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Rapid detection and isolation of Foot and Mouth Disease Virus in samples from clinically suspected animals in Egypt during 2016-2019

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ABSTRACT

Foot and Mouth Disease (FMD) is a highly contagious and economically important disease of susceptible cloven-hoofed animals, mostly for cattle, buffalo and pigs. This study was designed to isolate and identify the serotypes of FMD virus from clinically suspected animals in different localities in Egypt that would be useful to detect the current strains present in Egypt to be used in the future vaccination program. Tongue epithelium, vesicular fluid and heart tissue samples were collected from FMDV clinically suspected cases representing four different governorates in Egypt (Qalubia, Sharkia, Gharbia and Behera). We found that (32) out of (45) submitted samples showed positive result in real time RT-PCR and about (26) of these positive samples were isolated on BHK-21 cells giving overt cytopathic effect of the virus. The isolated viruses were identified and serotyped using antigen detection ELISA and RT-PCR that confirmed the three different serotypes A, O and SAT2 with different ratio for their prevalence in Egypt. We found that SAT2 was the predominant circulating serotype in the field, followed by serotype O and serotype A. Hence, this work demonstrates the co-occurrence of three different FMDV serotypes in Egypt. Consequently, further molecular analyses are recommended to confirm these findings to determine the molecular epidemiology of the isolates and to update the nature of future vaccine strains for successful preventive strategies.

1. INTRODUCTION

Foot and mouth disease virus (FMDV) is contagious virus affecting cloven-hoofed animal, such as cattle, buffalo sheep and goat. The disease is characterized by fever, vesicular eruptions on the feet, buccal mucosa and the mammary glands of females (Bronsvoort and Radford, 2004; OIE, 2009). FMD is caused by Foot and Mouth disease Virus (FMDV) of the genus Aphthovirus, in the family picornaviridae. There are seven immunologically district serotypes; O, A, C, South African Territories 1 (SAT1), SAT2, SAT3 and Asia 1. FMD virus is non-enveloped small RNA virus (Cooper et al., 1978). The RNA genome of approximately 8.5 kb comprises four structural protein genes 1A, 1B, 1C and 1D which make up the viral capsid and eight non-structural protein (NSP) genes L, 2A, 2B, 2C, 3A, 3B, 3C and 3D responsible for proteolytic cleavage and viral replication (Clavijo et al., 2004). The presence of seven serotypes and multiple subtypes and variants has added to the difficulty of laboratory diagnosis and control of FMDV. The rise of new variants is inevitably caused by continued circulation of the virus in the field and quasi- species nature of RNA genome (Domingo et al., 2003). Transmission is generally affected by contact between infected and susceptible animals to the excretions and secretions of acutely infected animals (Knight-Jones and Rushton, 2013). FMD Virus is reported to be endemic in Egypt since 1950, with the introduction of serotype SAT2. The SAT2 virus was again recorded in 2012 and appeared in two lineages, Alx-12 and Ghb12, which both belong to topotype VII (Ahmed et al., 2012). Serotype O is the most prevalent and was responsible for outbreaks in 1987, 1989, 1990, 1991 and 1993 (Samuel et al., 1999). It was the only serotype reported in Egypt between 1964 and 2005, except for an outbreak involving serotype A in 1972 (Rady et al., 2014). However, serotype A was introduced again in 2006 through animal importation, causing the economic loss of about one million cattle (Knowles et al., 2007). The three serotypes O, A and SAT2 have been cocirculating in Egypt since 2013. (Sobhy et al., 2014; Ahmed et al., 2016; Soltan et al., 2017). Virus isolation is the gold standard for FMDV detection in diagnostic procedures. This technique is reliant on the use of sensitive cells such as primary cultures of bovine thyroid and porcine kidney or BHK-21 and IR-P1 cell line for rapid and accurate detection of FMDV (Kabelo et al., 2020). RT-PCR is a strong and valuable tool concomitant with diagnostic routine methods facilitate monitoring the fields FMDV strains. real-time RT-PCR assays (rRT-PCR), targeting serotypic determinants encoded within variable VP1 (1D) sequences in the viral capsid, have been shown to be effective tools that can be used to detect and discriminate multiple FMDV lineages (Ahmed et al., 2012; Jamal and Belsham, 2015; Reid et al.,

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2014). The aim of our work here was to determine the situation of the circulating foot and mouth disease virus in Egypt between 2016 -2019 by isolation and identification of FMD virus serotypes causing outbreaks in four governorates in Egypt to design appropriate preventive measures including effective vaccination program.

2. MATERIAL AND METHODS

2.1. Samples

A total of 45 clinical samples were collected during 2016, 2017, 2018 and 2019 from four Egyptian governorates (Qalubia, Sharkia, Gharbia and Behera). The details of the samples including the location, year of collection and type of sample were mentioned in Table 1. The samples included vesicular fluid, tongue epithelium and heart tissue were prepared according to the OIE recommended protocols (OIE, 2018) and were stored at -80°C till used.

2.2. Processing of the collected samples

1-2 grams from the tongue epithelium tissue were grinded manually using sterile mortar and pestle with addition of some sterile sand. Then, 2-3ml PBS containing antibiotic [penicillin (10 U/ml), streptomycin (100 µg/ml) and amphotericin B (10 µg/ml)] and centrifuged at 5000 rpm for 5 min at 4 °C. The supernatants were collected and filtered through 0.22 µ Millipore filter and stored at -80°C till isolation on BHK-21 cells. The vesicular fluid samples were treated by antibiotic-antimycotic solution, filtered and also stored at -80°C till used.

2.3. Viral RNA extraction

The total RNA was extracted from the collected samples using Thermo scientific Gene Jet RNA purification (Thermoscientific, USA) according to the manufacturer's instructions. The extracted RNA was eluted in 30 µl of the kit elution buffer and stored at –80°C for further use**.**

2.4. Molecular detection of FMDV by Real Time RT-PCR

The Real time RT-PCR assay was used for FMDV detection in clinical samples recommended by (OIE, 2018). Primers and probe were synthesized by Invitrogen, USA and mentioned in Table 2. The 20 µl reaction volume consisted of 10 µl of SCRIPT One-Step RT-q PCR Probes Master with high ROX (Jena Bioscience, Germany), 1 µl of each primer, 0.5 µl of probe, 4 µl of RNA template and 3.5 µl RNAse free water The reaction was run on StepOne Real Time PCR instrument (Applied Biosystems, USA) using the following thermal conditions: reverse transcription at 50°C for 15 min then 95°C for 5 min followed by 45 cycles of denaturation step at 95°C for 15 sec and annealing and extension step at 60°C for 1 min.

2.5. Foot and Mouth Disease Virus isolation on baby hamster kidney (BHK-21) cell culture

FMDV positive samples by real time RT-PCR were inoculated on monolayer of BHK-21 cell culture as described by the (OIE, 2018). The cultures were examined microscopically (3-5) days for the development of a cytopathic effect (CPE). Specimens were considered negative for FMDV if no changes appeared after three successive blind passages on BHK-21 cells.

2.6. Serological detection of FMDV antigen by using double sandwich enzyme linked immune-sorbent assay (ELISA)

The positive viral isolates were serotyped by ELISA kit containing Serotype O, A, SAT1 and SAT2 FMDV antigens (Istituto Zoopro filattico Sperimentale della Lombardiae dell'Emilia Romagna [IZSLER], Brescia, Italy) according to the manufacturer's instructions.

2.7. Molecular serotyping of FMDV isolates by RT-PCR

Amplification of the partial VP1 coding region for serotype O, A and SAT2 was performed using the assay developed by Shehata et al. (2016) (Table 2) and 1-Step RT-PCR Reddy Mix kit (Thermoscientific, USA). The RT-PCR reaction was done in 25 µl reaction volume consisted of 12.5 µl of 2X 1-step PCR Reddy Mix, 0.5 µl of Verso Enzyme Mix, 1.25 µl RT- Enhancer, 1 µl of forward and reverse primer and 5 µl of RNA template. The cycling parameters were adjusted on Bio-Rad thermal cycler as following: 50°C for 30 min and 95°C for 15 min, then 35 cycles of denaturation at 95°C for 60 s, annealing at 60°C for 30 sec and elongation at 72°C for 1 min, followed by a final extension at 72°C for 5 min.

Table 1 Types and number of samples collected from suspected FMD infected cattle in four different governorates in Egypt (Qalubia, Sharkia, Gharbia and Behera) during (2016- 2019).

Gov	2016			2017			2018			2019			Total
	V.F	T.E	C.T	V.F	T.E	C.T	V.F	T.E	C.T	V.F	T.E	C.T	
Qalubia	2	1	-	1	3	-	1	2	1	1	2	1	15
Sharkia	1	-	-	1	1	-	1	1	1	-	2	2	10
Gharbia	1	-	-	1	1	-	1	1	1	1	1	-	8
Behera	1	3	-	-	3	-	1	2	-	1	1	-	12
Total	5	4	-	3	8	-	4	6	3	3	6	3	45

V.F: Vesicular fluid. T.E: Tongue epithelium. C.T: Cardiac tissue

Table 2 Primers and probe sequences used in real time RT-PCR and conventional RT-PCR in this study.

Name	Sequence 5'-3'	Amplicon	Reference
3D – F	5'-ACT GGG TTT TAC AAA CCT GTG A -3'	107bp	(Callahan et al., 2002)
3D – R	5'-GCG AGT CCT GCC ACG GA -3'		(Callahan et al., 2002)
3D Probe	5'FAM-TCC TTT GCA CGC CGT GGG AC-TAMRA-3'		(Callahan et al., 2002)
Primers used for RT-PCR			
Forward primers			
A-Egy-F	GGAATCWGCAGACCCTGTC	750	(Shehata et al., 2016)
SAT2-Egy-F	TGAYCGCAGTACACAYGTYC	666	(Shehata et al., 2016)
O-Egy-F	CCTCCTTCAAYTACGGT	283	(Bachanek-Bankowska et al., 2016)
Reverse primer Nk61*	GACATGTCCTCCTGCATCTG		(Knowles et al., 2005)

The RT- PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide for nucleic acid staining. A 100 base-pair (bp) DNA ladder, manufactured by GeneDireX, was run alongside the products to confirm the expected size of the bands.

3. RESULTS

3.1. Molecular detection of FMDV in clinical samples by real time RT-PCR

All clinically suspected samples were subjected for screening by the real time RT-PCR, 32 (71%) out of 45 samples were positive for FMDV by one step real time RT-PCR. 11 from 15 samples collected from Qalubia, 7 from 10 samples collected from Sharkia, 7 of 8 samples collected from Gharbia and 7 of 12 samples collected from Behera governorate. The cut off was adjusted at cycle threshold (ct) value close to 32 according to Shaw et al. (2007) (Fig. 1).

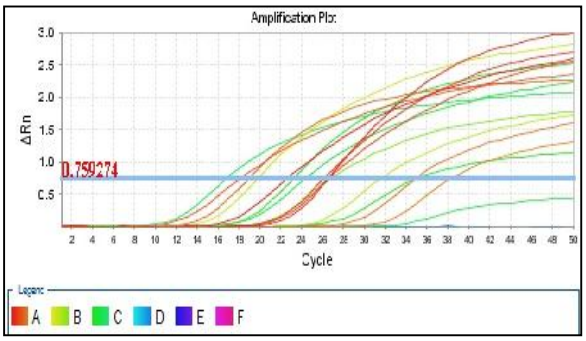

Figure 1 The amplification curve of the real time RT-PCR of collected samples from infected animals

3.2. Isolation and propagation of foot and mouth disease viruses on BHK-21 cells

Isolation of the FMDV from the positive samples by the real time RT-PCR (n= 32). 26 (75.7%) out of 32 samples showed positive results, 10 from 15 samples collected from Qalubia, 6 from 10 samples collected from Sharkia, 3 of 8 samples collected from Gharbia and 7 of 12 samples collected from Behera governorate. The characteristic cytopathogenic effect including cell rounding, degeneration and cytoplasmic granularity that followed by complete cell lysis 24-48 hrs post inoculation as shown in (Fig. 2 Panel B).

3.3. Serotyping by antigen detection ELISA:

Twenty-six of FMDV isolates were serotyped for A, O and SAT2 by antigen detection ELISA. SAT2 was the predominant serotype within 12 (46%) isolates followed by 7 (30%) for both serotype O and serotype A as shown in (Table 3).

3.4. Detection of the VP 1 gene from FMDV isolates by Polymerase Chain Reaction (RT-PCR):

RT-PCR (One-step Reverse Transcription) was performed using viral specific primers to detect the VP 1 gene from FMDV isolates. Consistent with the results obtained by antigen detection ELISA, 26 (81.2%) samples of 32 positive isolates were positive showing specific band size 283, 666, 750 bp for serotype O, SAT2 and A respectively (Fig. 3). As expected, there were no bands in the negative control samples as well as no cross reactivity was observed. Out of the total 26 positive samples by RT-PCR, 7 samples tested positive for serotypes A, 7 for serotype O, and 12 for Serotype SAT2 (Table 3).

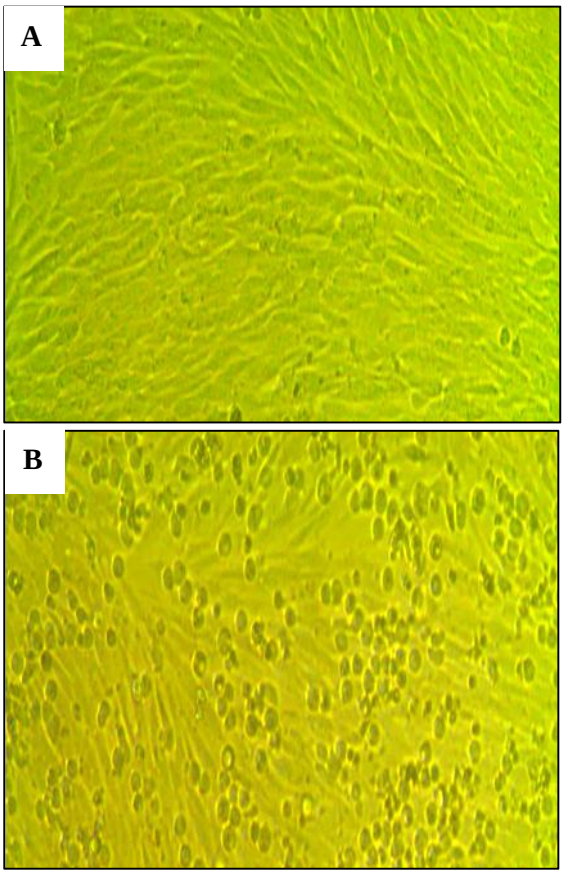

Figure 2 Representative pictures for the cytopathogenic effect of the FMDV on the inoculated BHK-21 cells. (Panel A) Normal cells and (Panel B) Cells showing CPE.

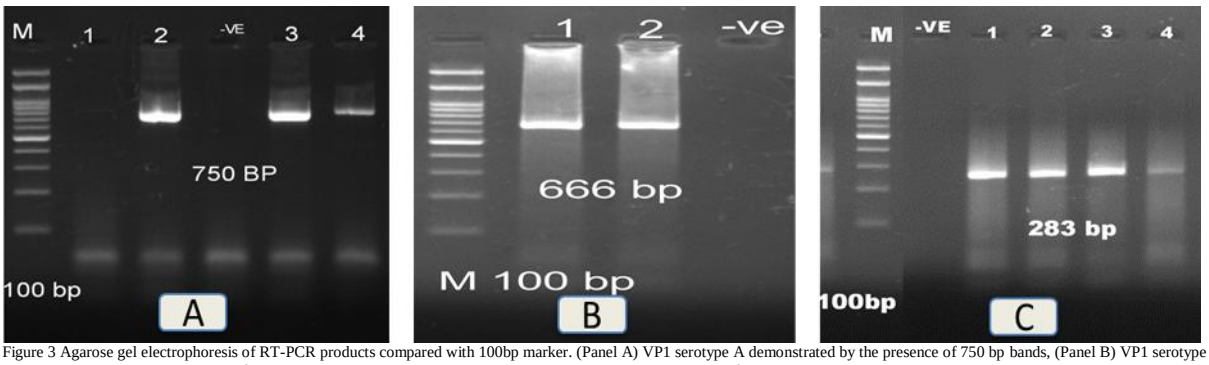

Figure 3 Agarose gel electrophoresis of RT-PCR products compared with 100bp marker. (Panel A) VP1 serotype A demonstrated by the presence of 750 bp bands, (Panel B) VP1 serotype SAT2 demonstrated by the presence of 666 bp band and (Panel C) VP1 serotype O demonstrated by the presence of 283 bp band.

Table 3 Prevalence of FMDV infection in clinical samples collected from four governorates in Egypt (2016-2019) as assayed by real time RT-PCR and isolation then serotyping by ELISA and confirmation with RT-PCR for the isolates.

GOV	Year	Sample	Real time RT-PCR	Isolation on BHK-21	ELISA	RT-PCR
Qalubia	2016	V.F	1/2	1/2	O	O
		T.E	1/1	1/1	A	A
	2017	V.F	1/1	0/1	-	-
		T.E	2/3	2/3	(2) SAT2	(2) SAT2
	2018	V.F	0/1	0/1	-	-
		T.E	1/2	1/2	SAT2	SAT2
		C.T	1/1	1/1	A	A
	2019	V.F	1/1	1/1	SAT2	SAT2
		T.E	1/2	1/2	O	O
		C.T	1/1	1/1	SAT2	SAT2
Sharkia	2016	V.F	1/1	1/1	A	A
		T.E	-	-	-	-
	2017	V.F	0/1	0/1	-	-
		T.E	1/1	1/1	SAT2	SAT2
	2018	V.F	1/1	1/1	SAT2	SAT2
		T.E	1/1	0/1	-	-
		C.T	1/1	1/1	SAT2	SAT2
	2019	V.F	-	-	-	-
		T.E	2/2	2/2	O + SAT2	O + SAT2
		C.T	1/2	1/2	SAT2	SAT2
Gharbia	2016	V.F	1/1	0/1	-	-
		T.E	-	-	-	-
	2017	V.F	1/1	0/1	-	-
		T.E	0/1	0/1	-	-
	2018	V.F	1/1	0/1	-	-
		T.E	1/1	0/1	-	-
		C.T	1/1	1/1	O	O
	2019	V.F	1/1	1/1	O	O
		T.E	1/1	1/1	SAT2	SAT2
Behera	2016	V.F	1/1	1/1	O	O
		T.E	2/3	2/3	(2) A	(2) A
	2017	V.F	-	-	-	-
		T.E	1/3	1/3	O	O
	2018	V.F	1/1	1/1	A	A
		T.E	1/2	1/2	SAT2	SAT2
	2019	V.F	0/1	0/1	-	-
		T.E	1/1	1/1	A	A
Total		45	32/45 (71.1%)	26/32 (81.2%)	12 (80.7%) SAT2, 7(26.9%) A, 7(26.9%) O	

V.F: Vesicular fluid. T.E: Tongue epithelium. C.T: Cardiac tissue

4. DISCUSSION

The real time RT-PCR is an extremely sensitive and specific tool for detection and identification of the FMDV serotypes in clinical samples (Kitching, 1992; King et al., 2006). The rRT-PCR has been used in this study for testing 45 clinical samples collected from different localities of some governorates in Egypt. The results revealed that 32 samples were positive in the represented governorates with different ratio. In addition, the sensitivity of rRT-PCR assay for detection of carrier cattle is 100% compared with virus isolation suggesting its suitability for screening FMDV carrier animals as reported by Zhang and Alexandersen (2003), who also stated that cell culture technique detects virus infection while rRT-PCR detects a small segment of virus RNA. In the present study, BHK-21 cell line succeeded to support the growth of FMDV from 26 out of 32 positive FMDV samples with the characteristic CPE. It is clear that six samples obtained from the four governorates detected by rRT-PCR and gave negative results using viral isolation.

Real-time RT-PCR is more sensitive and reliable test for detection of FMDV in vesicular fluid, tongue epithelium and heart tissue than viral isolation on BHK-21. These results came consistent with results obtained by Knowles and Davies (2002), Dukes et al. (2006), and Mohopatra et al. (2007). These results may be due to viral isolation is dependent up on presence of the complete infectious FMDV in the clinical samples while the rRT-PCR has the ability to detect both infectious and non-infectious virus as concluded by Shaw et al. (2004) and Mandour et al. (2014). The rRT-PCR generated results in less than one day in contrast to up to four days to define some positive and all negative samples by use of virus isolation. Antigen detection ELISA is the preferred procedure for FMDV serotyping, as stated by Ferris and Donaldson (1992) and Marquardt and Freiberg (2000), Our study revealed the co-circulation of serotypes A, O and SAT2 in Egypt during 2016- 2019. FMDV serotype SAT2 was the predominant circulating serotype in the field, followed by serotype O and serotype A. This was nearly in agreement with those reported by Mohamed et al. (2013) and El-Bagoury et al. (2018), who pointed out that the outbreak of FMD occurred

with new serotype SAT2 (75%) of tested samples started in February 2012. VP1 has a significant role in the host humoral immune response because it is considered as a major immunodominant neutralizing epitope (Sobrino et al., 2011). In this study, the VP1 gene was chosen for amplification by conventional RT-PCR because of its significance in the genetic characterization of FMDV strains and for confirmation of typing the FMDV isolates. The results came consistent 100% with the ELISA serotyping.

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5. CONCULSION

It is evident from the obtained results that the rRT-PCR is highly sensitive and specific tool for detection and identification of the FMDV serotypes in clinical samples. SAT2 serotype was the predominant circulating serotype in the field, followed by O and A. further molecular analyses are recommended to confirm these findings to determine the molecular epidemiology of the isolates and to update the nature of future vaccine strains for successful preventive strategies.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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