-to-use reagents mix containing RT-G-mix-1, RT-mix, and Revertase (MMIv). The mixtures were incubated in a thermocycler (Bio-Rad, USA) programmed at 37°C for 30 min. The mixture was then added to a 20 µL DNA buffer.

Polymerase Chain Reaction (PCR)

PCR reaction was performed to amplify VP1 gene of FMDV. The PCR reactions were carried out in 0.2 ml tubes.

The reaction mixtures contained 2.5 µL cDNA template, 1 µL (10 pmole) of forward primer C612F (TAGCGCCGGCAAAGACTTTGA), 1 µL (10 pmole) of reverse primer K61 (GACATGTCCTCCTGCATCTG), 12.5 µL Taq DNA Polymerase Master Mix RED (Ampliqon, Cat NO: A190301, Denmark) and 3 µL ultra-pure water in 20 µL total volume. The PCR cycle began with 3 min at 94°C, followed by 40 cycles consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 68°C for 30 s then a final extension at 68°C for 10 min.

The amplification products were separated electrophoretically in a 1% agarose gel in the presence of TBE buffer at a constant voltage of 75 V. The gel was stained with red safe nucleic acid staining solution (Biofact, Cat No: A190301, Korea) in UV light. The presence of PCR products was determined by comparison of their localization concerning to a mass marker (100 bp DNA ladder, Promega, Cat No: G2101, USA). The result was considered positive when the DNA band (814 bp) was seen on the gel.

Absolute Real-Time qPCR (rRT-PCR)

one primer series of VP1 gene regions recommended by OIE was used (11). The primer sequence used has been mentioned in RT-PCR. Quantitative real-time PCR results are generally normalized using endogenous control genes. In this study β-actin`s primer series: forward primer β-actin F (GATCTGGCACCACACACCTTCT) and, reverse primer β-actin R (GGGTCATCTTTTCACGGTTG) were used as endogen control housekeeping gen. These reference genes should be expressed at a constant level across all samples in the study.

Real-time qPCR was conducted using 2X SYBR Green I Master mix (Ampliqon, Cat NO: A325402, Denmark). Each reaction was run in duplicate and contained at a volume of 20 µl comprising: 12.5 µl Sybergreen master mix, 3 µl RNase free water, 1 µl of each primer, and 2.5 µl RNA sample.

The tube was placed into Rotor-Gene TM 6000 PCR machine and the amplification was done at the following temperature cycle: Initial denaturation (one cycle), at 95°C for 5 minutes, 40 cycles at 95 °C for 15 seconds, and 58 oC for 30 seconds. The fluorescence was acquired at the end of the 58ºC annealing/

extension step. The cycle threshold (Ct) value for each sample was 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 FMDV-purified RNA.

The RNA was extracted from a viral stock with a titer of 10-7 /ml TCID50. Then RNA of each dilution of the virus was extracted and analyzed for the sensitivity of RT–PCR.

Results

Enzyme-Linked Immunosorbent (ELISA)

All suspected epithelial samples from animals were tested for FMD antigen detection by ELISA. Sixty-six tested samples (55.99%) were positive for universal FMD antigen.

Conventional RT-PCR

Results of the detection of FMDV by the RT-PCR showed the presence of genetic material of the virus in 86 tested samples (73.16%), (Figure 1). PCR products was 814 bp. No amplification was found in negative controls.

Real-Time RT-PCR

The results of the rRT-PCR assay were evaluated by the CT value. Eighty-six FMDV isolates were tested by rRT-PCR recognized as positive (Figure 2). Calculation of CT-value in FMDV samples was performed by rRT-PCR. No CT value was detected in the negative sample. Also, an appropriate correlation was achieved between the results of RT-PCR and ELISA among all samples. It should be mentioned that all positive samples were with CT of less than 37.0, thus false negative results with rRT-PCR were not achieved and on the other hand, all negative samples with RT-PCR were negative with ELISA. Also, the reference gene (β-actin) was expressed at a constant level across all samples in the study. A 10-fold serial dilution of the titrated virus FMDV was prepared to determine the sensitivity of RT-PCR. Three subsequent dilutions up to the detection limit of rRT-PCR were positive considering CT. The viral RNA detection limit was achieved by RT-PCR 0.01 in TCID50/ml (Table 1, Figure 3).

An appropriate correlation was achieved between the results of PCR (rRT-PCR, RT-PCR) and ELISA among all samples. The information about the samples is given in Table 2 and 3.

The percentage of positive and negative samples detected by ELISA and PCR (rRT-PCR, RT-PCR) methods were compared with statistical methods. This comparison shows a significant difference between the efficiency of the methods ($P<0.05$), (Figure 4).

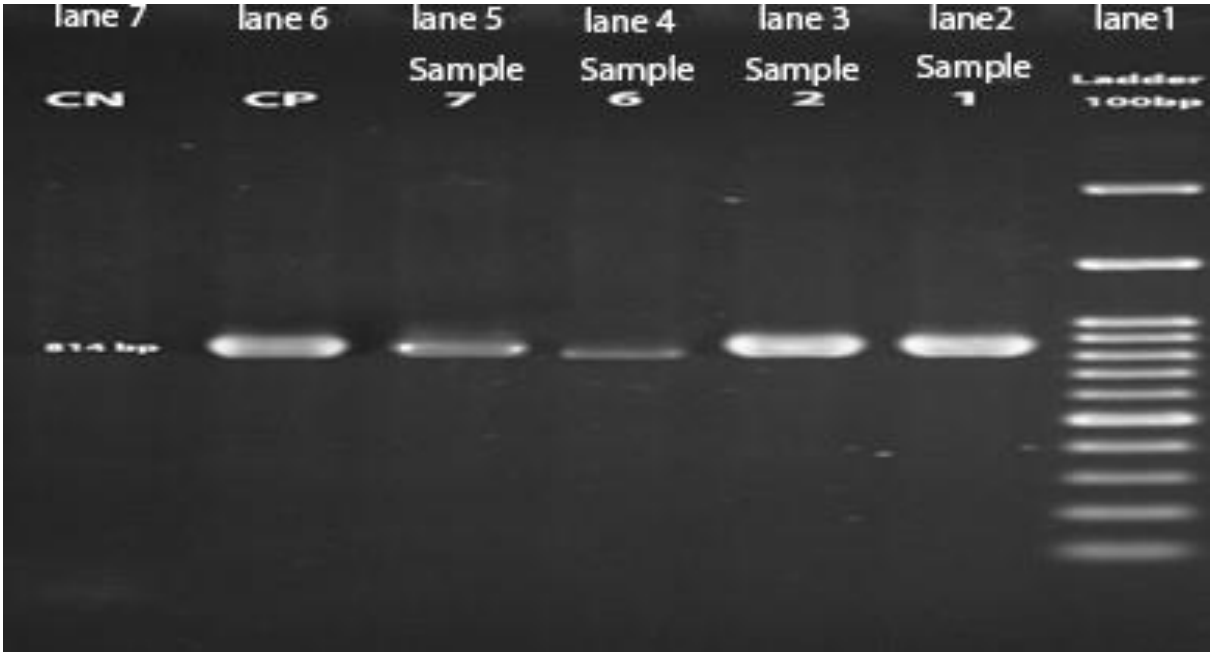


Fig 1. RT-PCR amplification of conserved part of VP1 gene of FMDV serotype A ~814 bp amplicon; from right to left: Lane 1: 100 bp DNA ladder, Lane 2-5: different samples, Lane 6: positive sample, and Lane 7: negative sample

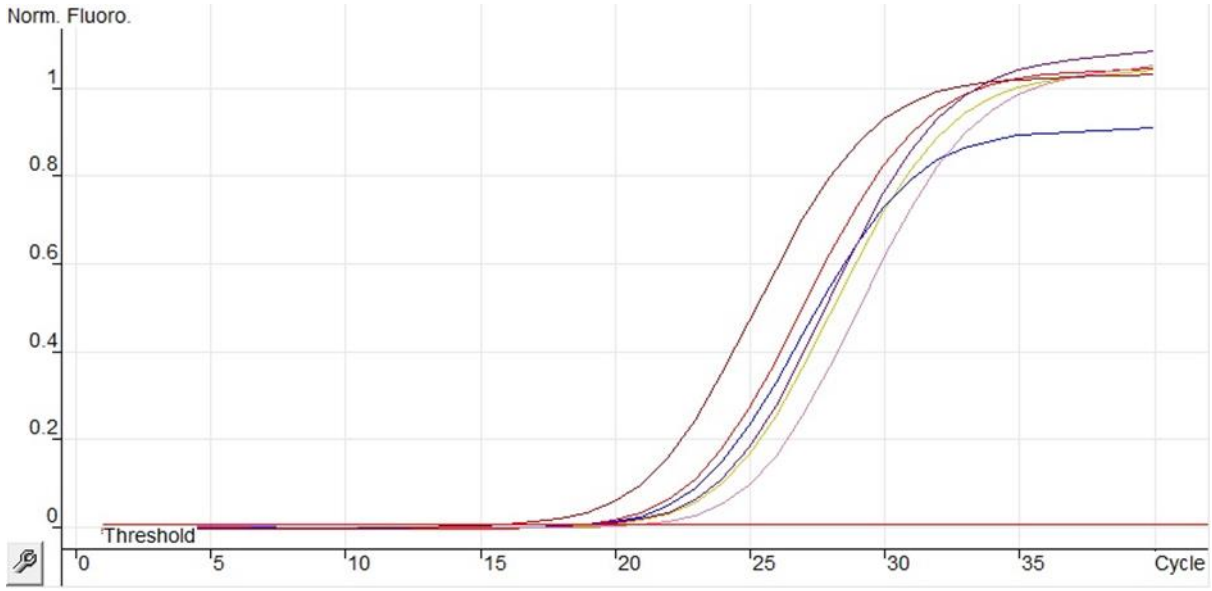


Fig 2. Logarithmic fluorescence plots versus cycle number resulting from the determination of FMDV RNA in tested samples

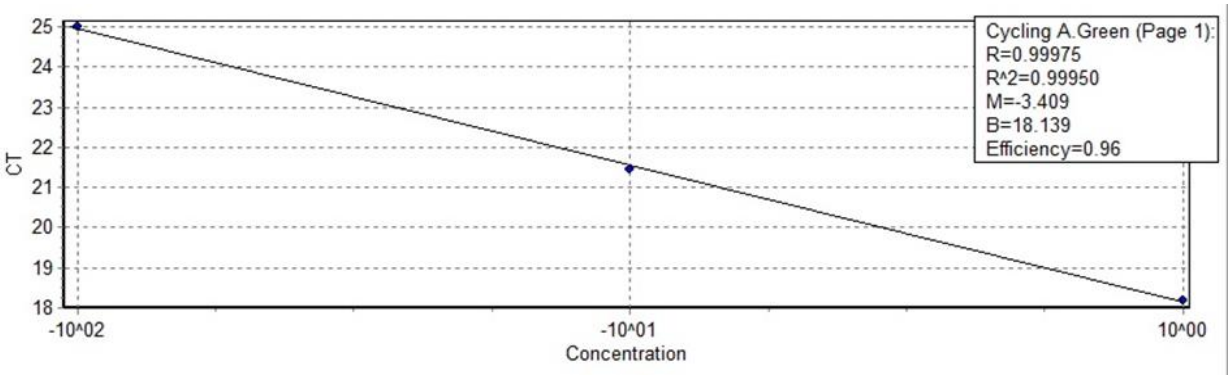


Figure 3. Standard curve

Table 1. Sensitivity of Real-time RT-PCR assay by a 10-fold serial dilution of FMD RNA. Which obtained from Rotor- Gene software

No	Name	Type	CT	Given Cnoc (copies/ul)
1	S 1	Standard	18.8	1
2	S 0.1	Standard	21.26	0.1
3	S 0.01	Standard	25.00	0.01

Table 2. Frequency and percentage of positive and negative samples detected by ELISA and PCR (rRT-PCR, RT-PCR)

ELISA	**Frequency**	**Percent**
Positive	66	55.93
Negative	52	44.07
PCR (rRT-PCR, RT-PCR)	**Frequency**	**Percent**
Positive	86	72.88
Negative	32	27.12

Table 3. Consensus table comparing the results of ELISA tests and PCR (rRT-PCR, RT-PCR)

ELISA				
Total	**Negative**	**Positive**		
86 (72.88%)	20 (19.95%)	66 (55.93%)	**Positive**	**PCR (rRT-PCR, RT-PCR)**
32 (27.12%)	32 (27.12%)	0 (0.00%)	**Negative**	
86 (100.00%)	52 (44.07%)	66 (55.93%)	**Total**	

Table 4. The results of the test using the molecular method assuming that the standard test is ELISA

Diagnosis status		**Number in the PCR (rRT-PCR, RT-PCR) method considering the ELISA method as the standard method**
EISA + PCR +	True positive	66
EISA - PCR -	True negative	32
EISA - PCR +	False positive	20
EISA - PCR +	False negative	0
Total		118

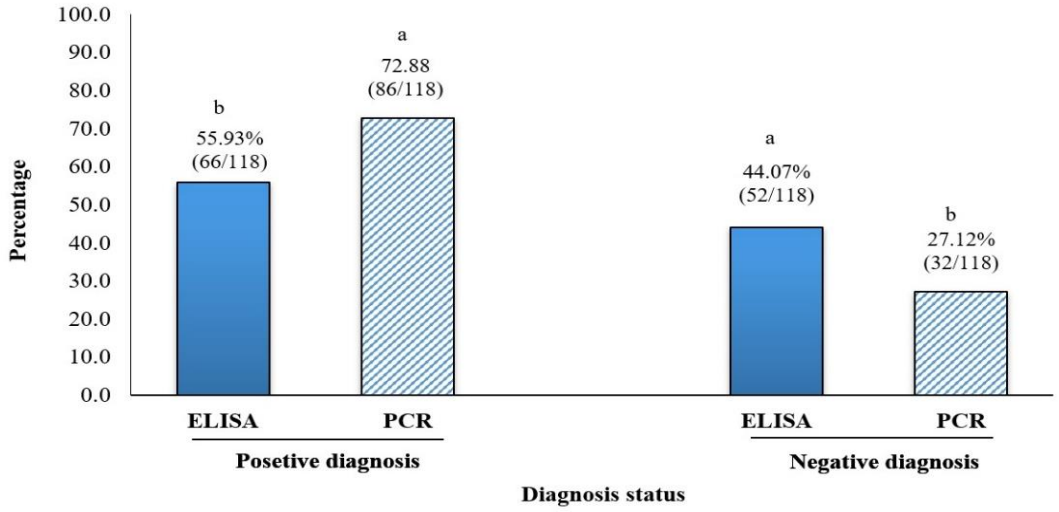


Fig 4. Statistical comparison of the percentage of positive and negative samples diagnosed by ELISA and PCR (rRT-PCR, RT-PCR) (note, the percentages with different significant letters have a significant difference $P < 0.05$)

Table 5. Kappa index and comparison of agreement between ELISA and PCR (rRT-PCR, RT-PCR) methods with McNemar's test

Simple Kappa Coefficient for Table of WRT by RBT	
Kappa	0.6416
ASE	0.0682
95% Lower Conf Limit	0.5079
95% Upper Conf Limit	0.7752

McNemar's Test for Table of WRT by RBT	
Statistic (S)	20.0000
DF	1
Pr > S	<.0001

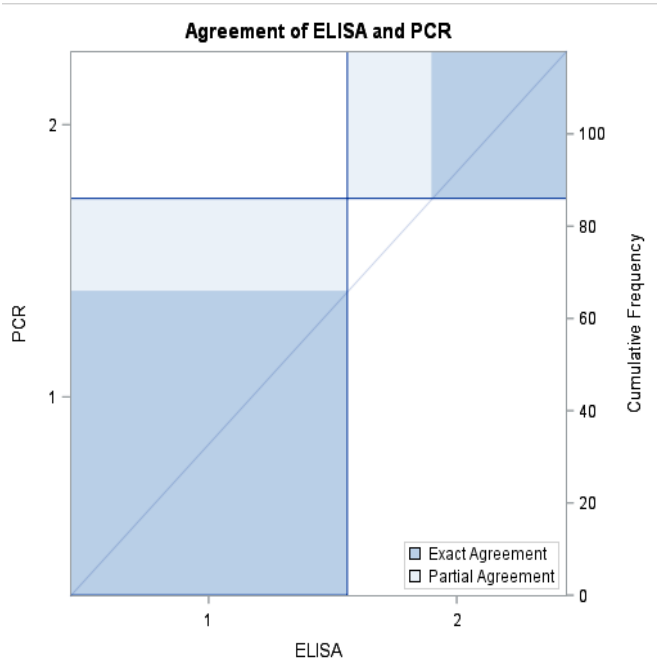


Fig 5. Cumulative detection frequency of positive and negative samples detected by two methods, ELISA and PCR (rRT-PCR, RT-PCR)

Table 6. Calculation of the accuracy, sensitivity and specificity of the PCR (rRT-PCR, RT-PCR) method considering the ELISA method as the standard method

Parameter	**The ability of the test to distinguish positive from negative cases**	**calculation method**	**Result (%)**
Precision	Ability to detect positive or contaminated cases	The sum of true positive and true negative to all states	83.05
Sensitivity	Ability to detect negative or uncontaminated cases	True positives are the sum of true positives and false negatives	100
Property	The ability of the test to distinguish positive from negative cases	True negatives are the sum of true negatives and false positives	61.53

According to the consensus table comparing the results of the tests (Table 3), the percentage of samples that were declared positive by both molecular method and Immune assay was 55.93% and the samples that were negative by both methods was 27.12%.

Also, 19.95% of the samples that were reported negative by the ELISA were reported positive by the PCR (rRT-PCR, RT-PCR), and on the other hand, there were no samples that were reported positive by the ELISA and negative by the PCR (rRT-PCR, RT-PCR) (0%).

The obtained kappa index (Table 5) shows that the agreement between the two methods in terms of distinguishing positive samples from negative samples is 64%. It means 64% of the samples agree with each other in the diagnosis, and there is a difference of 36% in a diagnosis. This indicator is cumulatively shown in the Figure 5, which shows that the percentage of samples that are positive for PCR (rRT-PCR, RT-PCR is higher than that of ELISA, and vice versa for negative ones.

Based on the results of Table 6, the accuracy, sensitivity, and specificity of the PCR (rRT-PCR, RT-PCR) method were calculated and compared considering the ELISA method as the golden standard method recommended by OIE. The method of calculating and defining each parameter is mentioned in Table 6.

Discussion

Foot and mouth disease (FMD) is the most contagious vesicular disease that affects domestic and wild ruminants with great potential for causing severe economic loss in susceptible cloven-hoofed animals. There are direct losses due to deaths in young animals, loss of milk, meat, and a decrease in product performance.

Also, the indirect economic losses caused to the livestock industry are treatment costs, reduction of milk production, lack of return of milk production, and the prohibition of the consumption of milk produced during antibiotic therapy. The costs of eradication or control are too high (12).

Early detection whit rapid and sensitive methods are essential for the effective control of the disease (13). The results of the present study showed a significant difference between antigen and molecular detection methods ($P<0.05$). As mentioned, the PCR method showed many false positive results 19.95%. This may be due to the fact that in the ELISA method, the amount of virus antigen in the epithelial sample taken from the suspected animal was not enough, but in the molecular method, even the smallest amount of the genetic material of the virus can be detected. The overlapping of a large part of the positive and negative results obtained in this study (Figure 5) shows that the PCR method is similar but more powerful than ELISA.

Although ELISA is the gold standard test for the diagnosis of FMD (14) RT-PCR (15-17) and r RT-PCR (18-20) is considered confirmatory, more advantageous, reliable, fast, and sensitive method of early FMD diagnosis. To confirm this, in this study, the positive samples detected by RT-PCR (73.16%) were more than those detected by the ELISA test (55.93%).

Also, the use of ELISA assay enabled obtaining results in a shorter time in comparison to those obtained by the RT-PCR and rRT-PCR assay methods, but by the RT-PCR and rRT-PCR assay were obtained more definite and persuadable results. This indicates a high and comparable sensitivity and usefulness of these methods.

Several authors reported a higher sensitivity of the rRT-PCR assay in compari-son to that of the virus isolation and Ag-ELISA combined (21-24). The data from the literature have highlighted that PCR assays are more frequently used for FMD diagnosis, whereas virus isolation is becoming rarely applied.

Conclusion

The presented results show that the PCR (rRT-PCR, RT-PCR) assays permit a more definite and accurate detection of FMDV in biological materials.

The use of molecular methods (PCR-based techniques) instead of antigen detection methods (such as ELISA) may require more specific tools, but more valuable results will be obtained.

Considering the sensitivity of early diagnosis of this disease, especially in endemic areas, it

is logical to use molecular methods to evaluate the prevalence and incidence of FMDV to facilitate the design of future strategies and programs to control and manage.

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Conflict of Interest

No conflict of interest is declared.

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None

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