Mansoura Veterinary Medical Journal

MOLECULAR DETECTION AND SEROTYPING OF FOOT AND MOUTH DISEASE VIRUS IN TONGUE EPITHELIUM SAMPLES

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ABSTRACT

The aim of the present study is molecular identification and typing of FMDV circulating in Dakahlia governorate in Egypt, 2014. The study was accomplished on pooled nine tongue epithelium samples collected from diseased cattle and buffaloes expected to be infected with FMDV. The diseased animals showed fever , excessive salivation and ulceration on tongue and gum. Conventional reverse transcription polymerase chain reaction (RT-PCR) was used for identification of FMDV using universal primers. Then serotyping of the identified virus was done using RT-PCR for serotype O and A and Real time RT-PCR for serotype SAT2 . FMDV was identified in all the examined nine samples. Five of them were of serotype O, two of serotype A, one of serotype SAT2 and one sample was neither O, A nor SAT2. In conclusion, molecular techniques are sensitive and rapid methods for FMDV detection. In addition, serotypes O, A and SAT2 are circulating in Dakahlia Governorate, Egypt, 2014.

Keywords: *Foot and Mouth Disease Virus, Egypt, RT-PCR, Real time PCR, SAT2.*

INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious vesicular viral disease of even-toed animals **(Brooksby, 1982 and Sobrino et al., 2001).** Caused by a virus that is classified under the genus *Aphthovirus* in the family *Picornaviridae*, order *picornavirales* **(Reid et al.,2002).** There are seven serotypes of foot and mouth disease virus (FMDV),namely, O, A, C, Asia1, SAT1, SAT2 and SAT3 with no cross protection between them **(Belsham, 1993).** In Egypt, the disease have been reported since 1950s and is still enzootic till today showing mild to severe outbreaks from time to time in different locations in Egypt **(Mousa et al.,1974, Ibrahim et al.,2015)**.

Type O was the most prevalent since 1960 and till now (**Zahran,1960 and Farag et al.,2005**). Serotype A was introduced to Egypt since 2006 through life animals imported from Ethiopia, causing severe clinical signs occurred among cattle and buffaloes **(Abdel-Rahman et al.,2006 and Knowles et al., 2007)** while type SAT2 was firstly detected in 1950 and re-emerged in 2012 and made an outbreak all over the country **(Ahmed et al.,2012).**

FMDV expresses itself in infected animals with fever, excessive salivation, tongue and gum ulceration. Tongue epithelium is a good source of the virus for sampling and for

virus diagnosis either serologically or molecularly **(OIE manual, 2012)**.

FMDV had a single stranded positive sense RNA , of about 8.3Kb in length with a 5' untranslated region (5' UTR) of about 1300 nucleotides. While at the 3' end , also a 3' untranslated region(3'UTR) exists. In between the two untranslated regions there is a single open reading frame (ORF) of about 7000 nucleotides, translated into proteins. The ORF consists of , a leader sequence (L) , 1A, 1B, 1C, 1D, 2A, 2B, 2C , 3A, 3B, 3C and 3D. The 1A, 1B, 1C and 1D sequences are translated into viral structural proteins , while L, 2A, 2B, 2C , 3A, 3B, 3C and 3D are the viral non structural proteins **(Belsham, 2005).** FMDV RNA can be detected in tongue epithelium samples using conventional RT-PCR using universal primers targeting a highly conserved 5`UTR, that is present in all serotypes **(Reid et al. 2000)**. A Real time PCR assay designed for serotype SAT 2 detection in Egypt according to **(Ahmed et al. 2012)**.

The aim of the present work lies in molecular identification of FMDV in Dakahlia governorate in Egypt, 2014 using RT-PCR and further molecular typing of the identified virus by RT-PCR for O and A serotypes and real time PCR for serotype SAT2.

MATERIALS AND METHODS

1-Sample collection:

Pooled nine tongue epithelium samples (45 samples). Samples were collected from diseased cattle and buffaloes suspected to be infected with FMDV. The diseased animals showed fever, excessive salivation, vesicles around the mouth and on the dorsum of the tongue and on some cases an ulcerated area due to vesicle rupture. Tongue biopsies were also taken from three apparantly healthy buffaloes as negative control samples. All samples were transported to the lab on PBS: glycerol (1:1) mixture , then homogenized with PBS (pH 7.4) to make 10% suspension **(OIE manual ,2012).**

2-RNA extraction:

Viral RNA extraction was performed using Gena bioscience total RNA purification kits. A lysis buffer was mixed with sample homogenate ,put on the spin column, the column was washed with primary and secondary washing buffers, then RNA was eluted using elution buffer and stored on -20°c till use that was done according to **Reid et al. 1998.**

3- Molecular identification of FMDV

3.1. Oligonucleotide primers

The universal primer pair: (1F/1R) were used , that amplifies the highly conserved 5`UTR producing a 328 bp amplicon with FMDV of any serotype . The 1F primer sequence is : GCCTGGTCTTTCCAGGTCT , while 1R sequence is : CCAGTCCCCTTCTCAGATC. Primers were synthesized by the Metabion international, Germany according to **Reid et al. 1998.**

3.2. RT-PCR amplification:

Extracted RNA was amplified using Thermoscientific Verso one step RT-PCR kit according to **Reid et al. 1998**. The reaction mixture consists of 2.5 ul of sample RNA , 0.5 ul enzyme mix ,12.5 ul one step master mix, 0.5 ul of the forward primer, 0.5 ul of the reverse primer, 1.25 ul of RT-enhancer and 7.5ul of nuclease free water. The thermal profile used in RT-PCR reaction : cDNA synthesis occur at 50°c for 15 min, Verso inactivation at 95°c for 2 minutes then

denaturation at 95°c for 15 seconds, amplification occurs though primer annealing at 55°c for 30 seconds followed by extensionat 72°c for 1.5 minutes, the cycle repeats for 40 times followed by final extension at 72°c for 10 minutes.

3.3. Agarose gel electrophoresis for the amplified product:

Agarose gel was prepared with a final concentration of 1% in tris borate EDTA buffer , and Ethidium bromide stain was added to reach a final concentration of 0.5ug/ml. Samples and DNA marker (Thermoscientificbio 50-1000 bp and 100-1000 in molecular typing of serotype A)were loaded, allowed to migrate in the gel by electric power about 80 volt for about 40 mintes, then the gel was visualized and photographed by Molecular Imager® Gel Doc™ gel documentation system that was done according to **Amaral-Doel et al. 1993.**

4- Molecular typing of the amplified nucleic acid:

4.1. Molecular typing of O and A serotypes:

4.1.1. Oligonucleotide primers :

Using serotype O specific primer (O-1C283F/EUR-2B52R) for amplification of VP1, the sequence of O-1C283F primer is (5' GCCCAGTAACTACACACAGTACAG 3') while the EUR-2B52R sequence is (5' GACATTGTCCTCCTGCATCTGGTTGAT3') gives a band of 1142 bp according to **Knowles et al. 2005** and serotype A specific primer (A-1C612F/EUR-2B52R) that amplifies VP1 the sequence of the A-1C612F primer is: (5' TAGCGCCGGCAAAGACTTTGA 3') and gives a band at 814 bp according to **Knowles et al. 2007.**

4.1.2. RT-PCR amplification:

The reaction mixture used as previously mentioned. The thermal profile used in RT-PCR reaction : cDNA synthesis occur at 50°c for 15 min, Verso inactivation at 95°c for 2 minutes then denaturation at 95°c for 15 seconds, amplification occurs though primer annealing at 60°c for 30 seconds for serotype O primers and 55°c for 30 seconds in serotype A primers, followed by extension at 72°c for 1.5 minutes , the cycle repeats for 40 times followed by final extension at 72°c for 10 minutes.

4.1.3. Agarose gel electrophoresis for the amplified product: as mentioned previously

3.2. Molecular typing of serotype SAT2 by Real time RT-PCR:

4.2.1. Oligonucleotide primers and probe:

The specific oligonucleotides used were forward primer (5`-TGA AGA GGGCTGAGCTGTACG-3`), reverse primer (5`-CTCAACGTCTCCTGCCAGTTT-3`) and dual-labeled (FAM-TAMRA) TaqMan® probe (5`-ACA GAT TCG ACG CGC CCATCG-3`) Primer and probe were synthesized by the Metabione, Germany according to **(Ahmed et al., 2012).**

4.2.2. Real time PCR amplification:

In this assay, AgPath-ID™ One-Step RT-PCR Kit, was used in which 25 ul RT-PCRs contained 2ul of each primer, 1ul of TaqMan® probe, 12.5 ul of commercial mastermix and 5 ul of RNA extracted from a clinical sample and control samples. One-step reverse transcription and PCR amplification (Life Technologies™) was performed using the following cycling program: 60°C for 30 min and 95°C for 10 min, followed by 50

cycles of 95°C for 15 s and 60°C for 60 s. Ct values were detected for positive samples.

RESULTS

1-Results of molecular identification:

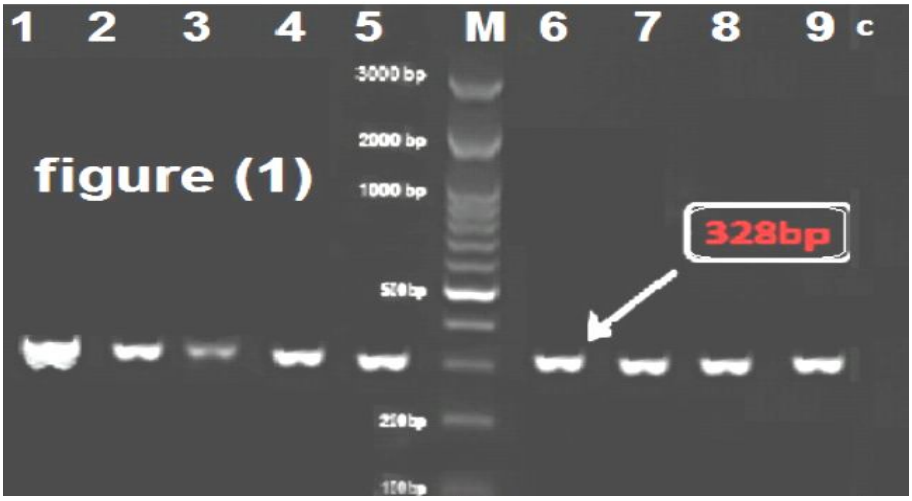
The pooled nine samples give positive results on RT-PCR by using universal primer and negative control samples gave no bands **(table 1 and figure 1).**

2-Result of molecular typing:

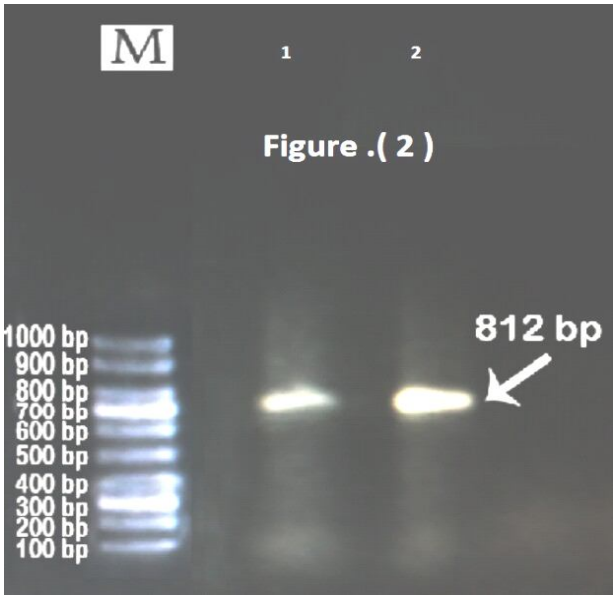
Five samples gave the 1142 bp bands with serotype O specific primer. Two samples gave bands with serotype A specific primers at 812bp. One sample gave a Ct value equals 22.92 with serotype SAT2 specific primers and probe. One sample gave no reaction with all used primers **as shown in table 1 figures (2,3,4)**

Table (1): The results of molecular identification by RT-PCR and molecular typing by RT-PCR and real time RT-PCR.

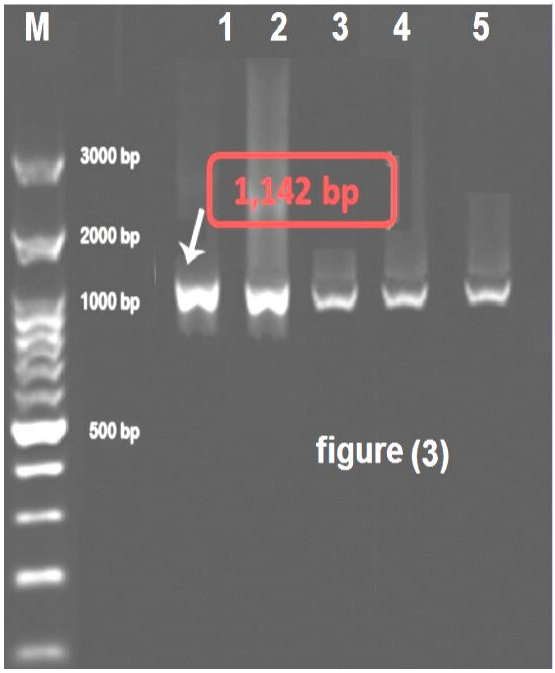
Sample	RT-PCR	Molecular typing
1	+	SAT2 (Ct=22.92)
2	+	O
3	+	O
4	+	Not typed
5	+	O
6	+	O
7	+	O
8	+	A
9	+	A



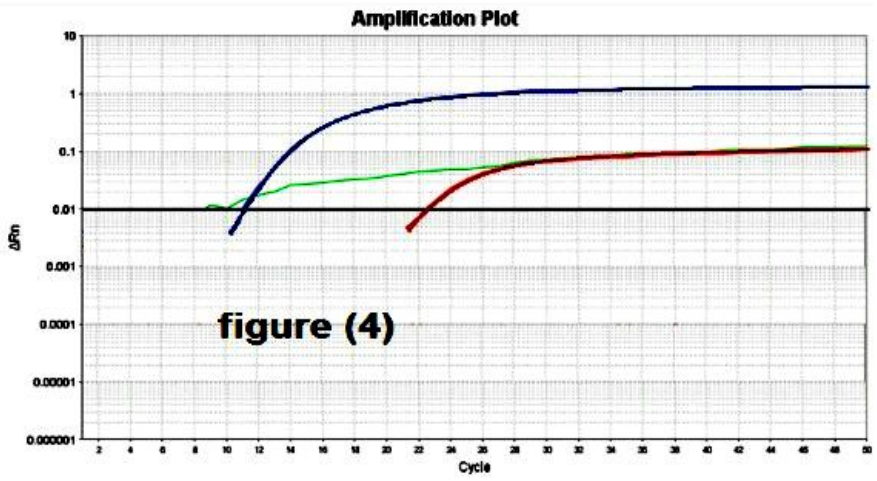
Figure(1):PCR products with FMDV universal primer, nine positive samples and the control negative samples.



Figure(2):PCR products with serotype A specific primers, two positive samples.



Figure(3):PCR products with serotype O specific primers, five positive samples.



Figure(4): The amplification blot of serotype SAT2, Ct value=22.9 (red curve) and control positive sample(blue curve) Ct value = 10.6

DISCUSSION

Foot-and-mouth disease (FMD) is a highly contagious disease affecting artiodactylae, mostly cattle, swine, sheep, goats, and many species of wild ungulates, caused by a virus of *Picornaviridae*, *aphthovirus*. FMD affects animals worldwide and is included as an OIE listed disease and one of the notifiable diseases **(OIE manual, 2012).**

RT-PCR is an important molecular technique used to identify RNA viruses. It is cheap, fast, easy to perform, relatively accurate and reproducible. Firstly, it was designed to identify FMDV regardless of it's serotype using primers targeting viral highly conserved sequences as 5' untranslated region and 3D sequences as in **(Reid et al.1998).** Afterwards it was necessary to make molecular typing to enhance control procedures using primers targeting VP1, in which nucleotide changes result in emergence of new types and subtypes. These primers detect all the seven serotypes separatly **(Knowles et al . 2005 and 2007).**

In the present study, nine pooled tongue epithelium samples were collected , prepared , RNA was extracted and tested by RT-PCR for detection of the virus using the universal primers. All the 9 samples gave the specific bands at 328bp. These results nominate RT-PCR to be used for diagnosis of FMDV with very high sensitivity as mentioned in **Amaral-Doel et al. 1993, Reid et al. 1998 and 1999** who used primers specific to the 5`UTR and 3D of FMD as a highly conserved regions in the virus genome. Using RT-PCR for FMDV diagnosis and serotyping. RT-PCR is the suitable means for FMD diagnosis and serotyping in comparison with virus isolation/antigen detection ELISA in diagnosis. The nine samples were tested by RT-PCR using the serotype O specific primers; only five of them were positive giving the 1.142 bp. These findings are in agreement with **Knowles et al. 2005** and this primer is recommended in the WRL reports, which are developed from the most recent Egypt field isolates.

Two from 9 samples were of serotype A using RT-PCR this results in agreement with **(Knowles et al. 2007)**

One sample produced a significant Ct value with the SAT2 specific primers and probe, this assay was performed by **(Ahmed et al., 2012)** who developed the technique, tailored to Egyptian isolates of serotype SAT 2 and it is highly recommeded to design real time PCR assays to each locality. In conclusion, conventional RT-PCR could be used as a reliable and sensitive method for laboratory confirmation for FMDV infection that reduce the dependence on tissue culture and time needed to isolate the virus. Serotypes O, A and SAT2 circulate in Dakahlia governorate in 2014. So, we recommend the use of the locally produced trivalent vaccine(O,A and SAT2) to protect cattle against the disease.

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