Scholars Journal of Applied Medical Sciences (SJAMS) **ISSN 2320-6691 (Online)**
Sch. J. App. Med. Sci., 2015; 3(8E):3103-3106 **ISSN 2347-954X (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublishers.com DOI: 10.36347/sjams.2015.v03i08.069

Research Article

Detection of Foot and Mouth Disease Virus in clinical samples by PCR-Elisa

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Abstract: Foot-and-mouth disease (FMD) is one of the highly contagious diseases of domestic animals. Effective control of this disease needs sensitive, specific, and quick diagnostic tools. In this paper seventy suspected field's samples to FMD were analyzed using virus isolation on cell culture, Enzyme Linked Immunosorbant Assay (ELISA), RT-PCR (Reverse Transcription- Polymerase Chain Reaction) and PCR-ELISA. In RT-PCR and PCR-ELISA detection of conserved and universal region of the virus was detected. For this purpose annealing primer to 2B region of the FMDV genome was applied. In our study ELISA used as a golden test and sensitivity of the other test was calculated according this test. Sensitivity of virus isolation, RT-PCR and PCR-ELISA was determined 85.7%, 96.6% and 100% respectively. The highest dilution of FMDV A_{2005IR} $10^{5.7}$ TCID50/ml (Tissue Culture Infectious Dose 50%/ Milliliter) that can be detected by ELISA, RT-PCR and PCR-ELISA was found 1/100, 1/10, 1/1000 viral stock dilutions respectively. Specificity of primer and probe that used in RT-PCR and PCR-ELISA evaluated by intact bovine epithelial tissue and after the PCR cycles no amplification was detected.
Keywords: PCR-ELISA, RT-PCR, Foot and Mouth Disease virus

INTRODUCTION:

FMD virus (FMDV) belongs to the Aphthovirus genus of the Picornaviridae and is considered to be the most contagious agent of infection for domestic animals. FMDV infects many different species and is excreted high level. This virus can be transmitted by multiple routes, including contaminated fomites, infected animals and products as well as by wind-borne transmission [1-2].

Clinical diagnosis of FMD may be difficult, especially for sheep and goats, in which clinical signs are often mild. Furthermore, several other vesicular virus infections, including those caused by vesicular stomatitis virus and other diseases like bluetongue cannot be distinguished easily by the clinical finding [3]. Thus, exact diagnosis must be carried out at specialized laboratories and due to the rapid spread of the infection it must be fast, sensitive and specific. Traditionally laboratory diagnosis by ELISA detects FMDV antigens. This technique often is done by cell culture isolation concurrently. ELISA is performed on epithelial suspensions and tissue culture supernatants to determine the serotype of the virus [4].

In recent years RT-PCR assay for diagnosis of FMDV infection have been developed. In clinical samples although various protocols have been published, but none of them have sufficient sensitivity, specificity and robustness for diagnostic work unless backed up by the other techniques [5].

The purpose of this study is establishing the highly fast, accurate and sensitive method based on PCR-ELISA for detection of FMD virus without focusing on serotype. Also the results of virus isolation, ELISA and RT-PCR compared with PCR-ELISA for evaluation of the sensitivity and specificity of this technique.

MATERIALS AND METHODS:

Sample preparation: during this study we evaluated 70 epithelial samples of suspected cattle to Foot and Mouth Disease. After preparation of the samples they were inoculated onto porcine kidney cell line (IB-RS-2 cells, ATCC: CRL 1835) for virus isolation. The cell cultures were examined for cytopathic effect (CPE) during 48hours. If CPE was not detected, the process repeated for second and third time.

ELISA:

Indirect sandwich ELISA used to typing of all samples, as described previously6. The results were read by ELISA reader (BDSL Immunoskan MS) at 492 nm wavelength.

RNA extraction: RNA samples consist of viral RNA which extracted from original epithelial suspensions by Guanidium-thiocyanate-phenol-chloroform method [7]. The purity and concentration of total RNA was determined by Nano drop (ND-1000 V3-1).

Oligonucleotide primers and probe: For conventional RT-PCR and PCR-ELISA, published primers (P32: 5'-CAGATGCAGGAGGACATGTC-3', P33: 5'-AGCTTGTACCAGGGTTTGGC-3') which can amplify 131bp from 2B region of FMDV nucleic acid were used. For PCR-ELISA, biotinated probe (5´-BIO-CCAGAGGCGCAAACTCCTTA-3´) that anneale to the internal part of 2B was applied.8 All primers and probe synthesized commercially (Cinagen Co. Iran).

PCR amplification: After RNA extraction, cDNA were created with reverse transcriptase enzyme by reverse primer (P33). RT mixture had been consist of 4 µl RNA, 2.5 µl P33 (10pmol) and 10 µl nuclease free water. The mixture was incubated in PCR machine for 5minute in 70ºC. Then 4 µl RT 5x buffer, 2 µl dNTP (10 µM each), 1 µl RNase inhibitor (40 U/µl, Fermentas Life sciences) and 1 µl Moloney Murine reverse transcriptase (80 U/µl, Fermentas Life sciences) was added. Final volume of the reaction was adjusted by nuclease free water to 40 µl. The RT reactions incubate for 60 minutes in 37ºC.

PCR master mix composed of 2 µl of 10x buffer, 2 µl each of P33 and P32 primers (10 pmol), 1.5 µl MgCl2 (50 mM), 1µl dNTP (10 mM each) , 1 µl Taq DNA polymerase (5 U/µl, Fermentas Life sciences) and 5 µl cDNA. Finally nuclease free water added up to 50 µl. PCR amplification were performed by 94ºC for 5min and 30 cycles at 94ºC for 45sec, 55ºC for 45 sec and 72ºC for 45 sec. the PCR cycles terminated by final extension at 72ºC for 10 min.

All PCR products were visualized by electrophoresis in 1.2% agarose gel, and stained for 20 min in ethidium bromide (1 µg/ml). The gels were examined using Gel Documentation System (Bio Doc-It Imaging system).

PCR-ELISA:

Diagnosis of FMDV by PCR-ELISA was performed using Dig detection kit according manufacturer instruction (Roche diagnostics GmbH). Briefly the technique is based on the incorporation of Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate, alkali-stable (Dig-dUTP) during PCR amplification. PCR reactions are performed with the supplied nucleotides by the kit in PCR-dig labeling mix. The mixture contains standard dNTPs (2 mM dATP, dCTP, dGTP, 1.9 mM dTTP) and 0.1mM Dig dUTP to label the fragments. PCR reaction in PCR-ELISA composed 5µl cDNA, 5µl PCR reaction buffer, 2 µl MgCl2 (25 mM), 2 µl dNTP (PCR DIG labeling mix), 2 µl each of target specific primers (P32 and P33, 10 pmol), 1µl Taq DNA polymerase (5 U/µl) and 31 µl nuclease free water. PCR cycling were performed by 94ºC for 5 min, 30 cycles at 94ºC for 45 sec, 55ºC for 45 sec, 72 ºC for 45 sec and final step at 72ºC for 10min.

The labeled PCR products were bounded to the Streptavidin coated microplate by biotin-labeled capture probe. PCR amplicons are detected by adding anti-Dig-peroxidase conjugate and ABTS (azino ethyl benzotiazoline sulfonic acid) as substrate. The quantitative determination was performed by measuring the variation of absorbance in 492 nm wave length by ELISA reader. The absorbance of more than 0.1 was determined as positive case.

Sensitivity of virus isolation, PCR and PCR-ELISA was calculated by considering the ELISA as golden test. For this purpose $\frac{\text{True Pos.}}{\text{True Pos.+Fuls Neg.}} \times 100$ formula was used. For evaluating the specificity of primers and probe that applied in PCR and PCR-ELISA intact bovine epithelial tissue were tested. Also In order to compare the threshold of virus detection by ELISA, conventional RT-PCR and PCR-ELISA 1/10, 1/100 and 1/1000 serial dilution of FMDV type A2005IR (105.7 TCID50/ml) were evaluated as a positive control.

RESULTS:

In our study 70 epithelial samples were analyzed by virus isolation, ELISA, RT-PCR and PCR-ELISA and the result was shown in table1. Among these techniques the highest and lowest percentage of detection was related to PCR-ELISA (78.6%) and virus isolation (48.6%) respectively.

In this report ELISA used as a golden diagnostic test for FMDV detection in clinically suspected animals and the other procedures were compared with it and sensitivity of each test was calculated and presented in table1.

Table 1- The result of evaluation of suspected samples to FMD by different diagnostic methods

	Positive number	% Positive number	% Sensitivity
ELISA	43	61.5	-
Virus Isolation	34	48.6	85.7
PCR	49	70	96.6
PCR-ELISA	55	78.6	100

The border line for virus detection by ELISA, RT-PCR and PCR-ELISA determined as mentioned in table2. As shown in this table PCR-ELISA is the most sensitive procedure in our study as it can distinguish nearly 500 FMD viral particles ($10^{2.7}$ TCID50/ml) in each ml of positive control. ELISA and PCR with ability to detect 5010 and 50100 FMDV/ml were the second and third sensitive methods. As depicted in figure1 after electrophoresis of PCR product from RT-PCR and PCR-ELISA only pure and 1/10 dilution of positive control can created visible band.

Table 2- Result of evaluation FMDV A_{2005} serial dilution by ELISA, PCR and PCR-ELISA

Method / Dilution	FMDV A_{2005} TCID50/ml	ELISA		PCR	PCR-ELISA	
		OD	Result		OD	Result
Pure	$10^{5.7}$	0.704	+	+	2.99	+
1/10	$10^{4.7}$	0.358	+	+	2.8	+
1/100	$10^{3.7}$	0.154	+	-	2.24	+
1/1000	$10^{2.7}$	0.057	-	-	1.68	+

Farther more in our study by ELISA, RT-PCR and PCR-ELISA FMDV types A, O and Asia1 were detected successfully (data not shown). It should be mentioned that these types of the virus are the endemic FMDV in our country. Also for evaluating the specificity of oligonucleotides that used as primers and probe in RT-PCR and PCR-ELISA bovine epithelial tissue were tested by these methods and no amplification was detected.

DISCUSSION:

One of the paramount necessities for successful FMD control in endemic area, like our country, is diagnosis of infected animals in outbreaks as soon as possible. To meet this purpose powerful and sensitive screening techniques were needed. In present study routine diagnostic methods like virus isolation, ELISA, conventional RT-PCR were used and sensitivity of them were compared with PCR-ELISA as a new assay.

In our study PCR-ELISA with 100% sensitivity showed maximum sensitivity in diagnosis of FMD among suspected animals. However virus isolation with 85.7% had lowest sensitivity in virus detection. Virus isolation on cell culture is a basic virological method and probably the reliable confirmation of existence of FMDV. But it has some limitation on practical experience in large scale sample analysis. For example it needs noticeable cell preparation facilities, confirmation post inoculation with another specific method like ELISA and time consumption process.2 Furthermore negative result in first passage should be fallowed up to three times. Also countries without high-security laboratories as biosafety level III are not allowed to manipulate infected FMDV samples during virus isolation process [6].

In this report the sensitivity of PCR-ELISA was found more than conventional PCR and ELISA. It can easily predict that combination of PCR and ELISA in a single frame (PCR-ELISA) will have a more sensitivity than two other methods. Also this result was confirmed in examination of logarithmic serial dilution of FMDV. As shown in table2, 102.7 TCID50/ml of virus can be detected by PCR-ELISA but 103.7 and 104.7 TCID50/ml of virus have positive reaction by ELISA and RT-PCR respectively. In other word sensitivity of PCR-ELISA is 10 and 100 times more than ELISA and RT-PCR respectively. Low sensitivity of RT-PCR in comparison with ELISA may be due to the weakness of visual assessment of gel in conventional RT-PCR, but in ELISA the samples were examined more accurately by absorbance evaluation with ELISA reader.

ELISA in most laboratories recognized as a valuable test in pre and post inoculation of suspected samples in cell culture. But it has some defects; for example clinical samples with little amount of virus may be undetermined by this method. Molecular diagnostic techniques like gel based RT-PCR were recognized as a sensitive and specific assay in detection of FMD virus. But sometimes more sensitive method is required to capture very low titer of the virus. Furthermore little amount of virus after RT-PCR amplification may be having invisible band after electrophoresis. This can be critical for FMD free and endemic countries that have control the disease by intensive vaccination program. Very sensitive screening test will be helpful to determine the beginning of outbreaks in these kinds of regions. But increment of sensitivity may be not accompanied with specificity.

It should be noted that the yield of PCR product in both RT-PCR and PCR-ELISA from the dilutions of viral stock has been equal. Therefore after gel electrophoresis and staining, the density of PCR product in both methods was similar (figure1). But in final step of PCR-ELISA, optical density (OD) of the

viral stock (1/1000 dilution) placed in positive range (>0.1). In our study in accordance with previous research, PCR-ELISA was 100 times more sensitive than conventional RT-PCR.

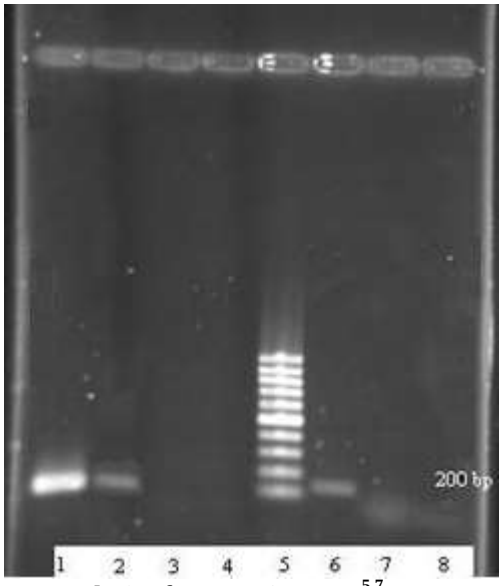

Fig-1: Analysis of FMDV (A_{2005} $10^{5.7}$ TCID50/ml) serial dilution by RT-PCR and PCR-ELISA. In both methods the PCR products were stained with ethidium bromide after electrophoresis. Lanes 1, 2, 3 and 4 show the RT-PCR product of pure, 1/10, 1/100, 1/1000 serial dilutions; Lane 5 is DNA marker and Lanes 6, 7 and 8 are PCR-ELISA amplicons from 1/10, 1/100, 1/1000 viral dilution respectively

PCR-ELISA has been already used by other researchers for diagnose of FMDV, Swine Vesicular Disease and human hepatitis A, as a reliable and sensitive method [3, 9, 10]. In our work this technique does not have any non specific background that usually has seen without probing protocols [11, 12]. Also by detecting conserved sequences of the 2B region, all seven serotype of FMDV could be determined. In this study we found that nucleotides substitute of 3721-3740 and 3832-3851 were the most suitable sequences for designing capture probe to detect all serotypes of the virus. It should be mentioned that these parts of the genome are annealing location for P32 for P33 primers that used in PCR examination.

Furthermore the PCR-ELISA offers the preference of elimination the need for nested PCR, with its consequent risk of sample-to-sample contamination and removal of the staining by carcinogenic dye (ethidium bromide) in gel agarose method. Other advantages of this method are limited expense, rapid and high throughput to perform by capacity of handling up to 96 samples per test.

REFERENCES:

1. Donaldson A; Aerobiology of foot-and-mouth disease (FMD): an outline and recent advances. Rev Sci Tech Off Epiz 1986; 5:315-321.
2. Donaldson A, Alexandersen S, Sorensen J, Mikkelsen T; Relative risks of the uncontrollable (airborne) spread of FMD by different species. Vet Rec 2001; 148:602-604.
3. Alexandersen S, Forsyth M A, Reid S M, Belsham GJ; Development of reverse transcription-PCR (oligonucleotide probing) enzyme-linked immunosorbent assays for diagnosis and preliminary typing of foot-and-mouth disease: a new system using simple and aqueous-phase hybridization. J clin micro 2000; 38:4604-4613.
4. Reid S, Forsyth M, Hutchings G, Ferris N; Comparison of reverse transcription polymerase chain reaction, enzyme linked immunosorbent assay and virus isolation for the routine diagnosis of foot-and-mouth disease. J viro meth 1998; 70:213-217.
5. Remond M, Kaiser C, Lebreton F; Diagnosis and screening of foot-and-mouth disease. Comp immo micro infec dis 2002; 25:309-320.
6. OIE T; Manual of standards for diagnostic tests and vaccines for terrestrial animals 2009. Vol 1, Pt 2, Chapter 1.2.5. 2009.
7. Harris E, Kadir N; A low-cost approach to PCR: appropriate transfer of biomolecular techniques: Oxford University Press, USA; 1998.
8. Vangrysperre W, De Clercq K; Rapid and sensitive polymerase chain reaction based detection and typing of foot-and-mouth disease virus in clinical samples and cell culture isolates, combined with a simultaneous differentiation with other genomically and/or symptomatically related viruses. Arch viro 1996; 141:331-344.
9. Callens M, De Clercq K; Highly sensitive detection of swine vesicular disease virus based on a single tube RT-PCR system and DIG-ELISA detection. J viro meth 1999; 77:87-99.
10. Tahk H, Lee M H, Lee K B, Cheon D S, Choi C; Development of duplex RT-PCR-ELISA for the simultaneous detection of hepatitis A virus and hepatitis E virus. J viro meth 2011; 175(1): 14-20.
11. Barlic-Maganja D, Grom J; Highly sensitive one-tube RT-PCR and microplate hybridisation assay for the detection and for the discrimination of classical swine fever virus from other pestiviruses. J viro meth 2001; 95:101-110.
12. Whitby J, Heaton P, Whitby H, O'sullivan E, Johnstone P; Rapid detection of rabies and rabies-related viruses by RT-PCR and enzyme-linked immunosorbent assay. J viro meth 1997; 69(1):63-72.