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RESEARCH

Circulation of FMD serotypes SAT2 (VII-Ghb-12), O (EA-3) and A (G-IV) in cattle population in Egypt, 2018

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ABSTRACT

Background: Foot and mouth disease virus (FMDV) is the causative agent of multiple outbreaks and severe economic losses in the cattle population since its introduction in Egypt. Although vaccination is practiced to control the disease, it is still endemic. Moreover, emergence of new strains is possible through the international borders of the endemic countries. Consequently, there is a dire need for routine molecular characterization of the circulating FMDV strains to ensure the rapid detection of any new or mutant strains and update the used vaccine accordingly.

Methods: Twelve epithelial samples were collected from cattle showing signs suspecting of FMD. The samples were tested by real time RT-PCR using universal primers for detection of all seven serotypes of FMDV. The positive-resulted samples were then tested using conventional PCR and pan FMD primers. The same samples were tested again using serotype-specific primers to amplify the VP1 coding region and the products were sequenced for phylogenetic analysis.

Results: FMDV RNA was detected in eight out of the 12 samples tested using real time RT-PCR. Conventional PCR revealed that Six of the eight samples were positive using pan FMD primers. However, using serotype-specific primers; only four samples tested positive; two samples for serotype O, one sample for serotype A and one sample for serotype SAT2 while two samples remained untyped. VP1 coding region sequencing and phylogenetic analysis showed that serotype O strain of this study belonged to EA-3 lineage while serotype A clustered with G-IV African strains and serotype SAT2 virus was related to the previously-reported Egyptian strains of Ghb-12 lineage of G-VII.

Conclusion: This study reports the co-circulation of serotypes O, A and SAT2 FMDVs in Egypt indicating the breach in the prevention and control measures. It also proves the competence of real time RT-PCR for the rapid and sensitive diagnosis of FMD in epithelial samples.

Keywords: Foot and Mouth Disease, RT-PCR, Molecular epidemiology, Phylogenetic analysis.

BACKGROUND

Foot and mouth disease (FMD) is a contagious and debilitating viral disease of cloven hoofed animals including cattle, buffalo, pig, sheep, goat and more than 70 species of wild animals. FMD endemic countries suffer from loss of productivity in the adult and high mortality in the young animals (James and Rushton, 2002).

Foot and mouth disease virus (FMDV) is non-enveloped, icosahedral in shape and about 30nm in diameter. It belongs to genus Aphthovirus of family Picornaviridae and consists of a genome enclosed within a protein capsid. The capsid consists of 60 copies of capsomers, each capsomer consist of four structural proteins; VP1, VP2, VP3 and VP4. VP1, VP2 and VP3 are exposed externally on the virus surface while VP4 is located internally (Fry *et al.*, 2005). The genome is a single strand positive-sense RNA which is approximately 8500 nucleotides in length and encodes both structural and non-structural proteins (Carrillo, 2012).

FMD is always referred to as a single disease, however, the causative agent (FMDV) presents in seven separate serotypes (A, O, C, Asia1, South African Territories (SAT) 1, 2 and 3). All seven serotypes cause indistinguishable clinical signs but they have different geographical distribution (Domingo *et al.*, 2002). Serotype O and A are widely distributed globally and occur in Africa, Asia and South America (Knowles and Samuel, 2003). Serotype Asia1 is restricted in

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Asia and the SAT serotypes are reported only in Africa. Serotype C has not been reported since the 2004 outbreak in Kenya and has probably disappeared (Brito *et al.*, 2017).

The high genetic and consequently antigenic variability of FMD viruses within each serotype further divide them into "topotypes". This term refers to FMDV strains with a limited genetic diversity that can be grouped into distinct clusters and have defined geographical regions (Tekleghiorghis *et al.*, 2016).

Egypt has been FMD endemic since 1950 when serotype SAT2 was first introduced. Serotype O was reported in the following years and caused several outbreaks in 1987, 1989, 1990, 1991 and 1993. Serotype A was responsible for an outbreak in 1972 and another in 2006 when it was introduced through the infected animals imported from Ethiopia and caused up to 80% mortality in newly born calves. In 2012, a northern expansion of serotype SAT2 from sub-Saharan Africa caused the economic loss of more than 4600 animals. Since then, the three serotypes O, A and SAT2 have been circulating in Egypt (Bazid *et al.*, 2014; Sobhy *et al*., 2014; Ahmed *et al*., 2016; Soltan *et al*., 2017). Egypt relies on quarantine and mass vaccination in the control program of FMD. Mandatory vaccination is performed using a locally produced trivalent vaccine (Aidaros, 2002).

This work was carried out for the molecular characterization of the FMDV strains responsible for the 2018 cases in Egypt.

MATERIALS AND METHODS

Clinical Samples

Epithelial samples were collected from 12 cattle showing signs suspecting of FMD in different Egyptian governorates (Beheira, Beni-Suef, Faiyum, Gharbia, Giza, Dakhlia, Qalyubia and Suez) during January and February, 2018. The samples were placed in a transport medium prepared according to OIE (2017), transported on ice to the laboratory and kept at -70ºC till testing.

RNA extraction

Total RNA was extracted from the samples using Patho Gene-Spin^{TM} DNA/RNA Extraction Kit (iNtRON Biotechnology, Korea) according to the manufacturer`s protocol and resuspended in 30µl of the kit elution buffer. Total RNA was also extracted from the epithelial tissue of a non-infected cattle and used as a negative control. The RNA was preserved at -20ºC till use.

Real time reverse transcription polymerase chain reaction (rRT-PCR)

Detection of FMDV RNA was done using the universal primers and probes (Callahan *et al*., 2002) listed in table I and QuantiNov Probe RT-PCR Kit (QIAGEN, Germany). Each reaction was performed in a final volume of 10µl: 5µl 2x QuantiNova Probe RT-PCR Master Mix, 0.5µl QN ROX Reference Dye, 1µl forward primer, 1µl reverse primer, 0.5µl probe, 0.3µl QN Probe RT-Mix, 0.3µl RNase/DNase free water and 2µl of template RNA. The real-time PCR machine (StepOne, Applied Biosystem, USA) was adjusted to the following cycling conditions; 45ºC for 10 min for RNA reverse transcription, 95ºC for 5 min for enzyme activation followed by 40 cycles of: 95ºC for 5 sec for denaturation and 60ºC for 60 sec for annealing/extension and the fluorogenic data collection.

Table (I): Universal primers and probe used in rRT-PCR.

FMD Serotype	Primer ID	Sequence 5'- 3'	Location
Common	Callahan 3DF	ACTGGGTTTTACAAACCTGTGA	3D gene
	Callahan 3DR	GCGAGTCCTGCCACGGA	
	Callahan 3DP probe	FAM-TCCTTTGCACGCCGTGGGAC-TAMRA	

Reverse transcription polymerase chain reaction (RT-PCR)

The positive-resulted samples by rRT-PCR were retested using the 1F/1R primer set for detection of all seven serotypes of FMD virus and Thermo Scientific Verso 1-Step RT-PCR Reddy Mix Kit. Serotype differentiation was done using VP1 gene-specific primers (Knowles *et al.,* 2016) (Table II). The cycling conditions were as listed in table III.

Table (II): Common and serotype-specific primers used in RT-PCR.

FMD serotype	Primer ID	Sequence 5'- 3'	Location	Amplified product
Common	1F	GCCTGGTCTTTCCAGGTCT	5'UTR	328 bp
	1R	CCAGTCCCCTTCTCAGATC	5'UTR	
O	O-1C244F	GCAGCAAAACACATGTCAAACACCTT	VP3	1165 bp
	EUR-2B52R	GACATGTCCTCCTGCATCTGGTTGAT	2B	
A	A-1C612F	TAGCGCCGGCAAAGACTTTGA	VP3	814 bp
	EUR-2B52R	GACATGTCCTCCTGCATCTGGTTGAT	2B	
SAT2	1D209F	CCACATACTACTTTTGTGACCTGGA	1D	715-730 bp
	2B208R	ACAGCGGCCATGCACGACAG	2B	

Table (III): Thermal conditions of RT-PCR.

Phase Step	Time	Temp	No. of cycles
Reverse transcription	30 min	50°C	1
Verso inactivation	15 min	95°C	1
Denaturation	60 sec	95°C	35
Annealing	30 sec	55-58-60°C*	
Extension	120 sec	72°C	
Final extension	5 min	72°C	1

*55ºC for common and serotype A FMD primer sets, 58ºC for serotype SAT2 primer set and 60ºC for serotype O primer set.

VP1 sequencing

Thermo Scientific GeneJET Gel Extraction Kit was used for purification of the PCR products according to the manufacturer's protocol. Sequencing of the PCR product was done using BigDye™ Terminator V3.1 Cycle Sequencing Kit and the same forward and reverse primers used in RT-PCR.

Phylogenetic analysis

The obtained VP1 sequences were compared with other FMDV sequences available in GenBank. The sequences were aligned using CLUSTAL W 1.4 tool (Thompson *et al.,* 1994) in BioEdit (Hall, 1999). Phylogenetic trees were constructed using the Neighbor-joining (ML) method in MEGA 7 software and 1,000 bootstrap replicates were performed to estimate the reliability of each tree branch (Kumar *et al.,* 2016).

RESULTS:

FMD virus detection by rRT-PCR

Out of 12 samples tested by real time RT-PCR; eight samples were positive using universal FMD primers and probe. The cycle thresholds were as shown in figure 1.

FMD virus detection by conventional RT-PCR

RT-PCR was able to detect FMDV in six out of the tested eight samples. The six samples gave the expected band size (328 bp) as shown in figure 2.

These six samples when tested with serotype-specific primers gave positive results for serotype A (2 samples) serotype O (1 sample) and serotype SAT2 (1 sample). However, two samples remained untyped giving negative result with the three primer sets (Figure 3).

Phylogenetic analysis:

Phylogenetic trees were constructed based on the nucleotide sequence alignment of the VP1 gene of this study strains together with other contemporary Egyptian isolates and reference prototypes.

Serotype O strain O/Egy/Dakhlia/2018 had an identity percent of 99% with the Egyptian strains of 2017 and 2016; O/Fayoum1/Egy/2017 (MF322688.1) and O/Alexandria/Egy/2016 (MF322680.1). However, it showed lower percent of identity with other Egyptian strains of 2015; O/18/Egy/2015 (KU217459.1) and with Sudan's 2008 strain; O/SUD/3/2008 (KR149728.1) which was 95% and 91%; respectively. The constructed phylogenetic tree placed this study strain with EA-3 prototypes (Fig.4).

The two serotype A strains in this study were designated A/Egy/Giza/2018 and A/Egy/Qalyubia/2018 and showed 99% relatedness to each other. They showed a homology of 97% to other serotype A Egyptian strains; A/ Beni-suef1/Egy/2017 (MF322693.1) and A/ Giza 1/Egy/2016 (KX447000.1). The constructed phylogenetic tree showed that they grouped with the African strains of genotype IV (Fig.5).

The nucleotide sequence analysis of the studied SAT2 strain; SAT2/Egy/Suez/2018 revealed that it was related to the Egyptian strains of 2012 and 2015; SAT2/cow/Qaliubia/2015 (KU897093.1) and SAT2/EGY/3/2012 (JX570618.1) with an identity percent of 98% and 99%; respectively. It also showed a homology of 98% to the SAT2 strain of Gaza; SAT2/PAT/1/2012 (JX014256.1). Phylogenetic analysis clustered the SAT2 virus with other related Egyptian strains and reference prototypes, all belonging to Ghb-12 lineage of genotype VII (Fig.6).

DISCUSSION

Molecular assays have been developed to complement the immunological diagnostic methods and overcome the limitation of FMD virus isolation and antigen detection (Reid *et al.*, 2000). For instance, virus isolation (VI) needs the presence of infectious virus which can't be detected within the samples of poor quality due to inappropriate storage or transportation (Alexandersen *et al.,* 2003). Also, antigen detection ELISA needs the presence of a certain concentration of virus or antigen (1-2 ng/ml) to be detected (Shaw *et al.,* 2004).

Real-Time Polymerase Chain Reaction (rRT-PCR) is currently considered the gold standard assay for nucleic acid detection. It has enabled real-time quantification and can detect as few as 10–100 RNA per volume tested. It decreases the risk of cross-contamination and hence superseded analysis by gel-electrophoresis (Longjam *et al.*, 2011). Moreover, it generates the results within two hours or even less and has a higher diagnostic sensitivity than that of VI/ag ELISA combined (Shaw *et al.*, 2004).

Vesicular epithelium is the sample of choice for FMD diagnosis as it has a high virus titer during the acute stage of the disease (Ferris *et al.*, 2006). Therefore, in this study, 12 epithelial samples were colloected from FMD-suspected cases and tested using rRT-PCR and universal primers for detection of all seven FMDV serotypes (Callahan *et al.*, 2002). Out of the 12 tested samples, eight samples (66.7%) tested positive with CT values ranging between 4 and 28. This result is in accordance with Longjam *et al.*, (2011) who demonstrated that real time PCR was able to detect FMDV in 65.47% of the tested samples.

Conventional RT-PCR is a reliable and easily performed diagnostic method. However, it is not sensitive enough to replace ELISA and virus isolation for the primary diagnosis of FMD but could be used in parallel with them (Longjam *et al.*, 2011).

Reid *et al.*, (2000) stated that the prime pair; 1F/1R was able to detect FMDVs of the seven serotypes although it detected the SAT1/2/3 serotypes FMDVs less effeciently. In this study, six out of eight samples were positive using this same set of primer and gave the expected band size of 328 bp.

RT-PCR amplification followed by nucleotide sequence analysis is useful for rapid characterisation of field starins and tracing the origin of outbreaks (Sobrino *et al.*, 2001). Given that the VP1 coding region varies according to FMDV serotype and lineage and contains major antigenic sites of the virus capsid, phylogenetic analysis of the VP1 coding sequences are routinely used to characterize field viruses for epidemiological purposes (Bazid *et al.*, 2014).

Serotype-specific primers were used for VP1 region amplification (Knowles *et al.*, 2016). Gel electrophoresis of the PCR products confirmed the specifity of the used primer sets as no non-spesific products were detected. Two samples remained untyped revealing negative results with the three primer sets which implies that more than one primer set should be used for each FMD serotype to overcome any probable substitutions in the annealing sequence (Callens and De Clercq, 1997).

Serotype O strain of this study grouped with the prototypes of EA-3 and showed close identity with the recent Egyptian strains reported in 2016 and 2017. This confirms the previously published literature (Soltan *et al*., 2017; Khodary *et al*., 2018) and indicates the continuous circulation of this genotype in Egypt since its introduction in 2012 (Rady *et al*., 2014).

The two studied serotype A strains were closely related to each other and both clustered with G-IV serotype A strains. This genotype was responsible for FMD outbreaks in February, 2012 and originated from sub-Saharan Africa (Tekleghiorghis *et al*., 2016). The studies of Khattab (2012), Sobhy *et al.*, (2014) and Attia *et al.*, (2017), however, reported serotype A strains of Iran-05 lineage and this suggests the co-circulation of both African and Asian type A strains.

Serotype SAT2 has high genetic diversity with fourteen documented genotypes (Bastos *et al.*, 2003). Genotype VII was the only one reported in Egypt and appeared in two different lineage showing 10% divergence in their nucleotide sequence namely; SAT2/VII/Ghb-12 and SAT2/VII/Alx-12 (Ahmed *et al.*, 2012). SAT2 viruses of both lineages were isolated several times from different Egyptian governorates (Sobhy *et al.*, 2014; Ahmed *et al.*, 2016; Attia *et al.*, 2017). The SAT2 strain of this study were found to be belonging to lineage SAT2/VII/Ghb-12 of G-VII confirming the reported data.

The relatedness of the studied strains in this work to Sudanese strains is consistent with the fact that Egypt depends on animal importation from Sudan and the sub-Saharan countries to compensate the shortage in meat production (Kandeil *et al.*, 2013) and implicates the inefficient quarantine measures in the setback of the FMD control efforts. This also endorses the need of reviewing the vaccination coverage of the cattle population in Egypt.

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