Phylogenetic analysis of Egyptian foot and mouth disease virus endemic strains

Nader M. Sobhy, [1]* Sunil K. Mor,[2] Mohammed E.M. Mohammed,[1] Iman M. Bastawecy, [3] Hiam M. Fakhry,[4] Christiana R.B. Youssef,[5] and Sagar M. Goyal[2]

[1*]Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia 44511, Egypt.
[2]Department of Veterinary Population Medicine, University of Minnesota, St. Paul, MN 55108, USA
[3]Department of Virology, Animal Health Research Institute, Dokki 12618, Giza, Egypt
[4]Veterinary Serum and Vaccine Research Institute, East of Cairo 131, Egypt
[5]Department of Microbiology, Faculty of Pharmacy, Zagazig University, Zagazig, Sharkia 44519, Egypt.
nadermaher@zu.edu.eg, yaaco003@umn.edu

Abstract: Egypt as a developing country has high rate of meat and live animal importation thereby increasing the chances of human and animal disease importation. One such disease is caused by foot and mouth disease virus (FMDV), which is already endemic in the country. Because of high mutation rates of FMDV, molecular epidemiological approaches are used to construct evolutionary relationships among virus strains over time and space. We detected and characterized recent FMDV strains from Egypt using primers from 3D and1D genes and found the presence of three serotypes e.g., SAT-2, A and O. The SAT-2 serotype showed the highest level of homology (99–100%) with Palestinian-Gaza virus in the 3D and 1D regions while serotype O shared lineage with some Asian O strains from Yemen, Iran and Turkey. Serotype A was also closely related to Asian strains especially those from Iraq and Bahrain. Our results indicate that serotypes A and O are still prevalent although a bivalent A and O vaccine is obligatory in Egypt.
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1. Introduction

Foot-and-mouth disease, caused by foot-and-mouth disease virus (FMDV), is an acute and highly contagious disease of domestic and wild cloven-hoofed animals. The disease has huge economic impact on infected countries mainly due to constraints on international trade in animals and animal products (Kasambula et al., 2012). The FMDV is a non-enveloped virus with icosahedral symmetry and contains a single-stranded, positive-sense RNA molecule of approximately 8,500 nucleotides (nt). The FMDV genome is classified into: (i) 5' untranslated region (5'-UTR), which contains non-coding nucleic acids that carry many regulatory elements; (ii) protein coding region (ORF), which codes for both structural and non-structural proteins; and (iii) 3' UTR or non-coding region, which carries our regulatory functions and has a poly A tail (Carrillo, 2012). There are four structural proteins namely 1A, 1B, 1C and 1D and 8 non-structural proteins; L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D (Grubman, 1984). The 3D non-structural protein is viral RNA-dependent RNA polymerase (RdRp), which is responsible for both positive- and negative-sense RNA replication and is highly conserved (94–99% similarity) (Fenga, 2004). Of the structural proteins, VP1 is the most important; it is located around the icosahedral five-fold axis and is responsible for virus variation (Knowles and Samuel, 2003). Seven serotypes of FMDV (A, O, C, Asia-1, SAT-1, SAT-2, and SAT-3) have been identified serologically and multiple subtypes occur within each serotype (Nagendrakumar et al., 2009).

Egypt has a long history of occurrence of FMDV outbreaks as the country is dependent on importation of live animals and meat from many countries all over the world. Some of these countries may be endemic for FMDV (Hamza and Beillard, 2013). Unfortunately, most of the importation occurs without proper sanitation and quarantine measures. Three serotypes of FMDV have been detected in Egypt: O, A and SAT-2. Serotype O is the most endemic since 1970 (Samuel et al., 1990; Kitching, 1998) while serotype A was isolated and identified in 2006 after importation of live animals from Ethiopia (Abed El-Rahman et al., 2006; El-Kholy et al., 2007; Knowles et al., 2007). The newest serotype is SAT-2, which was detected in 2012 (EL-Shehawy et al., 2012; Valdazo-González et al., 2012). The current belief is that the outbreak in 2012 was due only to SAT-2 serotype since most of the animals in Egypt are supposed to be vaccinated with a bivalent vaccine containing A and O serotypes. This study was undertaken to sequence the recent FMDV strains

from Egypt to reconstruct evolutionary relationships between virus strains and to better understand the causation and dynamics of the disease in Egypt.

2. Materials and Methods

2.1. Clinical samples

Five samples of sloughed tongue epithelium were collected from cattle showing fever, ropy salivation and anorexia in five governments in Egypt e.g., Al-Fayoum, Sharkia, El-Mania, Alexandria and Ismaalia. The samples were homogenized in Eagle's MEM followed by centrifugation. The supernatants were inoculated in baby hamster kidney-21 (BHK21) cells. The inoculated cells were observed daily for the appearance of cytopathic effects (CPE). After the appearance of CPE, the infected cells were frozen and thawed three times followed by centrifugation at 1,200 xg for 20 min.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Viral RNA was extracted from supernatants of infected cells using the QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA). Oligonucleotide primers are shown in Table 1. Conserved primer set was designed to amplify 881 bp of RdRp gene in 3D region of all FMDV serotypes. Differentiation of serotypes was done by using primers from the most variable genes in the 1D region. Previously published 1D primers were used for serotypes A and O while self-designed primers were used for serotype SAT-2 (Table 1).

Extracted RNA was subjected to RT-PCR using one step RT-PCR kit (Qiagen, Valencia, CA). The amplification protocol used 25µl reaction mixture and the cycling parameters were 50ºC for 30 min and 95ºC for 15 min in RT step; then 35 cycles consisting of 94ºC for 1 min for denaturation followed by annealing for 1 min at suitable temperature of each primer set as shown in Table 1. Elongation was done at 72ºC for 1 min followed by final extension cycle at 72ºC for 10 min. The RT-PCR products were analyzed by 1.2% agarose gel electrophoresis in Tris acetate EDTA buffer followed by staining with ethidium bromide. A single band of expected product size confirmed the presence of target FMDV serotype.

2.3. Sequencing of amplified RT-PCR products

The RT-PCR products were purified using QIAquick PCR purification Kit (Qiagen, Valencia, CA) as per manufacturer's instructions. The purified PCR products were sequenced using the same forward and reverse primers as used in RT-PCR. The obtained sequences were curated and aligned using "Sequencher 5.1" software (https://genecodes.com) followed by BLAST analysis in GenBank data base for comparing with other FMDV sequences. The compatible nucleotide sequences were aligned by using the Clustal W option in MEGA 6.0 (Molecular Evolutionary Genetic Analysis) computer program in order to obtain a consensus sequence. The phylogenetic correlation comparison and tree construction were also done by using MEGA 6.0. A phylogenetic tree of aligned sequences was constructed by selecting the best fit Maximum Likelihood model in Mega 6.0 based on lowest BIC score (Bayesian Information Criterion). The evolutionary distances were computed using the Tamura- 2-parameter +G (Gamma distribution with 5 rate categories) for 3D and Tamura 3-parameter+G for 1D as a best fit model with 1000 bootstrap replicate values (Tamura et al., 2013). The gene bank accession numbers for 3D genes are KJ210072, KJ210076, KJ210074, KJ210077 and KJ210080 while for 1D gene are KJ210071, KJ210075, KJ210073, KJ210078 and KJ210079.

Table 1. Forward and reverse primers with size and annealing temperatures.

Primer	Sequence (5' to 3')	Size (bp)	Reference	Annealing temperature
F 3RdRp R3RdRp	5-TTC GAG AAC GGC ACD GTC GGA-3 5-CAC GGA GAT CAA CTT CTC CTG-3	881	This work	54ºC
F A-1C612	5-TAG CGC CGG CAA AGA CTT TGA-3	815	El-Kholy et al., 2007	55ºC
F O-1C 124	5-ACC AAC CTC CTT GAT GTG GCT-3	1300	El-Kholy et al., 2007	52ºC
(A,O) R2B58 (NK61)	5-GAC ATG TCC TCC TGC ATC TG-3		El-Kholy et al., 2007	
F SAT-2 R SAT-2	5-ACG GTG GGA AYG TTC AAG AG-3 5-TTC AAG ACC GGT GTC AGC-3	931	This work	52ºC

3. Results

3.1. Virus isolation

The FMDV was isolated in BHK21 cells from all five samples. The identity of the virus was confirmed by RT-PCR and sequencing of the RdRp gene. Sequencing of 1D gene confirmed the presence of three different FMDV serotypes; serotype SAT-2 from Sharkia, serotype A from Al-Fayoum, and serotype O from one sample each from El-Mania, Alexandria and Ismaalia governments.

3.2. Phylogenetic analysis of FMDV serotypes based on 3D region sequencing

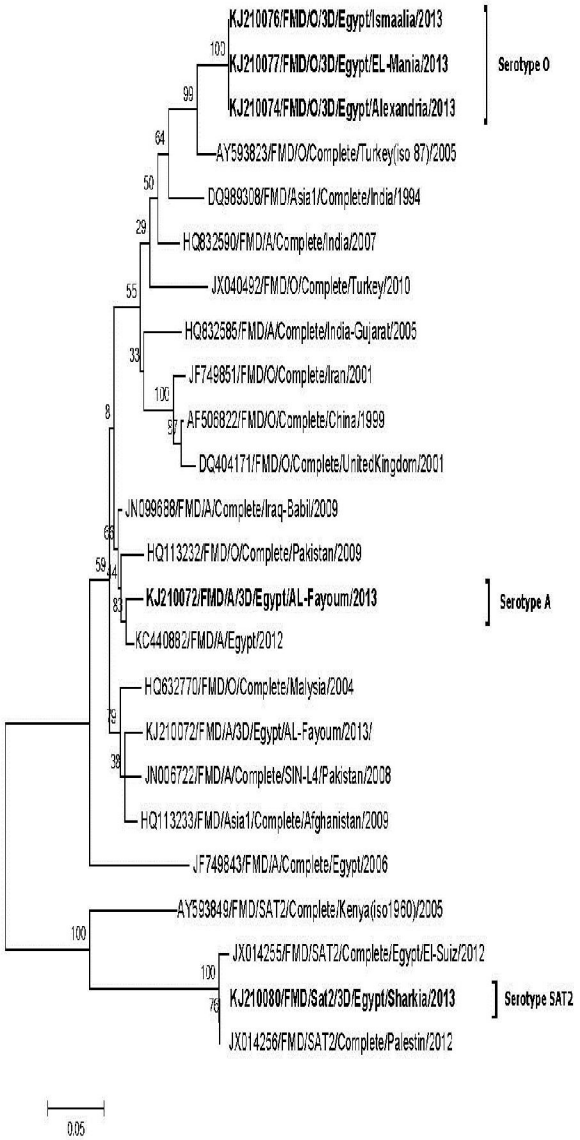

Figure 1. Phylogenetic analysis based on 800 nucleotides of the RNA polymerase (3D) gene. The tree shows relationship between different FMD virus serotypes and conserved character of the gene using Maximum Likelihood method with Kimura 2 parameter mode and 1000 bootstrap replicates.

The nucleotide (nt) identity based on sequence alignment and phylogenetic analysis of isolate FMD/A/3D/Egypt/Al-Fayoum/2013/ KJ210072 with previously published Egyptian FMDV serotype A [Egypt /2006 (JF749843)] sequences was 92%. The maximum nucleotide identity (98%) was with FMDV serotype A sequences from Egypt (KC440882) and Persian Gulf areas especially from Iraq (JN099688) and (JN099702). Phylogenetic analysis of serotype O isolates of this study from Ismailia (KJ210076), Alexandria (KJ210074) and El-Mania (KJ210077) showed 100% identity with each other and 92% nt identities with Asian type O sequences from Pakistan (HQ1132321), Israel (FJ175666), Iran (JF749851) and Turkey (JX040492). The isolate

FMD/SAT-2 /3D/Egypt/Sharkia/2013/ KJ210080 from this study showed 99% to 100% nt identity with sequences from Egypt (JX014255) and Palestinian-Gaza (JX014256), respectively, and only 88% nt identity with African SAT-2 serotypes from Kenya (AY593849) (Figure 1).

3.3. Phylogenetic analysis of serotype A based on 1D region sequencing

The nucleotide identity was 77% with both endemic 2006 Egypt serotype A (JF749843) and 2009 Egypt serotype A (KC888937). African A serotypes along with 2006 and 2009 Egyptian isolates were found in one cluster while our Egyptian isolate from this study (KJ210071) and Egypt (KC440882) strain were found in another cluster (with Asian isolates). The percent identity of our isolate with African strains [Ethiopia (FJ798150), Sudan (GU566066) and Kenya (EF208773)] was 77%-78%. Our isolate had closest identity with Asian Gulf area serotypes e.g., 95% identity with Iraq (JN099695) and Bahrain (FJ755010) and 93% to 94% with Iran (FJ755054), Turkey (FJ755116) and Saudi Arabia (FJ755087) (Figure 2).

3.4. Phylogenetic analysis of serotype O based on 1D region sequencing

All three serotype O sequences from Ismailia (KJ210075), Alexandria (KJ210073) and El-Mania (KJ210078) were 100% identical with each other. On comparison with previously published serotype O sequences from Egypt, all three isolates had 100% identity with Egypt/72(DQ164871) while only 83% with Egypt/2010 (KC565752) and (KC565753). The isolate identity was 87% with Ethiopian strain (AY283393), 93% with Turkey strain (AJ251477), and 86% to 87% with Yemen (AJ318859) and Iran (JF749852) (Figure 2).

3.5. Phylogenetic analysis of serotype SAT-2 based on 1D region sequencing

The isolate FMDV/SAT-2/3D/Egypt/Sharkia/

2013/KJ210079 from this study had 100% identity with Egyptian strains Domyat (KF112932),

Menia (JX570620), Suiz (JX570622) and Garbia (JX570618). The identity was 100% with Palestinian-Gaza (JX014256) strain while with other Egyptian SAT-2 serotypes e.g., Alexandria (JX570617) was only 91%. In addition, there was 89%, 92% identity with Libya (JX570634) and (JX570632) strains respectively (Figure 2).

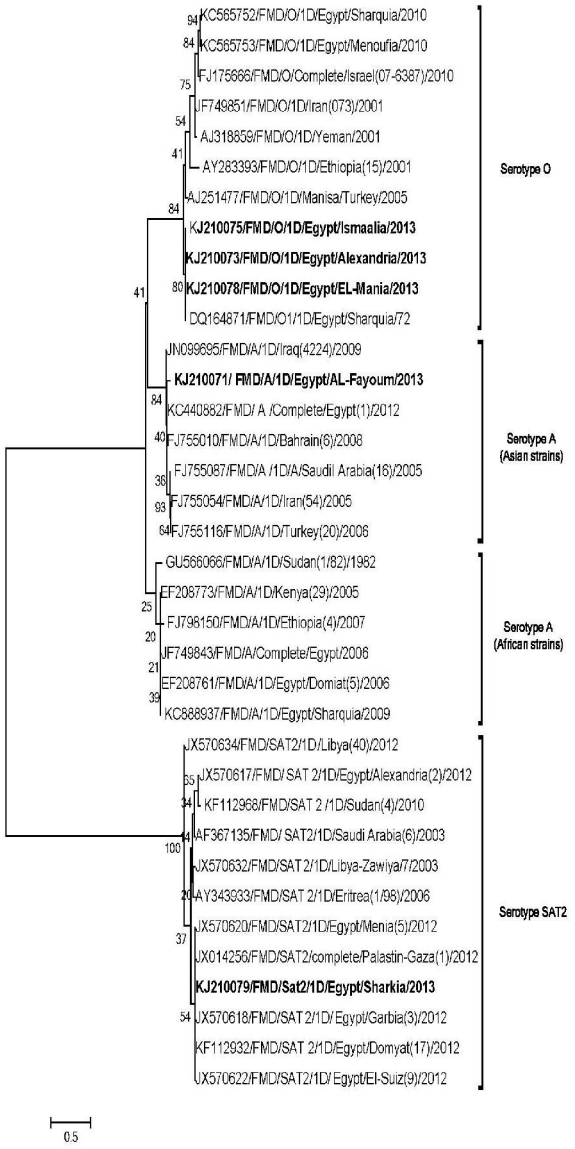

Figure 2. Phylogenetic tree of 1D gene partial sequences constructed by neighbor-joining method on basis of 491 nucleotide from 2677-3168 (Ref. JN099703) for serotype A, 61-552 (Ref. DQ164871|) for serotype O , 3300 -3791 (Ref. JX014256) for SAT-2 serotype. Tree shows relationship of Egyptian FMDV serotypes with different serotypes.

4. Discussion

The epidemiology of FMD in North Africa is complicated by co-circulation of endemic FMDV strains and by sporadic incursions of exotic viral strains from the Middle East and Sub-Saharan Africa (Ahmed et al., 2012). There is a need for routine molecular characterization of FMDV strains in Egypt because of the following factors: (i) Egypt is a developing country with a large gap between its domestic meat production and consumption. In 2010, production and consumption were 728.88 and 937.65 thousand tons, respectively, achieving only 77.73% self-sufficiency (Dawoud, 2005). The country tries to close this gap by importing meat or live animals, increasing the chance for animal diseases to enter the country; (ii) political unrest across North Africa has forced the migration of people and animals in large numbers, which increases the chance for diseases to cross national and international borders (Global Food Security, 2012); (iii) Egypt does not have a strong national database on the origin and/or genetic changes in animal pathogens including FMDV. This has a negative impact on the control strategy of the disease in Egypt (EL-Shehawy et al., 2011); and finally (iv) there are numerous variants within each serotype of FMDV allowing them to break through the existing herd immunity (Meyer et al., 1994; Balinda et al., 2010).

The changes in FMDV serotypes add up to approximately 1% per year in the VP1 gene (Abdul-Hamid et al., 2011). Hence, it is important to ensure rapid detection and characterization of any new or mutant strains to allow for vaccine modification, if necessary. This will ensure the development of effective strategies for the control and prevention of this disease. In this study, sequencing of viral isolates from clinically infected cattle in Egypt confirmed the presence of FMDV when primers from the most conserved 3D gene region were used. The isolates were further differentiated into three different serotypes, A, O and SAT-2 based on sequencing of 1D gene. This indicates that our primer set for 3D gene can be used as a diagnostic tool for the detection of FMDV in field samples as has been suggested by others (Chen et al., 2003; Manju et al., 2001). A high degree of sequence conservation was observed across the serotypes on doing phylogenetic analysis based on the 3D gene (Figure 1). These findings are in correlation with previous studies, which indicated that nonstructural proteins fail to produce the serotype-inclusive groups observed with capsid protein sequences (Carrillo et al 2005). Thus, 3D gene is the best for detection of FMDV but is not good for serotype differentiation.

Based on 1D gene sequence, we found three different serotypes (A, O and SAT-2). Serotype A from 2006 and 2009 in Egypt was more related to South African serotypes than the strain isolated in this study, which is more closely related to Asian A

strains from Iraq and Bahrain and recently identified in Egypt. This strain originated in Iran in 2005 and spread to the neighboring countries of Pakistan and Turkey and then to Bahrain in 2008 (Knowles et al., 2009; Upadhyaya et al., 2013). The finding of a similar strain in our study indicates that emerging Asian strains may pose a new risk for Egypt.

Serotypes O in this study, had the same lineage as Asian O strains from Yemen, Iran and Turkey and matched completely with O1 Egypt strain isolated in 1972. This is in agreement with previous reports (EL-Shehawy et al., 2011; Mandour et al., 2013), which indicated that these strains are still prevalent in the country and may play a role in disease outbreaks. However, the O serotypes in our study had only 89% identity with recent Egyptian strains from 2010 (KC565752 and KC565753) indicating that more than one subtype of O is present in the country.

Phylogenetic analysis and pairwise distances indicate that serotype SAT-2 in this study matched 100% with those isolated during 2012 outbreaks in Egypt and Gaza. The Egyptian and Gaza SAT-2 serotypes may have the same origin due to unrestricted, trans-regional animal movement which is common in Africa due to free Bedouin movement (Sangare et al., 2004). On the other hand the difference between our isolated strain and those isolated from Alexandria government that similar to Libyan isolates, also suggest that there are 2 different SAT2 subtype in the country. Our findings indicate that both serotypes A and O are still present in the country, which is in contrast to Kandeil et al. (2013) and Shawky et al. (2013) who isolated and confirmed only serotype SAT-2 in their survey. Discovery of both A and O serotypes, in spite of widespread vaccination against them, may indicate vaccine failures. It is important, therefore, to implement appropriate vaccination strategies including vaccination of animals in remote areas, farmer education, and financial commitment by the government (Loth et al., 2011). We also recommend that the country should exercise more precaution during importation of live animals and meat from various Asian countries. There is also a need to design epidemiological studies and a national FMDV knowledge bank reporting all the starting and spreading points of FMD. This along with successful vaccine campaign will help the country improve its FMD control strategy and minimize the chances for the emergence of new serotypes/subtypes.

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Corresponding author:
*Dr. Nader M. Sobhy
Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, Elzera'a Square, Zagazig, Sharkia 44511, Egypt.
E-mail: nadermaher@zu.edu.eg, yaaco003@umn.edu

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