Phylogenetic analysis of Egyptian foot and mouth disease virus endemic strains in 2013

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ABSTRACT

Foot and mouth disease virus (FMDV) is a long known virus but still a current threat in Egypt with devastating consequences as shown in 2012 in Egypt. One of the most important FMD features is high potential for genetic and antigenic variation. Therefore molecular study of FMDV is pre-requisite for amplification of FMDV specific genomic fragment and also copying specific serotypes O, A and SAT2 nucleotide sequence. In this work FMDV serotype O, A and SAT2 were isolated from cattle clinical samples (foot lesions). RNA extracted from clinical samples was subjected to reverse transcriptase polymerase chain reaction(Rt. PCR) nucleotide of FMDV ID and 3D genes were determined using standard automated sequencing technique. Phylogenetic analysis of FMD / SAT-2 /3D/Egypt/Sharkia/2013/ FMD / (KJ210080) revealed (99–100%) identity with Egypt 2012/ and Palestinian-Gaza virus. Phylogenetic analysis of serotype O of this study from Ismailia (KJ210076) , Alexandria (KJ210074) and El-Mania (KJ210077) showed 100% identity with each other and 92% identities with Asian type O sequences from Pakistan, Iran and Turkey while FMD/A/3D/Egypt/Al-Fayoum /2013/KJ210072 with serotype (A) sequences had closest identity with Asian Gulf area serotypes 95% identity with Iraq and Bahrain Also 94% with Iran/05. FMDV O, A and SAT2 strains were homologous with the vaccinal bovine strain.

Keywords: Foot-and-mouth disease; molecular epidemiology; phylogenetic analysis, Egypt.

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 regulatory functions and has a poly A tail (Carrillo, 2012). There are four structural proteins namely 1A, 1B, 1C and 1D and 10 non-structural proteins; L, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C, and 3D *(Grubman et al., 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). The structural proteins , VP1 is the

most important ; it is located around the icosahedral five-fold axis and is responsible for virus variation *(Mahy, 2005).* Seven serotypes of FMDV namely (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 allover 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-Gonzálezet al., 2012).* The current beliefs 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.

MATERIALS AND METHODS

Clinical samples

Eight samples of sloughed tongue epithelium were collected from cattle showing fever, ropy salivation and anorexiain six governments in Egypt e.g., Faiyum, Sharkia, El-Mania, Ismailia and Alexandria. The samples were homogenized in Eagle's MEM followed by centrifugation . The supernatant were inoculated in baby hamster kidney-21 (BHK21) cells .The inoculated cells were observed daily for the appearance of cytopathic effects *Huang et al., (2011) and Neeta et al., (2011).*

RNA extraction

Viral RNA was extracted from supernatants of infected cells using the QIAamp Viral RNA Mini kit (Qiagen) *Knowles and Samuel (1994) , EL-Shehawy et al., (2011) and Shawkey et al., 2013* .

Oligonucleotide primers

The primers used are shown in Table 1. Conserved primer set was designed to amplify 881 bp of Rd Rp 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).

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

Primer	Sequence (5' to 3')	Expected size (bp)	Reference	Annealing temperature
F 3RdRp R 3RdRp	5-TTC GAG AAC GGC ACD GTC GGA-3 5-CAC GGA GAT CAA CTT CTC CTG-3	881	This work	54
F A -1C612	5-TAG CGC CGG CAA AGA CTT TGA-3	815	El-Kholy et al., 2007	55
F O -1C 124	5-ACC AAC CTC CTT GAT GTG GCT-3	1300	El-Kholy et al., 2007	52

(A ,O) R2B58(NK61)	5-GAC ATG TCC TCC TGC ATC TG-3	1000	El-Kholy et al., 2007	(A,O) R2B58 (NK61)
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	52

F= forword R=reverse RdRp= RNA-dependent RNA polymerase

Reverse transcription-polymerase chain reaction (RT-PCR)

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 50°C for 30 min and 95°C for 15 min in RT step; then 35 cycles consisting of 94°C for 1min for denaturation followed by annealing for 1 min at suitable temperature of each primer set as shown in **(table 1)** Elongation was done at 72°C for 1 min followed by final extension cycle at 72°C 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.

Sequencing of Purified RT-PCR products

The RT-PCR products were purified using QI a quick PCR purification Kit (Qiagen) 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 "Sequencer 5.1" software (https://genecodes.com) followed by BLAST analysis in Gen Bank data base for comparing with other FMDV sequences. The compatible nucleotide sequences were aligned by using the Clustal W option in MEGA 5.0 (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 5.0. The evolutionary distances were computed using the Kimura 2-parameter model .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.

RESULTS

FMDV was isolated in BHK21 cells from all eight samples . The identity of the virus was confirmed by RT-PCR and sequencing of Rd Rp gene. Further sequencing of 1Dgene confirmed the presence of three different FMDV serotypes (O,A & SAT 2) . From (table2) and photo (1) **:** Three positive samples FMDV(O) collected from El-Mania , Alexandria and Ismailia governorates shown in Lanes 1-3 positive FMDV type (O) 422bp and three positive samples FMDV (SAT 2) shown Lane 4-6 positive FMDV type (SAT2) 732bp from Sharkia and two positive samples collected from Faiyum governorate shown in Lane 7&8 positive FMDV type (A) (863 bp) .

Phylogenetic analysis of FMDV serotypes based on 3D region sequencing

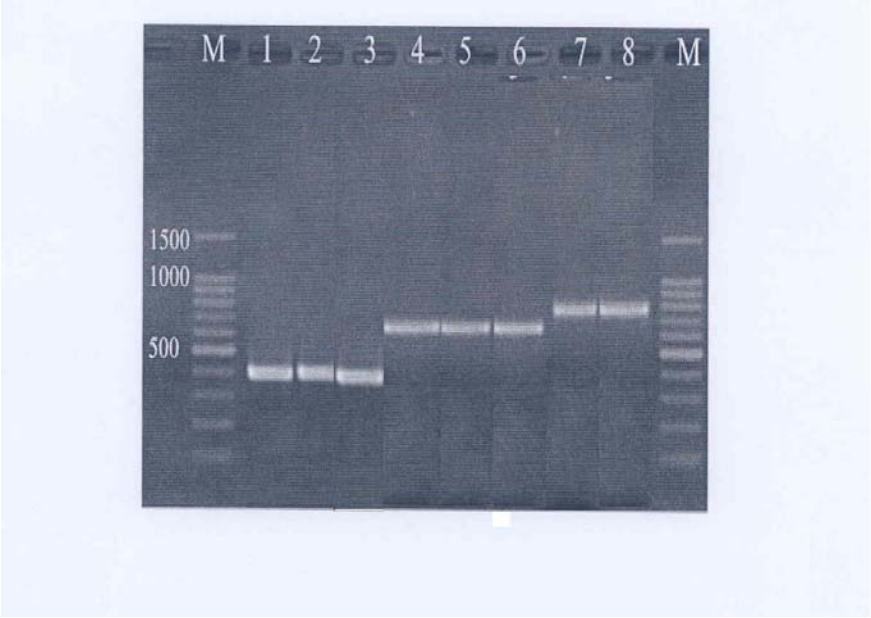
The nucleotide (nt) identity based on sequence alignment and phylogenetic analysis of isolate FMD/A/3D/Egypt /Al-Fayoum /2013/KJ210072 with serotype (A) sequences from Persian Gulf areas especially from Iraq (JN099688) and (JN099702). Phylogenetic analysis of serotype (O) isolates of this study from Ismailia (KJ210076) , Alexandria (KJ210074) and El-Mania (KJ210077) showed 100% identity with each other and

92% identities with Asian type O sequences from Pakistan (HQ1132321), Israel (FJ175666) , Iran (JF749851) and Turkey (JX040492) . The FMD / SAT-2 /3D/Egypt/Sharkia/2013/ FMD / (KJ210080) isolate from this study showed 100% identity with each other and 99% to 100% identity with sequences from Egypt (JX014255) and Palestinian-Gaza (JX014256) , respectively, and only 89% identity with African SAT 2 serotypes from Kenya (AJ251473) and Uganda (JF49862) ***(Fig. 1).***

Table 2. The identified FMD serotypes in different Egyptian governments.

Government	Detected Serotype
Sharkia	**SAT2**
Faiyum	**A**
El-Mania	**O**
Alexandria	**O**
Ismailia	**O**

Photo (1): Detection of FMDV genome by RT-PCR. M: 1500 bp DNA ladder.



Lanes 1-3 positive FMDV type (O) (422bp) ,
Lane 4-6 positive FMDV type (SAT2) (732bp) ,
Lane 7&8 positive FMDV type (A) (863 bp) .

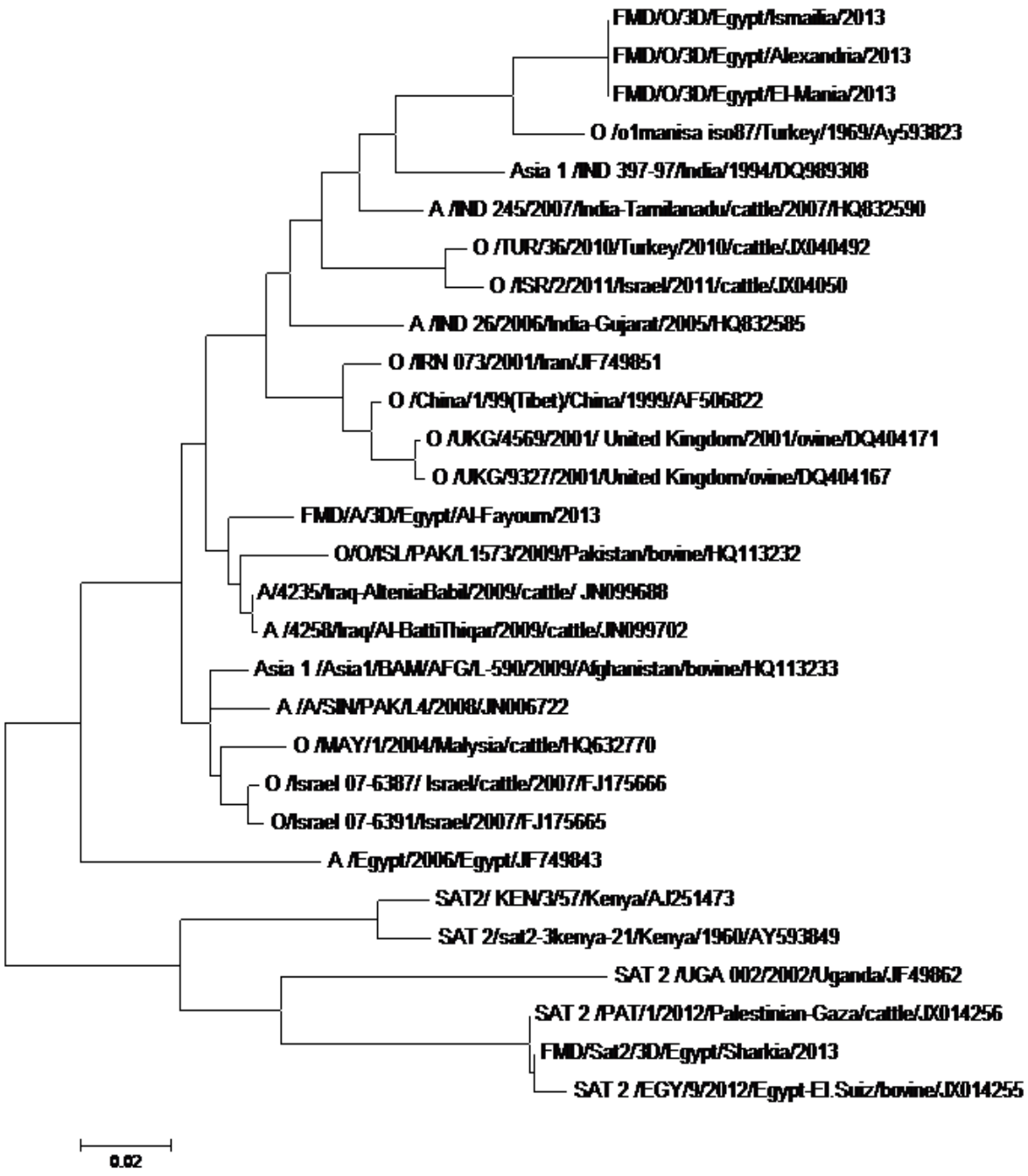


Fig. 1. Phylogenetic analysis of Egyptian FMDV isolates based on 800 nucleotide of 3D gene.

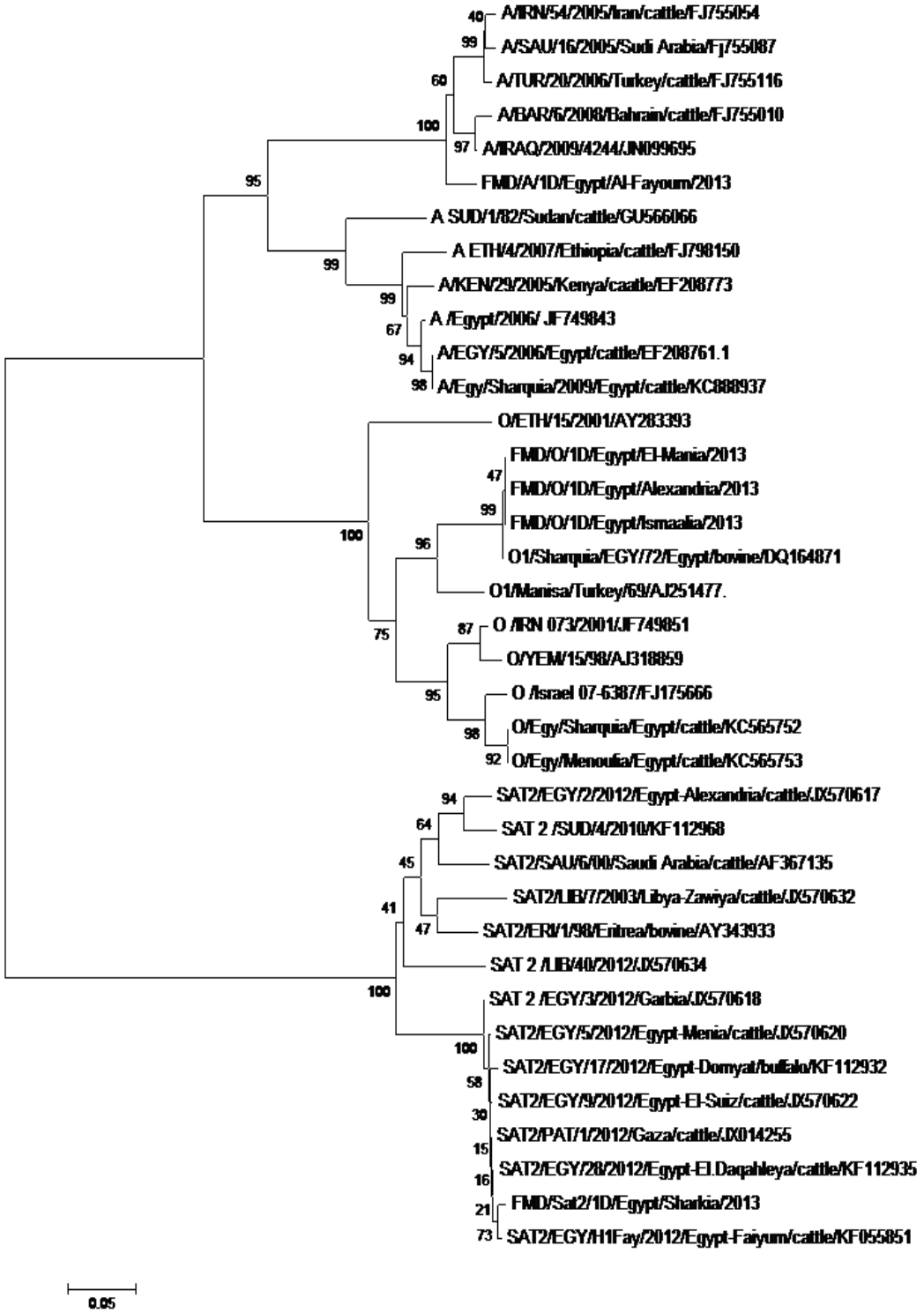


Fig. 2: Phylogenetic tree constructed by neighbor-joining method on basis of partial sequences 1D gene. Tree showed relationship of Egyptian FMDV serotypes with different serotypes.

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 89% with Egypt/2010 (KC565752) and (KC565753).The isolate identity was 87% with Ethiopian strain (AY283393), 93% with Turkey strain (EG251477), and 86% to 87% with those from Iran (JF749852) and Yemen (EG318859) ***(Fig. 2).***

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

FMDV/ SAT-2 /3D/Egypt/Sharkia /2013/KJ210079 isolate from this study had100% match with Egyptian strains Domyat (KF112932), El-Mania (JX570620), Qaliobia, Suiz (JX570622), Faiyum(KF055851)and Daqahleya (KF112935). The identity with other Egyptian SAT-2 serotypes e.g., Alexandria(JX570617) was only 91%.In addition, there was 100% and 89% identity with Palestinian-Gaza (JX014256) and Libya (JX570634), respectively ***(Fig. 2).***

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) was founding 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) *(Fig.2).*

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).* So, there is a need for routine molecular characterization of FMDV strains in Egypt because of the following factors. First, Egypt as a developing country has 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. Secondary, 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).* Thirdly, Egypt doesn't 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).* Finally, there are numerous variants within each serotype of FMDV allowing them to break through the existing herd immunity *(Meyer et al., 1994 ; Knowles and Samuel, 2003; 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 needed thereby leading to the development of effective strategies for the control and prevention of disease.

In this study, sequencing of viral isolates from clinically infected cattle in Egypt with two different primers (3D and 1D) revealed the presence of three different serotypes (A , O and SAT 2) . The 2006 and 2009 (A) serotypes were 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. This strain originated in Iran in 2005 and spread to neighboring countries (Pakistan and Turkey) and to Bahrain in 2008 *(Knowles et al.,2009 ; Upadhyaya et al.,2013).*

The finding of similar strain in our study indicates that emerging Asian strains may pose a new risk for Egypt. This is the first time to detect Asian serotype (A) in the country since the appearance of serotype (A) in 2006 *(Abed El-Rahman et al., 2006; El-Kholy et al., 2007; Knowles et al., 2007).*

Serotype O in this study, had the same lineage as Asian O strains from Yemen, Iran and Turkey and matched completely with O_1 Egypt strain that had isolated in 1972. This is in agreement with previous reports *(El-Shehawy et al., 2011; Mandour et al., 2013)* indicating that these strains are still prevalent in the country and may play a role in disease outbreaks *(Kitching, 1998; Vosloo et al., 2002 ; Ghoneim et al., 2010).* 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 pair wise distances indicate that the 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 animal movement which is common in Africa due to free Bedouin movement which provided the first indication of trans-regional virus transmission *(Sangare et al., 2004).* Our findings indicate that both serotype 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 & O) serotypes in spite of widespread vaccination against them may indicate vaccine failures that can be overcome by using proper 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 precautions 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.

Finally we concluded that FMD serotypes in Egypt during 2013 SAT-2 /3D/Egypt/Sharkia/2013 showed the highest level of homology with Egypt 2012/ and Palestinian-Gaza , (O) isolates from Ismailia, Alexandria and El-Mania identities with Asian type O from Pakistan, Iran and Turkey and (A) isolates from Faiyum sequences had

closest identity with Asian Gulf area serotypes 95% identity with Iraq and Bahrain Also 94% with Iran/05 .

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