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RESEARCH

Molecular characterization of foot and mouth disease virus (O-EA3) isolated during 2016 outbreak in Egypt

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ABSTRACT

Background: In January 2016, several cases of clinical signs and mortalities suggested foot and mouth disease (FMD) outbreaks among cattle and buffalo herds in Egypt. This study aimed to investigate the contribution of Foot and Mouth Disease Virus (FMDV) and characterization of the circulating strains associated with the outbreaks.

Methods: The collected samples of Oropharyngeal, oral epithelial tissue and/or vesicular fluids were tested by real time Reverse transcriptase polymerase chain reaction (rRT-PCR) for detection of FMDV. The highly viral loaded FMDV positive samples were typed by conventional reverse transcriptase polymerase chain reaction (RT-PCR). Sequencing and phylogenetic analysis of 6 representative samples were performed.

Results: Testing of 80 collected samples of Oropharyngeal, oral epithelial tissue and/or vesicular fluids by real time Reverse transcriptase polymerase chain reaction (rRT-PCR) revealed 65 out of 80 FMDV positive samples (81%).Typing of the highly viral loaded FMDV positive samples (40 out of 65) by conventional reverse transcriptase polymerase chain reaction (RT-PCR) revealed that all tested samples belonged to serotype O of FMDV. Sequencing and phylogenetic analysis of 6 representative samples clustered the detected viruses with O topotype East Africa-3 (EA-3) viruses.

Conclusion: The newly identified 2016 viruses clustered in distinct clade in the phylogenetic tree other than the serotype O viruses isolated in previous outbreaks in Egypt, indicating the likelihood of new incursions into Egypt. These strains were closely related to previously characterized strains circulating in Sudan and sub-Saharan Africa suggesting continued circulation of EA-3 topotype of FMDV in the region.

Key words:
Foot and Mouth Disease Virus (FMDV), Serotype O, Topotype East Africa-3(EA-3), PCR, Egypt.

BACKGROUND

Foot-and-mouth disease virus (FMDV) is one of the most economically important viruses to the livestock industry. Foot and mouth disease (FMD) is a contagious disease results in serious production losses and is a major constraint to international trade in livestock and its products. FMDV is a single-stranded RNA virus which affects cloven-hoofed animals including cattle, pigs, sheep, goats and buffalo. FMDV is a member of the genus *Aphthovirus* in the family *Picornaviridae* and exists in seven serologically distinct serotypes: O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1(Bachrach, 1968, Carrillo et al., 2005, Coetzer et al., 1994). The serotypes are further delineated into clades (topotyes) based on the sequence of the viral structural protein 1 gene (VP1), which reflects genetic, antigenic and geographical relationships among the strains. Serotype O can be grouped into 10 topotypes named Euro-South-America (Euro-SA),Middle East-South Asia (ME-SA), South East Asia(SEA), Cathay (Chy), West Africa(WA), East Africa 1(EA-1), East Africa 2(EA-2), East Africa 3(EA-3), Indonesia-1(ISA-1) and Indonesia-2 (ISA-2) (Knowles and Samuel, 2003).

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In Egypt, The history of FMDV goes back to 1950, when an outbreak caused by serotype SAT2 was reported. Between 1964 and 2005, only serotype O was reported in Egypt, with the exception of 1972 when type A was introduced from Sub-Saharan Africa (Knowles et al., 2007). Series of outbreaks predominantly caused by serotype O, and with a dramatic upsurge in FMD SAT 2 outbreaks during 2012 were reported (Ahmed et al., 2012). Serotypes O, A and SAT2 have been circulating in the country since 2013, and Serotype O is considered the predominant serotype (http://www.wrlfmd.org/fmd_genotyping/africa/egy.htm).

It has been shown that VP1 is the most variable protein of the structural capsid polypeptides thus, nucleotide sequencing of part or the entire genome region coding for VP1 is mainly used to study the epidemiology of FMD (Bittle et al., 1982, Beck and Strohmaier, 1987).

In Egypt, the importation of live animals is an increasing demand to face the continued shortfall in local supply of meat. The main suppliers for livestock to Egypt are mainly Sudan, Ethiopia, Uruguay, Ukraine, Australia and Hungary(Hamza, 2015).

In the beginning of 2016, outbreaks of foot and mouth disease had appeared among buffalo and cattle herds which demonstrated FMD clinical signs and mortalities.

In this study, we investigated the presence of FMDV and genetically characterized the circulating strains associated with the observed outbreaks.

MATERIALS AND METHODS

Sampling:

Samples used in this study were collected from eighty (80) animals (40 buffalos in Giza and 40 cattle in Beheira governorates) reared in farms that were suspected to be infected with FMDV in Egypt during an outbreak in January, 2016.

For clinically diseased animals, the samples were collected from epithelial tissue and/or vesicular fluid from recently ruptured or un-ruptured vesicles which were collected from tongue, buccal mucosa or feet. For healthy animals, the samples of Oropharyngeal (OP) fluid were collected by means of a probang (sputum) cup. The collected samples were placed in a transportation medium which is a mixture of equal amounts of sterile glycerol and phosphate-buffered saline (PBS) pH 7.2-7.4 with antibiotics and processed according to (Kitching and Donaldson, 1987) and OIE recommendations (Manual, 2012). Samples were kept on ice till reached to the lab and then stored in -80°C freezer till work.

Detection and characterization of circulating FMDV:

Extraction of RNA:

Total RNA was extracted from all collected samples using total RNA Purification Kit (Jena Bioscience, Germany) according to the manufacturer's instructions. The RNA was eluted in 50 ul of elution buffer provided with the kits and stored at -20°C.

Detection of FMDV by Real Time RT-PCR:

Amplification of 3D gene of FMDV by Real Time PCR (q PCR) described by (Callahan et al., 2002) was carried out using QuantiNova Probe RT-PCR Kit (Qiagen, GmbH, Germany) and the universal probe and primers as shown :

Forward primer: 5'-ACTGGGTTTTACAAACCTGTGA-3'
Reverse primer: 5'-GCGAGTCCTGCCACGGA-3'
TaqMan probe: 5'-FAM-TCCTTTGCACGCCGTGGGAC-TAMRA-3'

The thermal profile was adjusted according to the manufacturer's instructions of QuantiNova Probe RT-PCR Kit) (table 1). Cycle threshold (CT) for each sample was then determined according to (Reid *et al.*, 2001).

Table 1: Standard thermo cycling protocol used for qPCR amplification of the 3D gene of FMDV:

Step	**Time**
Temperature	
RT-step	10 min
45°C	
PCR initial heat activation	5 min
95°C	
2-step cycling	
Denaturation	5 sec
95°C	
Combined	30 sec
60°C	
annealing/extension	
Number of cycles	40

Typing of detected FMDV by conventional RT-PCR:

Forty samples of sixty five positive samples detected by real time RT-PCR (Ct values ≤ 25) were further tested for serotyping by reverse-transcription polymerase chain reaction (RT-PCR) using a panel of 8 primer sets described by (Knowles et al., 2016) (**Table 2**) and ONE-STEP RT-PCR Pre Mix Kit (Intron Biotechnology, Inc., Korea,Cat.#25101). A thermo cycler (Bio-Rad, USA) was used and the relevant PCR cycling program was selected (according to the manufacturer's instructions of working kit **(Table 3)**. The PCR products were analyzed by electrophoresis on a stained ethedium bromide 1.5% Agarose-Tris-borate-EDTA (TBE) gel. DNA size markers (GeneRuler 100 bp DNA Ladder Plus, Thermo Scientific, Germany) were run alongside the samples to confirm the correct size of the products according to (Knowles and Samuel, 1998).

Table 2: List of oligonucleotide primers used for RT-PCR and Sequencing of FMDV

Serotype	**Name**	**Sequence (5′ – 3′)**	**Gene**	**Product size**
O (Forward)	O–1C244F	**GCAGCAAAACACATGTCAAACACCTT**	VP3	1165
	O–1C283F	**GCCCAGTACTACACACAGTACAG**	VP3	1124
A (Forward)	A–1C562F	**TACCAAATTACACACGGGAA**	VP3	866
	A–1C612F	**TAGCGCCGGCAAAGACTTTGA**	VP3	814
O/A (Reverse)	EUR–2B52R	**GACATGTCCTCCTGCATCTGGTTGAT**	2B	
SAT2 (Forward)	SAT2–445F	**TGGGACACMGGIYTGAACTC**	VP3	1145
	SAT2–1223F	**TGAACTACCACTTCATGTACACAG**	VP3	1279
SAT2 (Reverse)	SAT2–2B208R	**ACAGCGGCCATGCACGACAG**	2B	
All serotypes (Sequence)	NK72	**GAAGGGCCCAGGGTTGGACTC**	2A/2B	

Table 3: Thermo cycling protocols used for RT-PCR amplification of the VP1 and other adjacent regions of FMDV

Step	Temperature	Time	Number of cycles
1. Reverse transcription	45°C	30 min	1
2. Inactivation	94°C	5 min	1
3. Denaturation	94°C	30 sec	Repeat steps
4.Primer annealing	50–55 – 60°C*	60 sec	3 to 5
5. Extension	72°C	60 sec	35 times
6. Final extension	72°C	5 min	1

*Primer annealing temperatures for the different FMDV serotypes are as follows:
Serotype O at 60°C; serotypes A at 55°C and serotype SAT 2 at 50°C.

Sequencing of 1D (VP1) gene of FMD Virus:

The PCR products of representative six detected FMDV samples were subjected to Macrogen Incorporation (Seoul, Korea) for purification and sequencing. For sequencing, we used O-1c 244 as forward primer which designed to anneal within the VP3 coding region while the reverse primer was the universal primer NK72 which annealing the 2B coding region and thus, the full length of the FMDV VP1 coding region could be amplified (Table 2).

Phylogenetic analysis:

The obtained VP1 nucleotide sequences were compared to previously published strains in GenBank using BLAST. In addition, representative nucleotide sequences of FMDV topotypes within the identified FMDV serotype were obtained from GenBank and aligned with the sequences in current study using BioEdit v7.1.3 (Hall, 1999), which uses CLUSTAL W 1.83 method (Thompson et al., 1994). Midpoint-rooted Neighbor-joining phylogenetic trees were constructed and visualized using MEGA 6.06 software (Tamura et al., 2013). The trees were assessed using 1000 bootstrap replicates.

RESULTS

FMDV detection, serotyping and genetic characterization:

For FMDV detection, Real time RT-PCR (using universal primers and probe of FMDV) was performed on all 80 collected samples. Samples with Ct values ≤ 25 which indicate high viral genomic load were considered FMD-positive. Only sixty five (65) samples showed positive results (Fig.1).

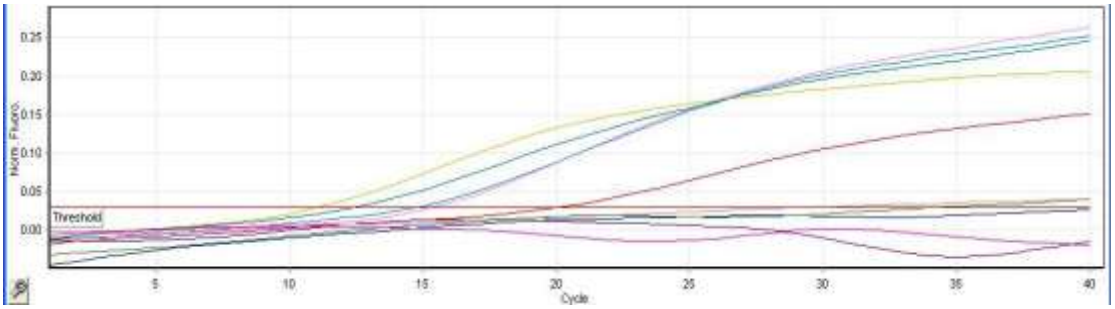

Figure 1: Illustrates some Representative results obtained from real time RT-PCR.
*FMDV Positive (above the threshold line) and negative (below the threshold line) samples.

For serotyping of the FMDV positive detected samples, conventional RT-PCR using serotype-specific primers was performed on selected 40 samples. All the amplified positive samples revealed O serotype with the used forward primer O-1c 244 which was demonstrated by the presence of 1100-bp band (Fig.2).

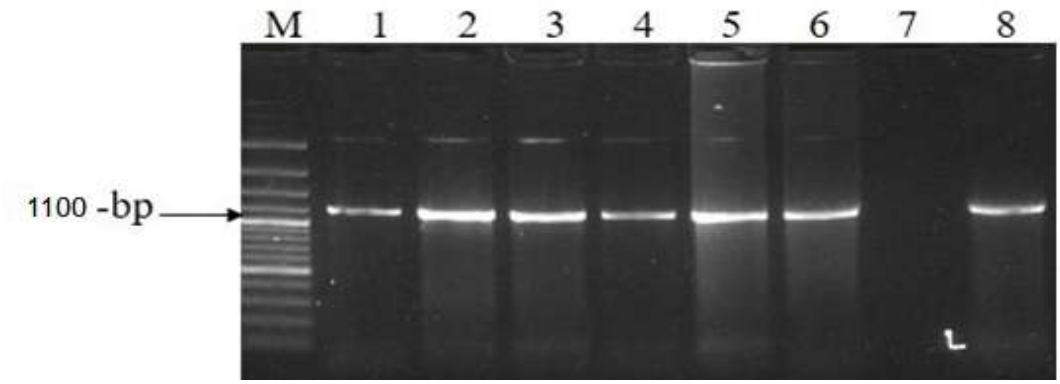

Figure 2: Agarose gel (1%) electrophoresis of RT-PCR products of FMDV VP1 and adjacent regions. **M**: 100 Plus bp ladder. **Lane** 7: control negative sample. **Lanes 1**-6 and 8: 1100bp PCR product of targeted fragment.

The sequence results of the selected six FMDV samples in this study (VP1 and adjacent coding regions) were compared with other published sequences (GenBank and http://www.wrlfmd.org/fmd_genotyping/prototypes.htm) which represent a list of reference sequences for FMDV serotype O. The constructed phylogenetic tree placed the newly detected strains in this study with the East Africa 3 (EA-3) topotype (Fig. 3). The obtained sequences were submitted to GenBank and the accession numbers are shown in (Table 4).

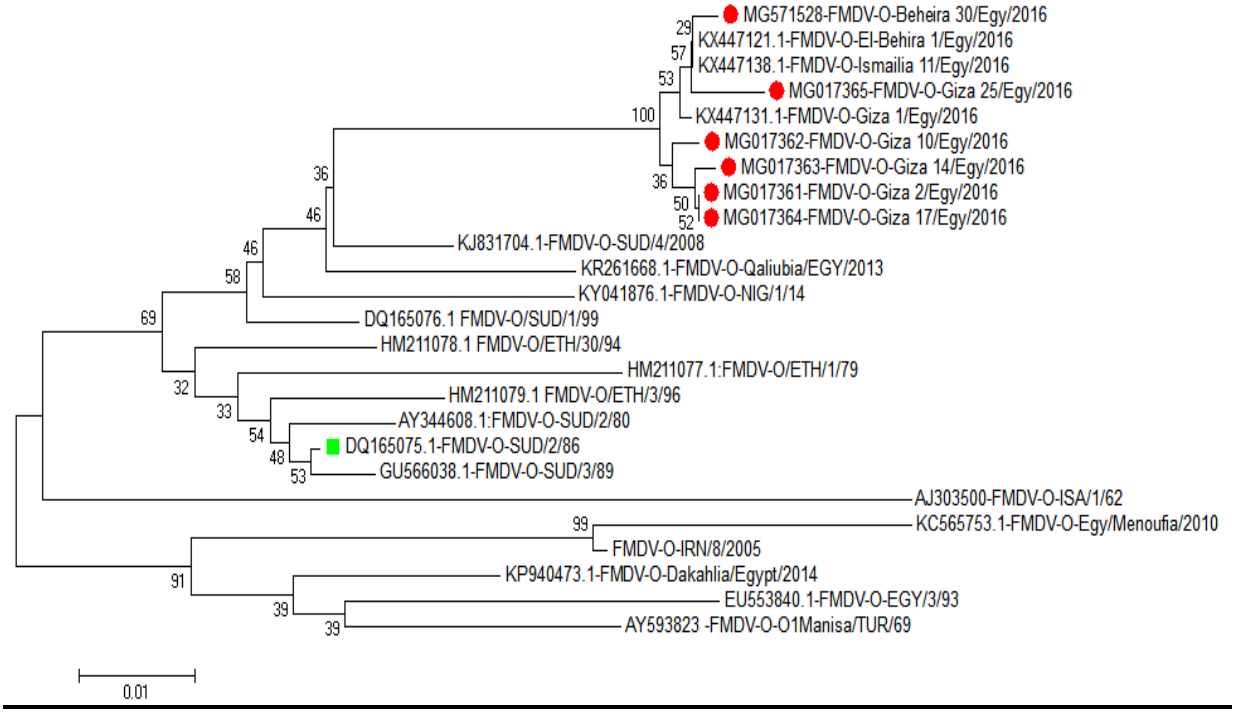

Figure 3: Phylogenetic analysis of the detected viruses compared with other FMD Viruses serotype O

- Blotted branches in the figure indicate the detected viruses in this study.

Table 4: Details of samples and strains characterization

Animal species	Location (province)	Strain characterization	Accession numbers
Buffalo	Giza	Serotype O topotype EA-3	MG017361
Buffalo	Giza	Serotype O topotype EA-3	MG017362
Buffalo	Giza	Serotype O topotype EA-3	MG017363
Buffalo	Giza	Serotype O topotype EA-3	MG017364
Buffalo	Giza	Serotype O topotype EA-3	MG017365
Cattle	Beheira	Serotype O topotype EA-3	MG571528

DISCUSSION

Foot-and-mouth disease virus has been known as one of the most economically important viral pathogens of animals, which is highly contagious among 70 species of cloven hoofed mammals (Bachrach, 1968).

Despite the presence of a locally developed trivalent vaccine against serotypes A, O and SAT2, annual outbreaks are reported and usually associated with animal movement throughout the country that increases the spread of FMDV originating from clinically infected or carrier animals imported from abroad (Rweyemamu et al., 2008, Nampanya et al., 2013). Early and accurate diagnosis of FMDV represents an essential tool for the control of the disease (Samuel and Knowles, 2001).

In the present study, we investigated the circulating FMD virus during an outbreak at the beginning of 2016 in Egypt to determine whether this outbreak occurred due to the infection with FMDV types reported in the previous outbreaks in Egypt or due to a new incursion of FMD virus.

The results showed that the detected FMD viruses were belonging to serotype O. The obtained specific PCR bands which include viral VP1 and adjacent regions were sequenced for genetic characterization of the circulating FMD virus. Comparison of the obtained VP1 and partial nucleotide sequences of serotype O viruses with those of other related viral sequences obtained by blast of nucleotide sequence in gene bank revealed that the detected viruses in this study were closely related to (FMDV - type O isolate Giza 1/Egypt/2016 VP1 gene, partial cds with identity 99%, FMDV - type O isolate Ismailia 11/Egypt/2016 VP1 gene, partial cds with identity 98% and FMDV - type O isolate O/SUD/4/2008 capsid protein gene, partial cds with identity 93%) which are all included in topotype East Africa-3 (EA-3) that differs from the previous topotype Middle East-South Africa (ME-SA) with lineage Panasia2 (O Panasia2) which was prevalent in Egypt from 2010 to 2012(Rady et al., 2014).

The detected strain mainly circulates in sub-Saharan Africa; however, these topotypes were previously detected in Egypt in 2012 (FAO, 2012). Detection of this topotype in this study confirms the previously published reports of widespread circulation of serotype O topotype EA-3 in the eastern African countries (Ayelet et al., 2009, Habiela et al., 2010) and suggests the continued circulation of these topotypes in the region.

The unique location of Egypt between different continents increases its vulnerability to incursion of the FMDV strains that circulate in both African and Asian countries. Moreover, previous studies highlighted the threat of incursion of FMDV strains predominate in sub-Saharan Africa to North Africa (Knowles et al., 2007).

In this study, serotype O topotype EA-3 was detected; this result is in accordance with the previously published reports of the world reference laboratory of FMDV (Wrlfmd) describing

the predominant circulation of serotype O topotype EA-3 strains in Egypt since its introduction in 2012. No other serotypes were characterized in this study and this might be attributed to the limited number of investigated outbreaks. However, OIE and wrlfmd reported circulation of SAT2 and A serotypes in other outbreaks in Egypt during 2016 (FAO, 2012).

Although serotypes O and A of FMDV are endemic in Egypt, the detected strain in current study during 2016 outbreak clustered independently from the previously identified viruses. It must be considered that not all the sequences from topotypes identified in Egypt have been deposited in GenBank. The percentages of identity with the other available strains in those topotypes suggest that strains circulating in Sudan are the most closely related ones.

Importation of livestock from Sudan and sub-Saharan countries creates a warning alarm for the continuous introduction of trans boundary animal diseases, in particular FMDV (Kandeil et al., 2013). Despite the exerted efforts by the Egyptian government to control FMD, the shortage in proper quarantining facilities, boarder-based slaughter houses and the breakdown in security forces, puts the country at high-risk of continuous disease introduction. Such pressing conditions may potentially hinder the ability of the vulnerable local market to expand and impact success in FMD control and eradication.

CONCOLUSION:

This study confirmed the characterization of FMDV topotype O EA-3 associated with outbreaks in both buffalo and cattle populations in Egypt during 2016.

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