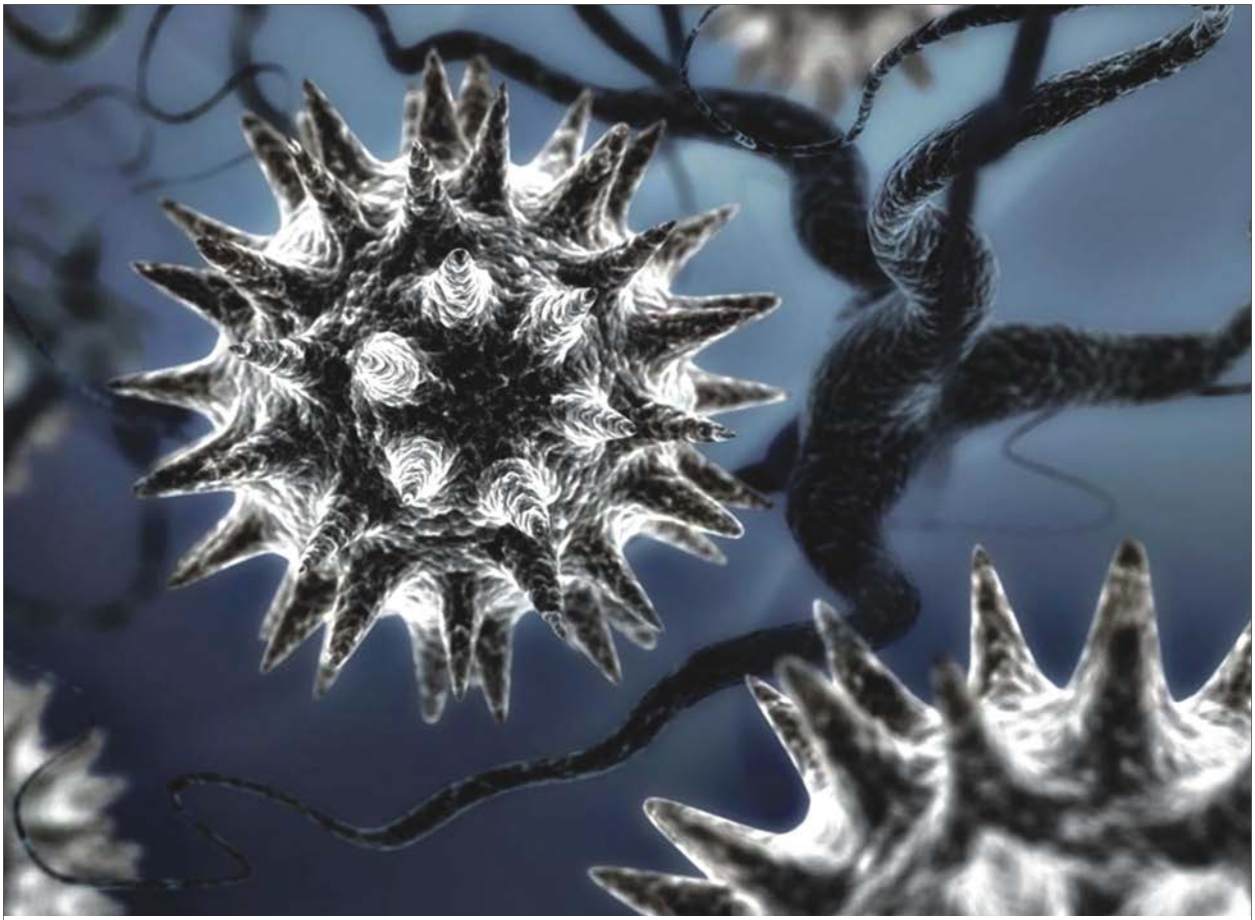

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Phylogenetic Analysis of Foot and Mouth Disease Virus Type O in Egypt (2009)

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ABSTRACT

This study describes the sequence analysis and molecular characterization of foot-and-mouth disease virus type O responsible for FMD outbreaks in Egypt in 2009. Phylogenetic analysis of partial VP1 nucleotide sequences demonstrated that the examined FMDV type O was related to Pan Asia strain within ME-SA topotype, rather than to O1 serotype used in vaccine preparation in Egypt.

Key words: FMD serotype O, PanAsia starins, partial VP1 sequencing

INTRODUCTION

Foot and mouth disease virus belongs to *apthovirus* genus of the *picornavirus* family (Rueckert, 1996). FMDV consisting of ssRNA plus-sense genome of approximately 8500 bases surrounded by 60 copies each of four structural proteins (VP1, VP2, VP3 and VP4) to form a naked icosahedral capsid (Rueckert, 1996).

Antigenically, FMDV is recognized as seven distinct serotypes namely: South African territories (SAT) 1, SAT 2 and SAT 3 are usually restricted to Africa; Asia1 is restricted to Asia; while O, A and C are present in Africa, Asia, South America and occasionally Europe (Knowles and Samuel, 2003).

Genetically, FMDV can be classified based on their geographic origin "topotype" e.g., the serotype O can be grouped into 10 topotypes have named Europe-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 *et al.*, 2004).

The molecular characterization of virus isolates is important requirement in control of FMD. Sequencing the region of the FMDV genome encoding the capsid proteins of the virus, provide the most detailed information about isolates as this region is variable between serotypes and subtypes (Domingo *et al.*, 1990). The hyper-variable region of FMDV genome that responsible for this antigenic diversity lies in the VP1 gene segment (Grubman *et al.*, 1993). Therefore, the vast antigenic diversity of FMDV is a result of mutations in VP1 seqence (Beck and Strohmaier, 1987). Sequencing of VP1 and phylogenetic analysis is a cutting edge technology for diagnosis and identification of different FMD viruses (Knowles and Samuel, 1995). Beck and Strohmaier (1987) first employed VP1 sequencing to study the epidemiology of FMDV strains A and O in Europe over a period of 20 successive years. After that sequencing became the main tool for characterizing FMD viruses and their epidemiology worldwide (Knowles *et al.*, 1998).

Understanding the epidemiology of a disease is essential for the formulation of the most effective control strategies. Nucleotide sequencing represents a core component for tracking outbreak sources and epidemiological investigations. So, this study aimed to analyze VP1 sequence and this analysis allows the study of viral biodiversity and evolution and thereby to track transmission events and sources, as well as to assure vaccine coverage of corresponding field FMDV lineages.

MATERIALS AND METHODS

Samples: Clinical materials from 4 cases (collected from 3 separate governorates in Egypt) were collected. The epithelial samples were placed in transport medium prepared according to (OIE, 2009). The samples were collected on ice until transported to the laboratory. In the laboratory, all samples were stored at -80°C till used.

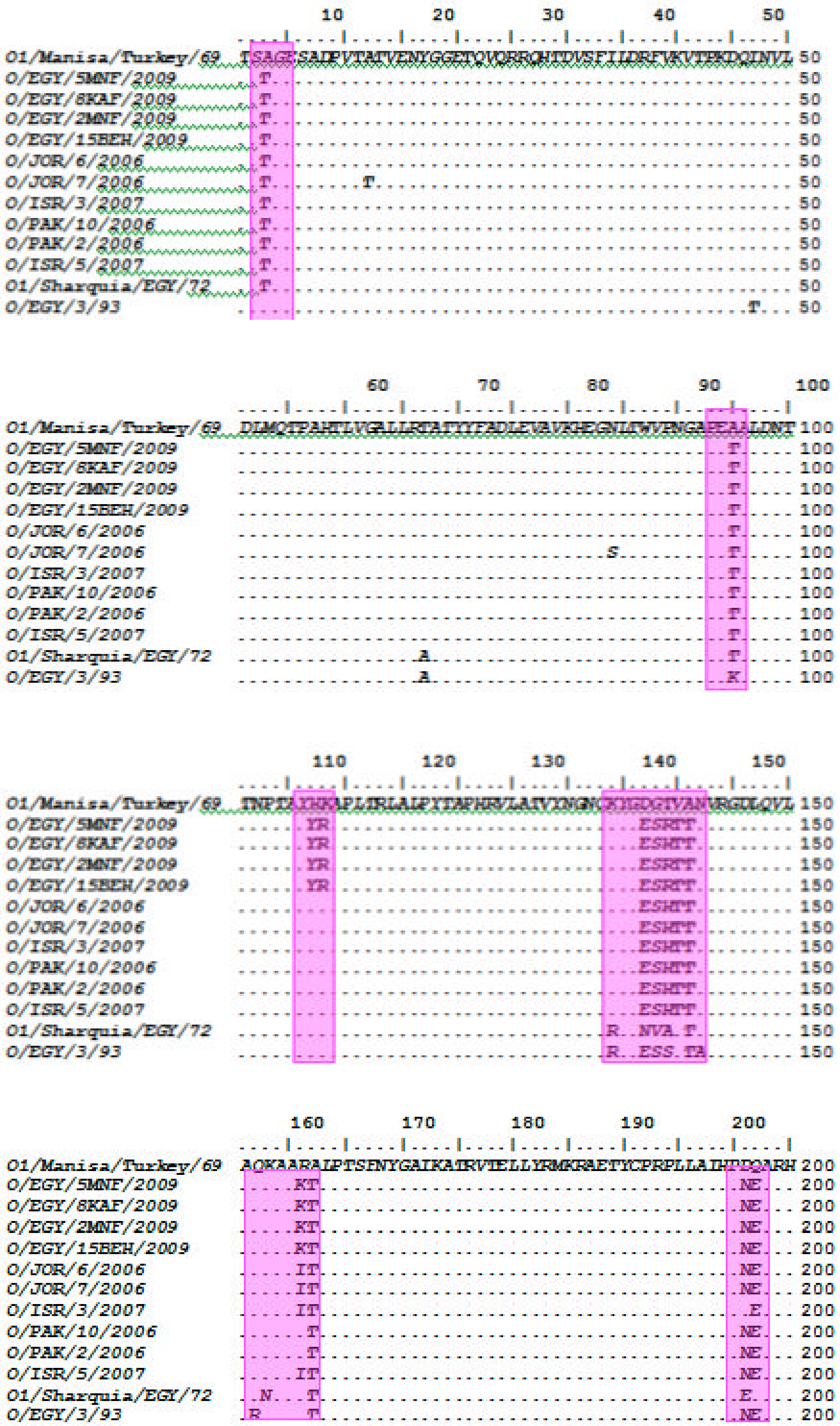
Viral RNA extraction and RT-PCR: In the laboratory, the viral RNA was extracted from the field samples (epithelial tissue scraping) using QIAamp viral RNA mini kit (catalog No. 52904, QIAGEN, Crawley, UK) from 4 samples. The complete VP1 region of the genome was amplified by RT-PCR using Verso^{TM} 1-step RT-PCR kit by using 2 primer sets (A-1C562F/EUR-2B52R and A-1C612F/EUR-2B52R) theses primers sets were designed according to Knowles *et al.* (2007) by the following thermal profile: For the first set 50°C for 15 min; 95°C for 2 min; 40 cycles of 95°C min^{-1}; 51°C for 30 sec and 72°C for 1 min, followed by a final extension of 72°C for 10 min. While, the thermal profile for the 2nd set of primers: 50°C for 15 min; 95°C for 2 min; 40 cycles of 95 for 1 min, 58°C for 30 sec and 72°C for 1 min followed by a final extension of 72°C for 10 min.

Sequencing and phylogenetic analysis: The PCR products of four samples were sent to gene sequence in Lab Technology company local agent for Korean sequencing unit (MACROGEN) Company, But with the primers NK72, A-1C612F and A1D-523R according to Knowles *et al.* (2007). The sequence data was analyzed and used in construction of phylogenetic tree of the detected FMDV using BioEdit program version 7.0.8 and MEGA program version 5.05.

RESULTS

Phylogenetic analysis of FMDV type O in Egypt (2009): The complete nucleotide sequence of VP1-coding region was determined for 4 type O viruses obtained from epithelium samples collected from three Egyptian governorates in august 2009. These sequences were aligned and compared with those of Egyptian type O viruses, including strains used for vaccine formulation. Representative strains of 8 topotypes of type O viruses were also included. A phylogenetic tree generated using the neighbor-joining method is shown in Fig. 2 and 3.

All viruses studied from the outbreaks in Egypt showed a limited degree of variation in the VP1 gene, with values of over 99.9% genetic relatedness among them with only one amino acid change observed as shown in Table 1 and in Fig. 1. Nucleotide sequence alignment of VP1 of examined samples in comparison with FMDV strains O1/Manisa/Turky/69 (Accession No. AJ251477.1), FMDV type O isolate O1/Sharquia/Egy/72 (Accession No. DQ164871.1) and FMDV type O isolate O/Egy/3/93 (Accession No. EU553840.1). Showing genetic relatedness



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                                10        20        30        40        50
                        ....|....|....|....|....|....|....|....|....|....|
O1/Manisa/Turkey/69     TSAGESADPVTATVENYGGETQVQRRQHTDVSFILDRFVKVTPKDQINVL 50
O/EGY/5MNF/2009         ..T............................................... 50
O/EGY/8KAF/2009         ..T............................................... 50
O/EGY/2MNF/2009         ..T............................................... 50
O/EGY/15BEH/2009        ..T............................................... 50
O/JOR/6/2006            ..T............................................... 50
O/JOR/7/2006            ..T........T...................................... 50
O/ISR/3/2007            ..T............................................... 50
O/PAK/10/2006           ..T............................................... 50
O/PAK/2/2006            ..T............................................... 50
O/ISR/5/2007            ..T............................................... 50
O1/Sharquia/EGY/72      ..T............................................... 50
O/EGY/3/93              ..............................................T... 50

                                60        70        80        90        100
                        ....|....|....|....|....|....|....|....|....|....|
O1/Manisa/Turkey/69     DLMQTPAHTLVGALLRTATYYFADLEVAVKHEGNLTWVPNGAPEAALDNT 100
O/EGY/5MNF/2009         ............................................T..... 100
O/EGY/8KAF/2009         ............................................T..... 100
O/EGY/2MNF/2009         ............................................T..... 100
O/EGY/15BEH/2009        ............................................T..... 100
O/JOR/6/2006            ............................................T..... 100
O/JOR/7/2006            .................................S..........T..... 100
O/ISR/3/2007            ............................................T..... 100
O/PAK/10/2006           ............................................T..... 100
O/PAK/2/2006            ............................................T..... 100
O/ISR/5/2007            ............................................T..... 100
O1/Sharquia/EGY/72      ................A...........................T..... 100
O/EGY/3/93              ................A...........................K..... 100

                                110       120       130       140       150
                        ....|....|....|....|....|....|....|....|....|....|
O1/Manisa/Turkey/69     TNPTAYHKAPLTRLALPYTAPHRVLATVYNGNCKYGDGTVANVRGDLQVL 150
O/EGY/5MNF/2009         ......YR............................ESRTT......... 150
O/EGY/8KAF/2009         ......YR............................ESHTT......... 150
O/EGY/2MNF/2009         ......YR............................ESRTT......... 150
O/EGY/15BEH/2009        ......YR............................ESRTT......... 150
O/JOR/6/2006            ....................................ESHTT......... 150
O/JOR/7/2006            ....................................ESHTT......... 150
O/ISR/3/2007            ....................................ESHTT......... 150
O/PAK/10/2006           ....................................ESHTT......... 150
O/PAK/2/2006            ....................................ESHTT......... 150
O/ISR/5/2007            ....................................ESHTT......... 150
O1/Sharquia/EGY/72      .................................R..NVA.T......... 150
O/EGY/3/93              .................................R..ESS.TA........ 150

                                160       170       180       190       200
                        ....|....|....|....|....|....|....|....|....|....|
O1/Manisa/Turkey/69     AQKAARALPTSFNYGAIKATRVTELLYRMKRAETYCPRPLLAIHPDQARH 200
O/EGY/5MNF/2009         .....KT......................................NE... 200
O/EGY/8KAF/2009         .....KT......................................NE... 200
O/EGY/2MNF/2009         .....KT......................................NE... 200
O/EGY/15BEH/2009        .....KT......................................NE... 200
O/JOR/6/2006            .....IT......................................NE... 200
O/JOR/7/2006            .....IT......................................NE... 200
O/ISR/3/2007            .....IT.......................................E... 200
O/PAK/10/2006           ......T......................................NE... 200
O/PAK/2/2006            ......T......................................NE... 200
O/ISR/5/2007            .....IT......................................NE... 200
O1/Sharquia/EGY/72      ..N...T......................................E.... 200
O/EGY/3/93              .R....T......................................NE... 200
```

Fig. 1: Amino acid alignment showing differences (similarities shown as dots) in comparison with FMDV strain O1/Manisa/Turky/69 (Accession No. AJ251477.1) as representative of FMDV vaccinal strain used in Egypt generated by BioEdit Bioinformatics program version 7.0.8

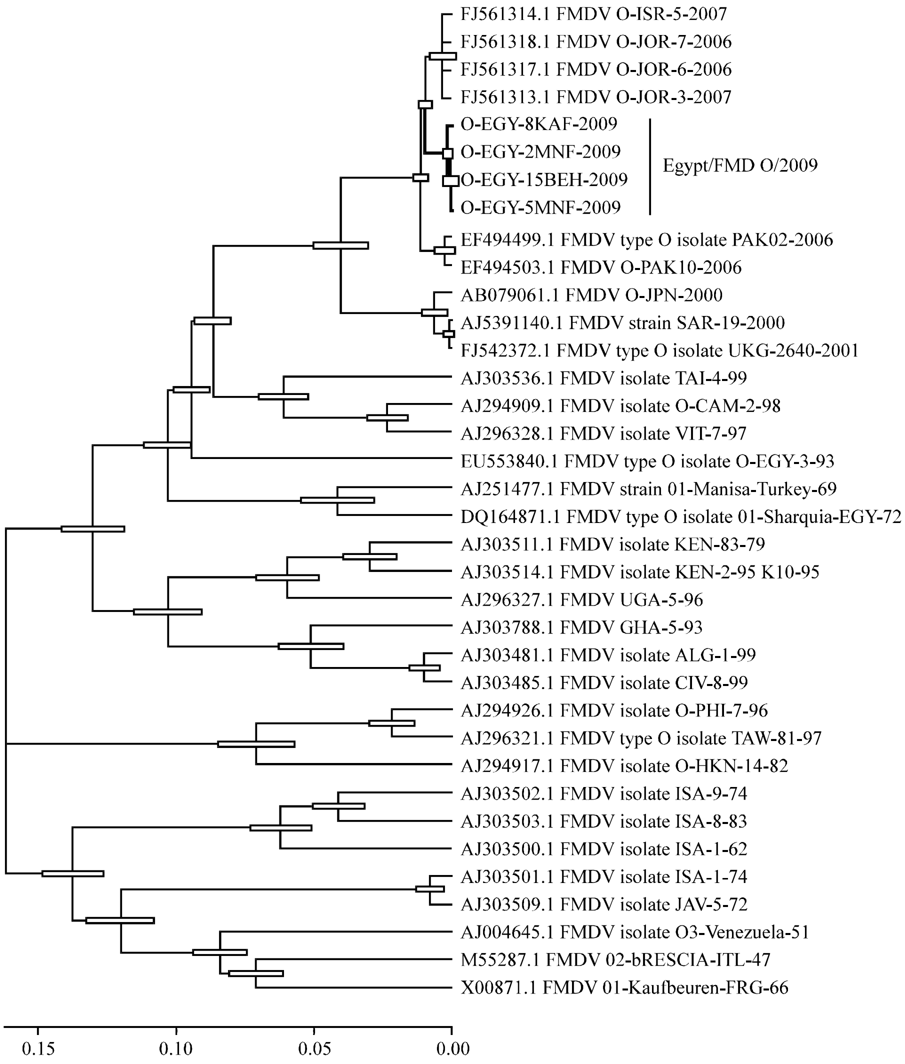

Fig. 2: Neighbor-joining phylogenetic tree of FMDV local isolates in comparison with the Egyptian isolates, the results of blast and representative isolates for eight topotypes of serotype o of FMDV

of about 87.9% in 632 nucleotides and differences in 13 amino acids of 210 amino acids in the VP1 protein as shown in Fig. 1.

From the results presented in Fig. 2, it became evident that all type O viruses sequenced from Egyptian outbreaks belong to the Middle East-South Asia topotype. Viruses in this topotype occurred in various lineages and, based on the phylogenetic analysis, it can be concluded that all detected viruses recovered from the Egyptian 2009 outbreaks fall within Panasian viruses rather than egyptian vaccinal strains.

When compared with the strains in the databank, they showed the closest relatedness with viruses O/ISR/5/2007, O/JOR/7/2006, O/JOR/6/2006, O/ISR/3/2007, O/PAK/2/2006 and O/PAK/10/2006, with which they have approximately 97.6% sequence identity as shown in Table 1.

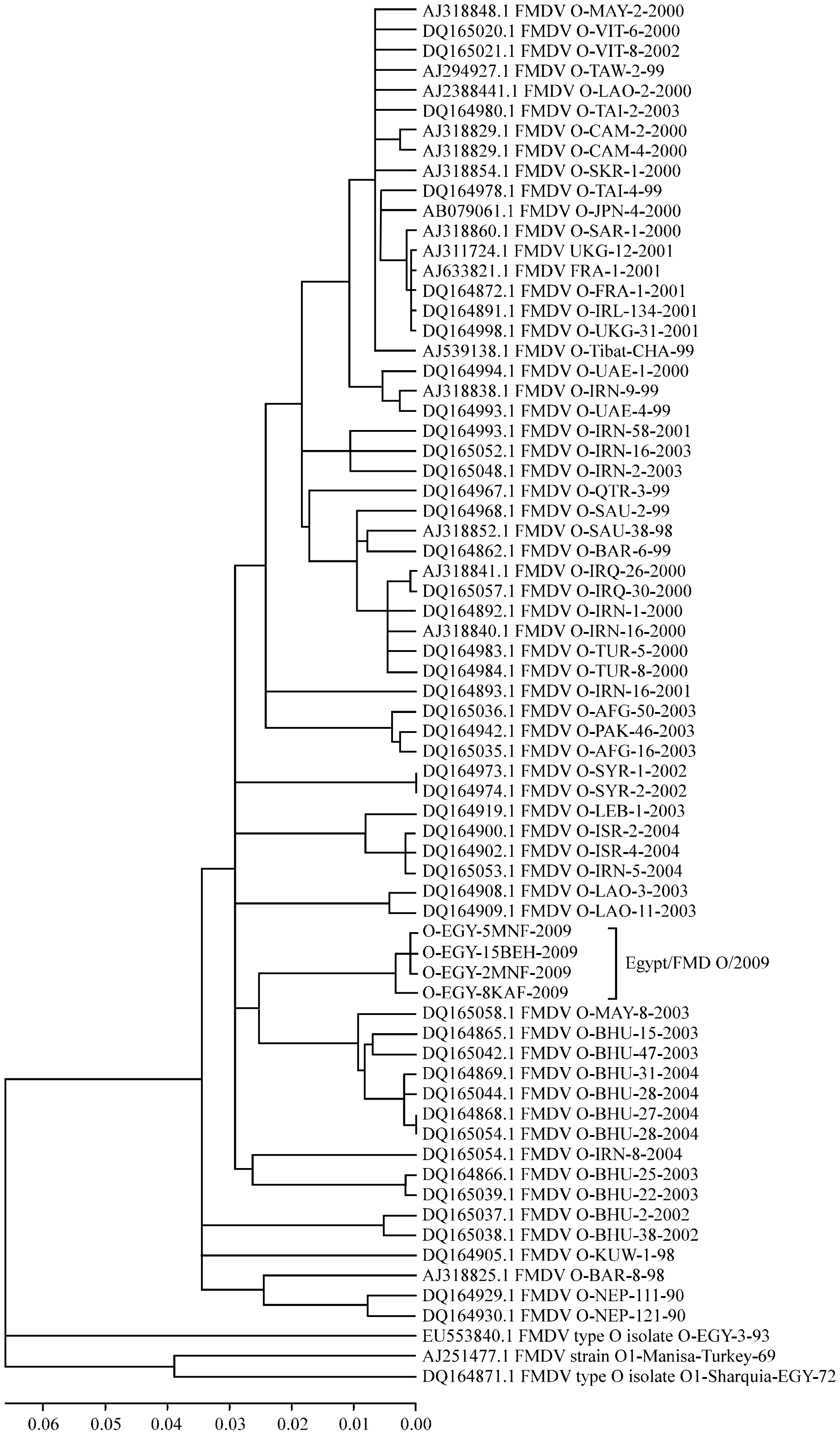

Fig. 3: Neighbor-joining phylogenetic tree of FMDV local isolates in comparison with the Egyptian isolates, the results of blast and representative isolates for PanAsia isolates within ME-SA topotype of serotype O of FMDV

Table 1: Deduced amino acid sequence homology percent of the obtained partial sequences of VP1 gene from the FMDV samples in the current study (columns) compared with other reference strains sequences (rows), homology percent generated by bioedit software version 7.0.8

	O/Egy/5MNF/2009* (%)	O/Egy/8KAF/2009** (%)	O/Egy/2MNF/2009*** (%)	O/Egy/15BEH/2009**** (%)
O1-Manisa-Turkey-69	93.8	93.8	93.8	93.8
O-JOR-6-2006	98.0	98.5	98.0	98.0
O-JOR-7-2006	97.1	97.6	97.1	97.1
O-ISR-3-2007	97.6	98.0	97.6	97.6
O-PAK-10-2006	98.0	98.5	98.0	98.0
O-PAK-02-2006	98.0	98.5	98.0	98.0
O-ISR-5-2007	98.0	98.5	98.0	98.0
O1-Sharquia-Egy-72	94.2	94.2	94.2	94.2
O-Egy-3-93	94.2	94.2	94.2	94.2

*O/Egy/5MNF/2009: Partial VP1 sequence obtained from sample No. 5 from Menofia governorate from Egypt (code is the isolate local designation), **O/Egy/8KAF/2009: Partial VP1 sequence obtained from sample No. 8 from Kafr Elsheikh governorate from Egypt (code is the isolate local designation), ***O/Egy/2MNF/2009: Partial VP1 sequence obtained from sample No. 2 from Menofia governorate from Egypt (code is the isolate local designation) ****O/Egy/5BEH/2009: Partial VP1 sequence obtained from sampleNo. 5 from Beheira governorate from Egypt (code is the isolate local designation)

When compared with the strain used for vaccine preparation in Egypt (O1/Manisa/Turky/69), all detected viruses were recorded values of 13.5% nucleotide sequence difference and were placed in a different group as Shown in Fig. 3.

DISCUSSION

Foot-and-mouth disease virus has been known as one of the most fearful viral pathogen of animals, since it is highly contagious among 70 species of cloven hoofed mammals (Carrillo *et al.*, 2005).

The VP1 genomic region that chosen for amplification contains the sequence for major antigenic sites of the virus capsid located between amino acids 138 and 160 VP1 (Strohmaier *et al.*, 1982). These sites which expose at the surface of the virion, is responsible for the induction of serum-nutralizing antibodies and represent the most variable part of the capsid (Robertson *et al.*, 1983). Thus sequence analysis of this region of the genome was expected to be suitable for the differentiation of FMDV isolates and the nucleotide sequence obtained from the VP1 gene will provide information on the structure of the antigenic domain in VP1 polypeptide using a computer program (Becker, 1987). Accordingly, the present study conducted to sequence the full length VP1 coding region of four samples. The results demonstrated the VP1 coding region was 633 nt in length and encoded 211 aa as shown in Fig. 1.

A VP1-based sequence similarity tree of several ME-SA FMDV-O isolates showed that the detected virus was most closely related to FMDV isolates O/JOR/2006, O/ISR/5/2007 and O/PAK/10/2006 by high percent of relatedness as shown in Fig. 1.

The genome of FMD is non segmented positive sense RNA strand with a high mutation rate about 1-8 nucleotides per replication cycle (Domingo *et al.*, 1995). In the present study nucleotide sequencing of whole VP1 genomic region coding for the outer capsid polypeptide VP1 the four examined samples. The results indicated that the presence of multiple nucleotide changes when compared with that of O1/Manisa/ Turkey/ 69 (Accession No. AJ251477.1).

Comparison of amino acid sequences of whole VP1 of the examined samples with that of O1/Manisa/Turkey/69 revealed that the presence of multiple amino acid changes especially at the major immunogenic site (positions 137 to 41) as well as positions 3, 95, 107, 108, 156, 157 and 197 as shown in Fig. 1. Most mutations were clustered within two distinct regions corresponding to the antigenic site 1 as described previously by kitson *et al.* (1990) they showed that the main antigenic site 1 comprising G-H loop (amino acid positions 133-158 and C terminus region (amino acid positions 194-211)).

FMDV can attach to cells via argine-glycine-aspartic acid (RGD) binding integrin molecules (Neff *et al.*, 1998). This motif is located in a flexible loop on the virion surface (Logan *et al.*, 1993) and is found on all seven serotypes. The results of the study carried out by Xiansheng *et al.* (2004) they reported that from analysis of P1 polypeptide was found that the RGDL tetra-peptides motifs are found in all types Os. This results agreed with that of the present study where the RGDL tetrapeptides motifs are found at positions from 144 to 147 in all samples as shown in Fig. 1. The presence of mutations in the main antigenic site 1 (amino acid positions 133-138) in the VP1 gene of FMDV as presented in the results of amino acid graphic view as shown in Fig. 1 exclude the possibility that improbably inactivated vaccines have been responsible for any of the outbreaks.

However, the percent of relatedness to the Egyptian FMDV isolates were 96% as shown in Table 1. In terms of phylogenetic interpretations, FMDV that differ in 2-5% from each other are generally believed to originate from the same epizootic (Samuel *et al.*, 1997). From that and the results of the present study there are seven amino acid changes at positions 138-158 as shown in Fig. 1 placed on the main antigenic site which explain the relatedness of the detected virus to vaccinal strain (O1/Manisa/Turkey/69) although, there are vaccinal failure and the presence of outbreaks. The common and major epitope of FMDV is located within the surface protein VP1 containing the immuno-dominant G-H loop and RGD integrin binding motif, essential for cell attachment (Fox *et al.*, 1989) changes in this protein may cause vaccine failure and changes in host specificity (Hernandez *et al.*, 1996). This agreed with presented results.

The four VP1 sequences reported in the present study were compared to a number of previously published. Aboot strapped neighbor joining trees containing all listed sequences were constructed by using MEGA 5. Figure 2 indicated that the four detected FMDV as well as the Egyptian reference strains were places in ME-SA topotype in two separate sublineage but in Fig. 3 indicated that detected virus more related to PanAsian strain than Egyptian isolates.

Viruses of the PanAsian strain (within the ME-SA) showed a limited degree of variability of the VP1 gene during the outbreak in 2001 in the United Kingdom. The results of the present study show that the degree of genetic variability of VP1 gene between the four samples representative the three governorates of Egypt was 0%.

PanAsian strain represents a new sublineage of FMDV emerged first in India and then transmitted to Middle East and Europe years later. This strain apparently confined to India for long time and then spread much faster than previously believed (Knowles *et al.*, 2005). Viruses of the PanAsian strain emerged first in Iran, Gulf area and Yemen in 1998, then in the United Arab Emirates, Turkey and Israel at 1999, Malysia and South Africa in 2000 (Knowles *et al.*, 2005). The present study demonstrates the existence of PanAsia strain in Egypt and this strain may be responsible for outbreaks in Egypt 2009.

PanAsian viruses dominated and out-competed other viruses in the Middle East area (Samuel *et al.*, 1997) and there was a substantial increase in the number of PanAsian isolates from Middle East (Knowles *et al.*, 2005).

This domination could be confirmed by the absence of other strains that were circulating in Middle East. Large scale outbreaks and persistence were characteristic to this strain, also induction of FMD outbreaks in three large dairy cattle farms in KSA, 2000. The cattle on these farms are regularly vaccinated with good vaccine which laboratory tests indicate should protect the cattle but in spite of vaccination and high security of the farms, the new virus was able to invade and induce clinical disease (Knowles and Samuel, 2003).

There is no evidence of increased or altered trade in region that could explain the sudden spread of the PanAsia virus. Additionally, the lack of efficacy of existing FMDV vaccines does not seem to be responsible for the spread of this strain in countries in which vaccination is practiced. Indeed, antigenic matching analysis has shown good cross-reactivity between field isolates of the PanAsia and current vaccine strains such as O1/Manisa/Turky/69 and this finding has confirmed for O/UKG/2001 virus by cross-protection studies (Cox *et al*., 2005). This may have agreed with the results of the present study where the percent of relatedness between the detected viruses and FMDV serotype O present in Egypt as Shown in Table 1.

CONCLUSION

Despite the strict control policies and quarantine measures at the borders, PanAsian viruses were able to evolve and get established in the Middle East area. Such emergence event sounds the alarm for how important is monitoring and tracking of FMD outbreak sources especially for endemic countries and the necessity to shift to more effective prevention and control programs to be implemented.

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