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Application of Conventional and Real Time Reverse Transcription Polymerase Chain Reaction Assays to Serotype False Negative Un-Typed Foot and Mouth isease Virus in Egypt

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Abstract: In Egypt where Foot and Mouth Disease Virus (FMDV) is endemic, rapid accurate diagnosis and serotyping of the causative virus genotypes is significant for vaccine matching and tracking FMDV evolution. In this study, real-time and conventional reverse transcription polymerase chain reaction (RT-PCR) assays using primer/probe developed from the 3D and VP1 coding regions of the virus genomes were used for diagnosis and serotyping of 31 clinical samples from Egypt during Mach 2012 to May 2015. The results revealed that 31 samples were positive with 3D rRT-PCR. Likewise, samples could be serotyped with rRT-PCR as 9 samples were serotype SAT2, 9 samples were serotype A, 8 samples were O and 5 samples were un-typed with rRT-PCR and could be serotyped by RT-PCR after several trails with different primers sets. FMDV is highly variable, serotyping of FMDV needs nucleotides sequencing and continuous monitoring of primers sensitivity and specificity.

Key words: FMDV · Diagnosis · Serotyping · Un-Typed FMDV

INTRODUCTION

Foot and mouth disease (FMD) is one of the most contagious viral diseases caused by FMDV. FMDV is a member of the Aphthovirus genus within the family Picornaviridae with 8.4 kilobases positive single-stranded RNA genome. Moreover, it has seven different serotypes: O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1 with various antigenic spectrum of virus genotypes within each serotype [1-3]. In fact, being a trans-boundary viral disease of livestock, FMDV threatens over 100 countries around the world [1, 4]. Recently, FMDV reported severe epidemics, as in Egypt 2012 with SAT2 serotype; in Saudi Arabia and Libya 2013 and in Tunisia 2014–2015 with O/ME-SA/Ind-2001 serotype which was used to be normally restricted in India [5].

Economically, FMDV has been dramatically spread, and continues to cause huge losses on the level of animal trade business. The losses are estimated around 10 million US $ per year on a global basis. This magnitude of losses could occur in a single country, if it was normally FMDV free, as in the UK in 2001 [5-8]. The mass culling of animals has generated awareness in the development of sensitive diagnostic techniques to confine FMDV outbreaks [9]. FMDV can be detected by several techniques, for example; FMDV isolation, enzyme-linked immunosorbent assay (ELISA) [10, 11] and Reverse transcription polymerase chain reaction (RT-PCR) [12]. However, each of these current diagnostic techniques has some drawbacks [13]. A number of pan-serotype specific rRT-PCR techniques have been established to detect highly conserved regions within FMDV genome[14] to detect the seven serotypes of FMDV. Serotyping of FMDV can be expert by use of serotype-specific primers/probes and nucleotide sequencing [15]. In Egypt where FMDV is endemic, rapid accurate diagnosis and serotyping of the causative virus genotypes is significant for vaccine matching and tracking FMDV evolution. It is difficult to design serotype-specific primers able to detect genetic variety among all FMDV serotypes [16].

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In present work, we focused on the use of conventional RT-PCR different serotype-specific primers to type real time RT-PCR false negative un-typed Egyptian field samples during March 2012 to September 2015.

MATERIALS AND METHODS

Samples: Thirty one clinical samples (Unruptured and recently ruptured vesicles in the buccal cavity, vesicular fluid, epithelium and hearts) were collected from cattle, buffalos and calves in Egypt from March 2012 to September 2015.

Viral RNA Extraction from Samples: Using QIAamp viral RNA mini kit (Qiagen, Catalog No. 52904) according to the manufacture instructions.

Diagnosis and Serotyping of FMDV by rRT-PCR and RT-PCR: Diagnosis of FMDV was done using the common primers and probe published before [17] while serotyping of positive samples using rRT-PCR were done for serotypes A, O and SAT2 by primers and probes previously published [15] and RT-PCR serotyping using (IAH – Method/Protocol Sheet, 2012) as shown in table (1). Quantitect kit (Qiagen, Catalog No. 204343) was used for rRT-PCR using a real-time PCR machine (StepOne, Applied Biosestyme, USA) with the thermal profile according to the manufacture instructions. Cycle threshold (CT) for each sample was then determined previously [18]. QIAGEN OneStep (Qiagen) was used for RT-PCR assay according to the manufacture instructions using BioRad thermal cycler.

RESULTS

Diagnosis of FMDV using rRT-PCR: Diagnosis of FMDV from 31 clinical samples using rRT-PCR was done by the common primers and probe mentioned before by Callahan *et al*. [17] (Fig.1), rRT-PCR assay was showing amplification curve of conserved 3D gene which confirmed the presence of FMDV in each sample and could detect 31 positive samples.

Serotyping of FMDV using rRT-PCR: Serotyping of positive samples using rRT-PCR was done for serotypes-genotypes A/Asia/Iran-05, O/ME-SA/PanAsia-2 and SAT2 by primers and probes previously published [15] (Figs. 2 & 3) and (Table 2) where it was showing an increase in the amplification curve of 9 positive samples with A/Asia/Iran-05 primers/probe, 9 positive samples with SAT2 primers/probe, 8 positive samples with O/ME-SA/PanAsia-2 primers/probe and 5 un-typed samples.

Serotyping of FMDV using RT-PCR: For 5 un-typed positive samples, IAH – 2012 Method/Protocol for RT-PCR was carried out for serotyping with several primers combination (Forward primers as O583, O283, O272, O244, A 562 and A 612 & reverse primers as EUR and NK61) [6]. After several trails, serotyping of 5 samples were done as illustrated in (Fig. 4), 4 samples were serotyped with EUR reverse primer and O 244 forward primer which giving specific band of 1100 bp and 1 sample was serotyped with NK-61 reverse primer and O 583 forward primer which gave specific band of 800 bp.

Table 1: Showing primers names and sequences used for diagnosis and serotyping.

No.	Name of primer	Primer Sequence 5'---_3'	Used assay	Reference
1	3D (F)	ACTGGGTTTTACAAACCTGTGA	rRT-PCR	[17]
2	3D (R)	GCGAGTCCTGCCACGGA		
3	3D (Probe)	TCCTTTGCACGCCGTGGGAC		

4	O (F)	CCGAGACAGCGTTGGATAACA		[15]
5	O (R)	CCATACTTGCAGTTCCCGTTGT		
6	O (Probe)	CCGACTTGCACTGCCTTACACGGC		
7	A (F)	ACGACCATCCACGAGCTYC		
8	A (R)	RCAGAGGCCTGGGACAGTAG		
9	A (Probe)	CGTGCGCATGAAACGTGCCG		
10	SAT2 (F)	TGA AGA GGG CTG AGC TGTACT G		
11	SAT2 (R)	CTC AAC GTC TCC TGCCAG TTT		
12	SAT2 (Probe)	ACA GAT TCG ACG CGC CCA TCG		
13	O-1C283F	GCCCAGTACTACACACAGTACAG	RT-PCR	IAH-Method/Protocol Sheet, 2012
14	O-1C244F	GCAGCAAAACACATGTCAAACACCTT		
15	EUR-2B52R	GACATGTCCTCCTGCATCTGGTTGAT		
16	NK61	GACATGTCCTCCTGCATCTG		

Table 2: Showing samples details with CT values.

No.	Serotype	Source	sample	CT (3D)	(Serotyping)
1	O (R)	Cattle – Monofia - 2014	Vesicular Fluid	12.29	12.65
2	O (R)	Cattle – Behera - 2014	Epithelium	13.54	15.57
3	O (R)	Cattle – Sharqia- 2014	Epithelium	26.48	22.39
4	O (R)	BaffaloKafrSheikh2014	Epithelium	27.44	29.71
5	O (R)	Cattle – Domiat - 2013	Tongue	14.26	16.28
6	O (R)	Cattle – Domiat - 2013	Epithelium	9.85	12.14
7	O (C)	Cattle – Domiat - 2013	Vesicular Fluid	15.89	---------
8	O (C)	Cattle – Behera - 2014	Epithelium	28.15	---------
9	O (R)	Cattle – Behera - 2014	Epithelium	14.09	16.88
10	O (R)	Buffalo KafrSheikh2014	Epithelium	29.66	25.34
11	O (C)	Cattle – Monofia - 2014	Vesicular Fluid	20.41	---------
12	O (C)	Cattle – Behera - 2013	Heart	21.25	---------
13	O (C)	Cattle – Behera - 2013	Epithelium	29.82	----------
14	A	Cattle – Giza - 2012	Vesicular Fluid	17	18.22
15	A	Cattle – Giza - 2012	Vesicular Fluid	14.1	20.34
16	A	Cattle – Banisuif- 2014	Epithelium	19.32	20.17
17	A	Cattle – Banisuif- 2014	Epithelium	15.85	18.05
18	A	Cattle – Sharqia- 2013	Vesicular Fluid	13.84	17.47
19	A	Cattle – Sharqia- 2013	Epithelium	23.65	22.19
20	A	Cattle – Sharqia- 2013	Vesicular Fluid	13.44	10.933
21	A	Cattle – Monofia- 2013	Epithelium	16.86	18.34
22	A	Cattle – Qena- 2013	Epithelium	27.49	26.56
23	SAT2	Cattle – Dakahlya- 2012	Heart	21.02	22.3
24	SAT2	Buffalo - Domiat- 2012	Vesicular Fluid	24.74	26.26
25	SAT2	Cattle – Gharbia- 2012	Epithelium	29.66	32.38
26	SAT2	Cattle – Alexandria2012	Epithelium	20.41	19.47
27	SAT2	Cattle – Dakahlia - 2012	Heart	21.25	23.77
28	SAT2	Cattle – Qena- 2014	Epithelium	17.6	17.93
29	SAT2	Cattle- Monofia- 2014	Epithelium	16.52	18.33
30	SAT2	Cattle- Monofia- 2014	Epithelium	17.84	18.39
31	SAT2	Cattle- Monofia- 2014	Vesicular Fluid	29.78	35.1

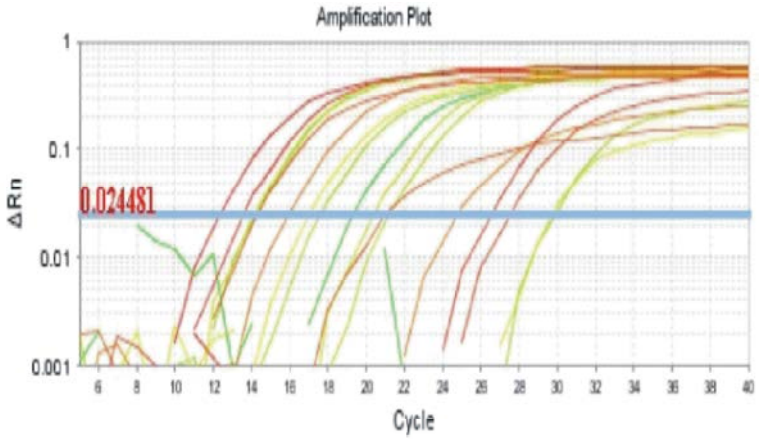

Fig. 1: Showing amplification curve of conserved 3D gene where 31 tested samples were positive for FMDV.

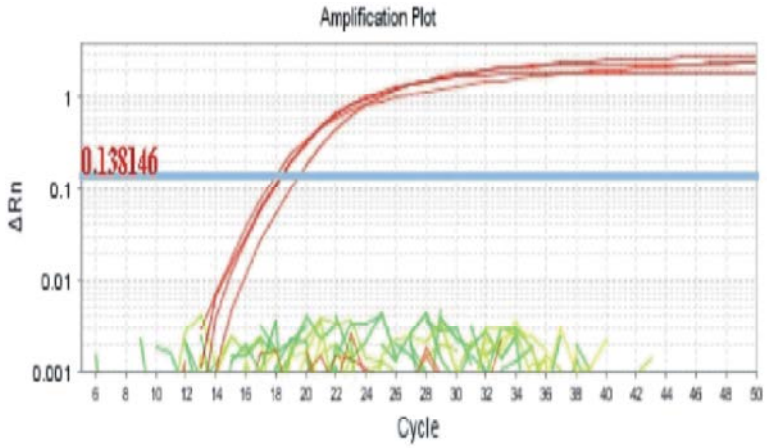

Fig. 2: Showing amplification curve of serotype SAT2 where 9 tested samples were positive for SAT2 serotype.

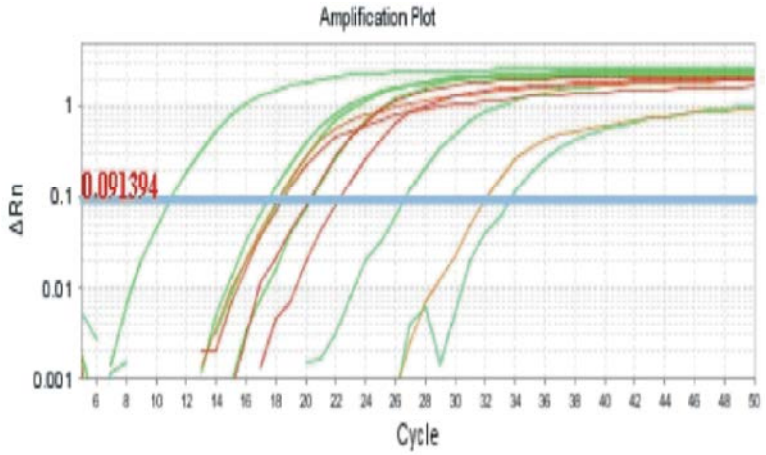

Fig. 3: Showing Amplification curve of serotypes A & O where 9 tested samples were positive for A and 5 tested samples were positive for O Pan-Asia serotype.

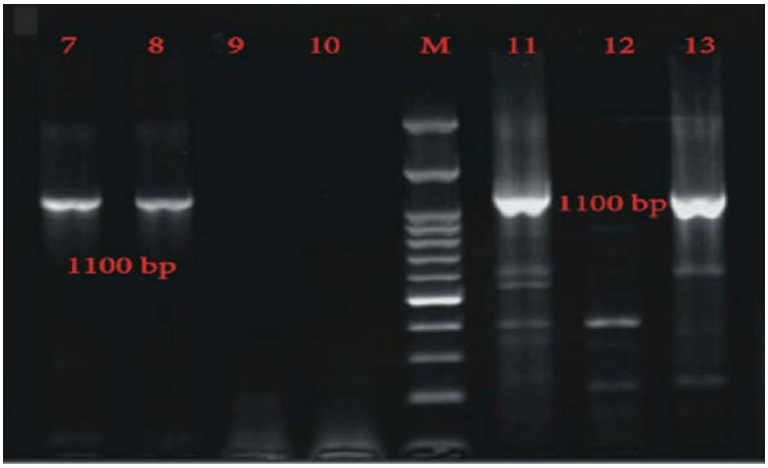

Fig. 4: Showing Ethidium bromide stained gel showing molecular marker 100bp and bands 1100bp (O) with primers (O244 forward & EUR reverse) which show positive samples (7, 8, 11& 13).

DISCUSSIONS

The current endemic state of FMDV in Egypt magnifies the importance of diagnosis and serotyping of virus genotypes for vaccine matching and tracking FMDV evolution. FMDV is highly variable and its evolution occurs at two levels; genetic and antigenic levels [19]. Due to the lack of proof reading of the viral RNA dependent RNA polymerase, a high mutation rate occurs during FMDV replication about one mutation per genome in each replication cycle [20]. FMDV evolution creates a wide spectrum of genotypes within each serotype which haven't cross protection for other genotypes even within the same serotype, so the molecular characterization of these genotypes is necessary to ensure appropriate vaccine selection [2]. Based on the nucleotide sequence of VP1 highly variable coding region along FMDV genome, FMDV was classified into topotypes within each serotype. Briefly, serotype O was categorized into eleven topotypes, serotype A and C was classified into three topotypes, serotype SAT 1 was classified into nine topotypes, serotype SAT 2 was classified into fourteen topotypes, serotype SAT 3 was classified into five topotypes and serotype Asia 1 was classified into 6 groups [21]. Egypt is endemic with 8 genotypes; O/ME-SA/Sharqia-72, O/ME-SA/PanAsia-2, O/EA-3, A/Asia/Iran-05, A/Africa/G-IV, A/Africa/G-VII, SAT2/Ghb-12 and SAT2/Alex-12 [22].

Several trials were carried out for designing rRT-PCR serotype-specific primers/probes[15]. It may be difficult with a highly variable FMDV genome and there is no constant sequences within all serotypes [17]. A number of rRT-PCR techniques have been designed to catch conserved parts of FMDV genome [14]. Serotyping of FMDV can be expert by use of serotype-specific primers/probes and nucleotide sequencing, but it is difficult to design serotype-specific primers to cover the genetic diversity within all FMDV serotypes [15]. Many efforts were done for serotyping as rRT-PCR assays using primer/probe developed from VP1 region for specific serotyping of O, A and Asia-1 FMDV circulating in Middle East. They were specific for serotype O lineage PanAsia, serotype A lineage Iran-05 and serotype Asia-1 [15].

In present work, RNA extracted from 31 clinical samples (Unruptured and recently ruptured vesicles in the buccal cavity, vesicular fluid, epithelium and hearts) were collected from cattle, buffalos and calves from Egypt during March 2012 to September 2015. Diagnosis of FMDV was done by the common primers and probe mentioned before by Callahan *et al.* [17] (Fig.1), the assay was useful in confirming FMDV diagnosis in each sample and could detect 31 positive samples. While serotyping of positive samples using rRT-PCR were done for serotypes-genotypes A/Asia/Iran-05, O/ME-SA/PanAsia-2 and SAT2 by primers and probes previously cited by Reid *et al*. [15] (Figs. 2,3) and (Table 2) where it was showing an increase in the amplification curve of 9 positive samples with A/Asia/Iran-05 primers/probe, 9 positive samples with SAT2 primers/probe and 8 positive samples with O/ME-SA/PanAsia-2 primers/probe. As primes/probe which were used in rRT-PCR assay were genotypic specific, so it could not serotype all samples. As Egypt is endemic with 8 genotypes; O/ME-SA/Sharqia-72, O/ME-SA/PanAsia-2, O/EA-3, A/Asia/Iran-05, A/Africa/G-IV, A/Africa/G-VII, SAT2/Ghb-12 and SAT2/Alex-12 [22] the previously published primers/probes sets for rRT-PCR [15] could only serotype O/ME-SA/PanAsia-2, A/Asia/Iran-05 and could not serotype SA/Sharqia-72 and O/EA-3 as FMDV is highly variable [19]. Moreover, due to the lack of proof reading of the viral RNA dependent RNA polymerase, a high mutation rate occurs during FMDV replication and about one mutation per genome per replication cycle [20]. Therefore, FMDV evolution creates a wide spectrum of genotypes within each serotype [2] and it is not easy to design serotype-specific primers to detect the genetic variety among all FMDV serotypes and genotypes [15]. For 5 non-serotyped positive samples, IAH – 2012 Method/Protocol for RT-PCR was carried out for serotyping with several primers combination (forward primers as O583, O283, O272, O244, A 562 and A 612 & reverse primers as EUR and NK61). After several trails, serotyping of 5 samples were done as illustrated in (Fig. 4), 4 samples were serotyped with EUR reverse primer and O 244 forward primer which giving specific band of 1100 bp and 1 sample was serotyped with NK-61 reverse primer and O 583 forward primer which giving specific band of 800 bp. Divergences between FMDV serotypes even within the same serotype due to the high variability of FMDV and mismatching of primers with some samples create the interest of nucleotides sequencing and continuous monitoring of primers sensitivity and specificity.

CONCLUSIONS

Serotyping of FMDV is not easy and need continuous monitoring of primers specificity and need new tools to increase the primer sensitivity as FMDV is highly variable.

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