

Isolation, Sequencing and Phylogenetic Analysis of FMD Strains During 2011-2012 Outbreaks in Egypt

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Abstract: Foot and mouth disease is a severe, highly contagious disease of cloven-hoofed animals. In this study, 1042 samples were collected from 423 clinically diseased, cross bred cattle of different ages in Behira and Sharkia governorates, Egypt; 136 samples were collected from cattle during June, 2011 and 906 samples were collected from cattle during March, 2012. Viral isolation was performed on BHK-21 cell line and revealed that 23.5% and 37.3% of 2011 and 2012 samples, were positive respectively. FMDV type A and O were identified during 2011 while type A, O and SAT2 were identified during 2012 by conventional RT-PCR.

Sequencing and phylogenetic analysis revealed that type A belongs to Asia topotype and Iran 05 lineage, type O belongs to ME-SA topotype and PanAsia-2 lineage while type SAT2 belongs to topotype VII and lineage Ghb-12. So these strains should be included in vaccine preparation. From the previous data we can conclude that molecular characterization of FMDV is essential for effective control strategies of the disease in Egypt and subsequently, FMD vaccines should be changed continuously due to continuous introduction and/or mutation of new serotypes of the virus.

Keywords : FMD, PCR, Phylogenetic Analysis .

1. INTRODUCTION

Foot-and-mouth disease (FMD) is an acute systemic infection affecting even-toed ungulates, both domesticated and wild, including cattle, swine, sheep and goats. FMD generally involves mortality rates below 5%, but even so it is considered the most important disease of farm animals since it causes important decreases in livestock productivity and trade (Woodbury, 1995 and Doel, 1996).

Cross challenge experiments in cattle have allowed the classification of FMD virus into seven immunologically distinguishable serotypes: O, A which exist in Asia, South America and Africa, and Asia-1 and the SAT types (South African Territories SAT1, SAT2 and SAT3) which occur in Asia and sub-Saharan Africa, respectively (Brooksby, 1982), type C currently occurs only in East Africa (Bastos, 2001).

The immediate determination and molecular characterization of the serotypes of field virus is essential in order to administer emergency vaccination with

appropriate antigen and to trace the source of an outbreak. (Remond *et al.*, 2002).

The aims of this study :

1. Viral isolation and typing of the viral isolates by RT-PCR.

2. Sequencing and Identification of the phylogenetic relationships between viral isolates, reference strains and vaccinal strains.

2. MATERIALS AND METHODS

1) *Animals:* The present study was performed on 423 clinically diseased, cross bred cattle of different ages from Behira and Sharkia governorates (during June, 2011 and March, 2012). In two localities animals were vaccinated with bivalent vaccine of FMD (type O and A) (Serum and Vaccine Research Institute, Abbasia).

2) *Sampling:* A total of 1042 samples were collected from animals included in this study. 136 samples were collected from cattle during June, 2011, which divided into 80 Saliva and 56 Buffy coat samples. 906 samples were collected from cattle during March, 2012, these samples were divided into; 100 oral vesicular fluid, 73 teat vesicular fluid, 129 oral epithelium, 157 saliva, 126 buffy coat, 138 serum, 129 plasma and 54 heart tissue according to Otto *et al.* 1996, Hui, 2004, Paixão *et al.*, 2008 and OIE, 2009.

3) *Viral isolation from vesicular fluid, oral epithelium, saliva and buffy coat samples on tissue culture:* The viral isolation was carried out according to Hanaa (1995) and Otto *et al.* (1996).

4) Baby hamster kidney (BHK-21) cells were distributed into a 24 well plate which were incubated at 37 °C and examined for the confluency of the monolayer sheet (80 %).

1. *Extraction of FMD viral RNA :*

RNA was extracted from tissue culture media (obtained after virus isolation), plasma and serum samples by QIAamp viral RNA mini kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's instructions.

RNA was extracted from heart tissue by RNeasy Mini Kit (Qiagen, Valencia, Calif., USA) according to the man-

manufacturer's instructions.

2. Typing of FMD viral RNA :

Type A, O and SAT2 were identified by conventional RT-PCR.

Reaction mix was performed according to the manufacturer's instructions of Qiagen One-Step RT-PCR

Kit (*Qiagen, Valencia, Calif., USA*). Electrophoresis was performed at 100 volts for 20 min. Positive result is indicated under ultra violet by the presence of the specific band corresponding to the sequence of 1D (VP1) region of the FMDV genome (*Knowles and Samuel, 1998*).

Table (1) : Primers used for typing of FMDV by RT-PCR { Knowles and Samuel, (1998) and IAH – Method/Protocol Sheet, (2012) :

Primer name	Sequence 5'- 3'	Serotype-size of amplified segment	
FMD-O-1C244F (Forward)	GCAGCAAAACACATGTCAAACACCTT	O 1165 bp	
FMD-NK72 (Reverse)	GAAGAAGGGCCCAGGGTTGGACTC		
FMD-A-1C612F (Forward)	TAGCGCCGGCAAAGACTTTGA		A 814 bp
SAT-1D209F (Forward)	CCACATACTACTTTTGTGACCTGGA	SAT2 716 bp	
SAT -2B208R (Reverse)	ACAGCGGCCATGCACGACAG		

3. Sequencing and phylogenetic analysis of FMD 1D (VP1) gene :

BLAST search (*Altschul et al., 1990*) was performed on the nucleotide sequences of 1D (VP1) gene obtained from this study within Gene bank to confirm the results of typing and to determine the closely related strains. Phylogenetic analysis was performed using MEGA 4 software (*Tamura et al. 2007*).

2.2. Typing of FMDV detected during, 2012 by RT-PCR :

Type O, A and SAT2 were identified during, 2012 by RT-PCR. Type A was not identified from Sharkia governorate. FMDV type SAT2 was identified on 20.1% from all samples collected during 2012, type O was identified on 11.4% while type A was identified on 3.5%.

3.RESULTS

1. Viral Isolation :

1.1 Viral isolation from Behira and Sharkia governorates during, 2011 :

From saliva and buffy coat samples collected during, 2011, 23.5% showed cytopathic effect (CPE) on BHK-21 cells which is characterized by rounding of the cells, granularity of the cytoplasm and complete cell lysis. 30% from saliva samples and 14.3% from buffy coat samples were positive for viral isolation.

1.2. Viral isolation from Behira and Sharkia governorates during, 2012 :

Viral isolation from samples collected during, 2012 on BHK-21 cells revealed that 37.3% showed (CPE). 61% from oral vesicular fluid samples, 49.3% from teat vesicular fluid samples, 35.7% from oral epithelial samples, 31.8% from saliva samples and 19.8% from buffy coat samples were positive for detection of (CPE).

2. Typing of FMDV by RT-PCR :

2.1. Typing of FMDV isolates during, 2011 :

Only type O and type A was identified during, 2011. Type O was isolated from 11.3 and 3.6% while type A was isolated from 15 and 8.9% of saliva and buffy coat samples respectively.

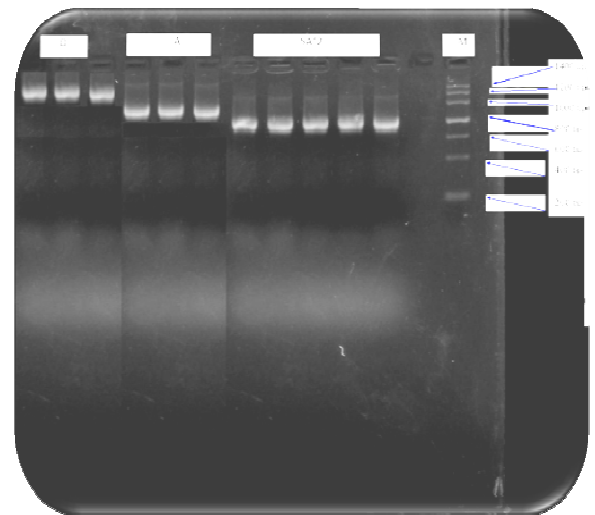


Figure (1) : Agarose gel electrophoresis of RT-PCR products compared with 200 bp marker. Type O demonstrated by the presence of 1165 bp band. Type A demonstrated by the presence of 814 bp band. Type SAT2 demonstrated by the presence of 716 bp band.

3. Sequencing and phylogenetic analysis :

3.1. Type O identified during, 2011 :

3.1.1. Percentage of identity with other strains :

The most closely related strains with type O identified during, 2011 are O/EGY/8KFS-2009, and O/EGY/2MNF-

2009 (with nucleotide divergence 4%), O/EGY/MNF-2009 and O/PAK04/2006 (with nucleotide divergence 5%). Nucleotide divergence with O1/Manisa/Turkey/69 is 17% and with O1/Sharquia/EGY/72 is 19% as shown in table (2).

Examination of deduced amino acids of VP1 of this isolate revealed 10 and 12 amino-acids substitution with O/EGY/8KFS-2009 and O/EGY/2MNF-2009, respectively and it is the lowest number of substitution with the analyzed strains. While amino-acids substitution with O1/Sharquia/EGY/72 is 39.

3.1.1.2. Phylogenetic tree :

Phylogenetic tree was constructed using MEGA 4 software and revealed that the present isolate and O/EGY/8KFS-2009, O/EGY/MNF-2009 and O/EGY/2MNF-2009 originate from the same ancestor, also revealed that this isolate closely related to the isolates of Pakistan, 2006, 2007 and 2008 which belongs to ME-SA Topotype and PanAsia-2 Lineage (figure, 2).

3.2. Type O identified during, 2012 :

3.2.1. Percentage of identity with other strains :

The most closely related strains with type O identified during, 2012 are O/EGY/8KFS-2009, O/EGY/2MNF-2009, O/EGY/15BH-2009 and O/EGY/MNF-2009 with 4% nucleotide divergence, OKAB/AFG/L2826/2009 and O/JOR/7/2006 with 5% nucleotide divergence. Nucleotide divergence with O1/Manisa/Turkey/69 and O1/Sharquia/EGY/72 is 16% and 19%, respectively while nucleotide divergence with O/EGY/3/93 is 15% (table, 3).

Amino-acid substitution is four with O/EGY/8KFS-2009, O/EGY/2MNF-2009 and O/EGY/15BH-2009 and three with OKAB/AFG/L2826/2009, while with O/EGY/3/93 is six.

3.2.2. Phylogenetic tree :

Tree examination revealed that the present isolate and O/EGY/8KFS-2009, O/EGY/MNF-2009, O/EGY/2MNF-2009 and O/EGY/15BH-2009 originate from the same ancestor and closely related to OKAB/AFG/L2826/2009 (figure, 3).

3.3. Type A identified during, 2011 :

3.3.1. Percentage of identity with other strains :

The most closely related strains with type A identified during, 2011 are A/EGY/IS/2011 (with nucleotide divergence 2%), A/EGY/KFS/2011 and A/EGY/GZ/2011 (with nucleotide divergence 3% and 4% respectively). Nucleotide divergence with A/SAR/AFG/L1434/2009 is 3%. While divergence with A/EGY/3/2006 is 22% (table, 4).

Comparison of amino-acids, deduced from the VP1 sequence of type A identified during, 2011 and other strains, revealed one amino-acid substitution with A/EGY/KFS/2011, two amino-acids substitutions with A/EGY/IS/2011, A/EGY/GZ/2011 and A/SAR/AFG/L1434/2009 and eight amino-acid substitutions with A/EGY/3/2006.

3.3.2. Phylogenetic tree :

Examination of phylogenetic tree revealed that the present isolate, A/EGY/KFS/2011, A/EGY/IS/2011,

A/EGY/GZ/2011 and A/SAR/AFG/L1434/2009 have the same ancestor (figure, 4).

3.4. Type A identified during, 2012:

3.4.1. Percentage of identity with other strains :

Nucleotide divergence between type A identified during, 2012 and A/EGY/IS/2011, A isolate 4234, A/KAB/AFG/L1163/2008 and A/SAR/AFG/L1434/2009 is 3% while divergence with A/EGY/3/2006 is 29% (table, 5).

Amino-acid substitution with A/EGY/IS/2011 is six, A isolate 4234 is three and A/EGY/2/2006 is 62.

3.4.2. Phylogenetic tree :

Phylogenetic tree revealed that this isolate, A/EGY/GZ/2011, A/EGY/IS/2011, A/EGY/KFS/2011, A/SAR/AFG/L1434/2009, A/KAB/AFG/L1163/2008 and A isolate 4234 have the same ancestor (figure, 5).

3.5. Type SAT2 identified during, 2012 :

3.5.1. Percentage of identity with other strains :

SAT2/EGY/9/2012 and SAT2/EGY/11/2012 have 2% nucleotide divergence with the isolate identified on this study while SAT2/EGY/4/2012, SAT2/EGY/5/2012, SAT2/EGY/13/2012 and SAT2/EGY/7/2012 have 3% divergence. The nucleotide divergence with SAT2/ERI/1/98 and SAT2/CAR/8/2005 is 13%. Nucleotide divergences with SAT2/LIB/7/2003 and SAT2/SUD/1/2007 are 12% and 14% respectively (table, 6).

Amino-acid substitution with SAT2/EGY/9/2012 and SAT2/EGY/11/2012 is four while substitutions with SAT2/ERI/1/98, SAT2/SAU/6/00, SAT2/CAR/8/2005 and SAT2/LIB/7/2003 are 14, 16, 17 and 18 amino-acid respectively.

3.5.2. Phylogenetic tree :

Examination of phylogenetic tree revealed that this isolate originates with other Egyptian isolates (except SAT2/EGY/2/2012) from the same ancestor and the most closely related foreign strains are SAT2/SAU/6/00 and SAT2/ERI/1/98 (figure, 6).

Table (2) : Nucleotide divergence (%) between type O isolated during 2011 and other closely related, reference and vaccinal strains using Maximum composite likelihood method of MEGA 4 software

Isolates name	Nucleotide divergence (%)																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1-FMDV type O identified in 2011																														
2-O/ISR/2/88 (DQ164899)	17																													
3-O/India/R2/75 (AF204276)	17	17																												
4-O1/Manisa/Turkey/69 (AJ251477)	17	16	10																											
5-O1/Sharquia/EGY/72 (DQ164871)	19	17	10	7																										
6-O/EGY/8KFS-2009 (JQ837834)	4	17	14	15	17																									
7-O/EGY/2MNF-2009 (JQ837835)	4	17	14	14	17	1																								
8-O/EGY/MNF-2009 (JQ837833)	5	17	15	15	17	1	0																							
9-O/PAK04/2006 (EF494500)	5	14	12	13	14	3	3	3																						
10-O/MAY/1/2004 (HQ632770)	7	16	12	13	14	5	5	5	2																					
11-O/ Israel 07-6380 (FJ175662)	6	15	12	13	14	3	3	3	1	3																				
12-O/TUR/926/2010 (JX040496)	9	17	14	16	17	7	6	7	5	6	6																			
13-O/TUR/27/2011 (JX040500)	10	17	15	16	18	7	7	7	5	6	7	1																		
14-O/BUL/30/2011 (JX040489)	9	17	15	16	17	7	7	7	5	7	6	1	1																	
15-O/BUL/1/2010 (JX040485)	9	17	15	16	17	7	7	7	5	7	6	0	1	0																
16-O/ISR/2/2011 (JX040501)	11	18	16	18	17	8	8	8	6	8	8	1	2	1	1															
17-O/NEP/002/2003 (HQ268519)	7	15	11	12	14	5	4	5	2	1	3	6	6	6	6	7														
18-O/Iran/1/2010 (HQ663879)	9	16	15	16	17	7	6	7	5	6	6	1	1	1	1	2	6													
19-O/EGY/3/93(EU553840)	15	14	10	11	11	15	15	15	12	12	13	14	14	15	15	16	12	14												
20-O/IRN/073/2001 (JF749851)	10	14	12	10	11	9	9	9	7	6	7	9	10	9	9	10	6	10	9											
21-O/TAW/2/99 (AJ539136)	10	14	12	10	11	9	9	9	6	5	7	9	9	10	10	5	9	10	3											
22-O/PAK/2/2008 (FJ798191)	6	14	12	12	14	3	3	3	0	3	1	5	6	5	5	7	2	5	12	6	6									
23-O/PAK/53/2007 (FJ798179)	5	14	12	13	15	3	3	3	1	3	2	5	5	5	5	6	3	5	13	7	7	1								
24-O/JOR/7/2006 (FJ561318)	5	15	12	13	15	2	2	3	1	3	1	5	6	5	6	7	3	5	13	7	7	1	1							
25-O/ISR/3/2007 (FJ561313)	6	15	12	13	14	3	3	3	1	3	0	6	7	6	6	8	3	6	13	7	7	1	2	1						
26-O/NEP/6/2003 (DQ165061)	6	15	12	12	14	5	4	5	2	1	3	6	6	6	6	7	1	6	12	6	5	2	3	3	3					
27-O/OKAB/AFG/L2826/2009 (HQ439238)	6	14	13	13	14	4	4	5	2	4	3	6	6	6	6	7	4	6	12	7	7	2	2	3	3	4				
28-O/BHU/47/2003 (DQ165042)	6	15	12	12	14	5	5	5	2	1	3	6	6	7	7	8	1	6	12	5	4	3	3	3	3	0	4			
29-O/UKG/1734/2001 (FJ542368)	12	15	13	11	13	11	11	11	8	7	9	10	10	11	11	11	7	11	11	3	2	8	9	9	9	7	9	7		
30-O/BHU/30/2004 (DQ165045)	7	16	13	13	15	5	5	5	3	1	4	7	7	7	7	8	1	7	12	6	5	3	3	3	4	1	5	1	7	
31-O/MAY/3/2006 (HQ116206)	7	14	12	13	14	5	5	6	3	2	4	6	7	7	7	8	3	6	11	6	6	3	3	3	4	3	4	3	8	3

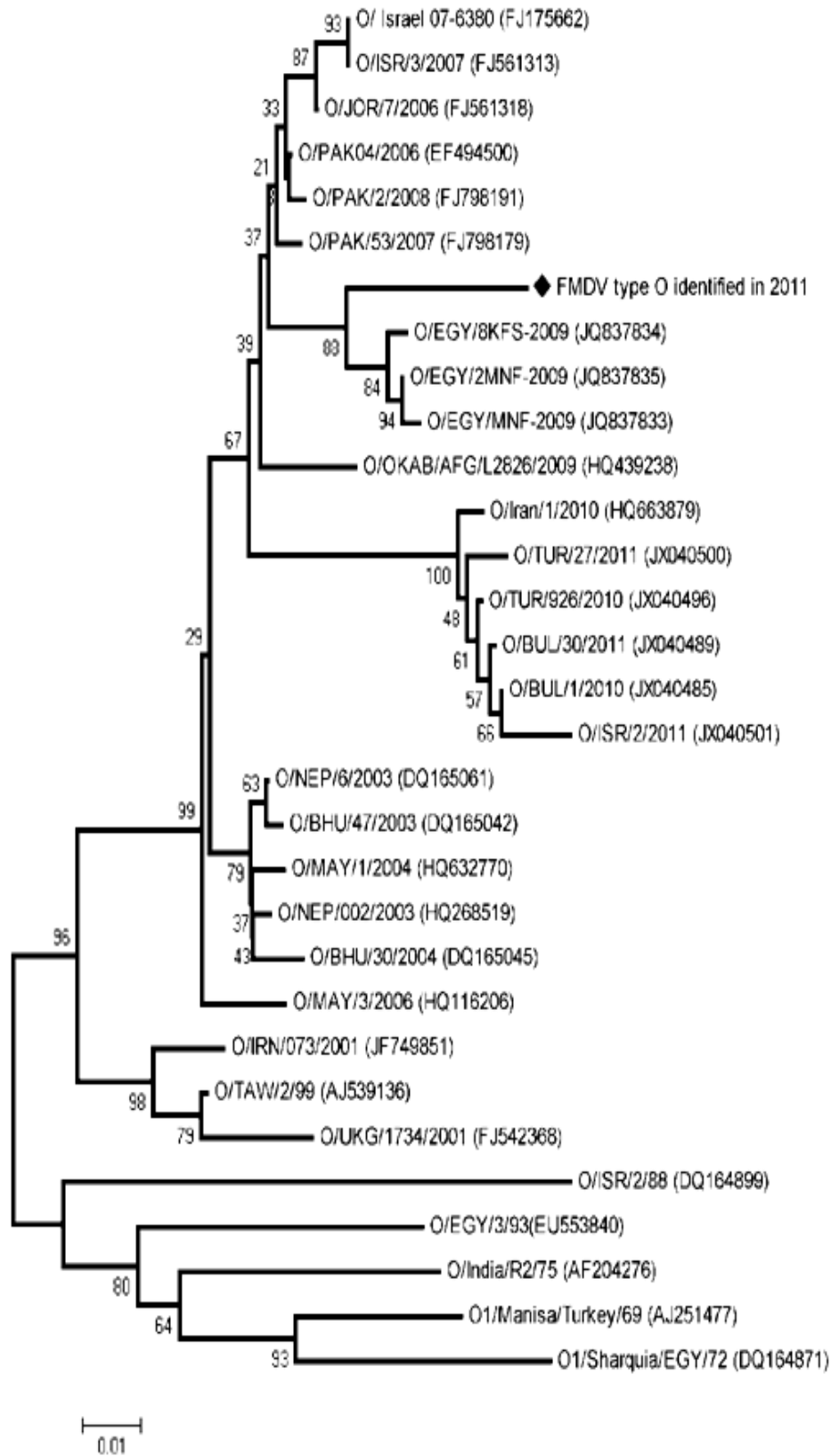


Figure (2) : Illustrates rooted neighbor-joining (NJ) tree of type O isolated in 2011 (marked) with other closely related, vaccinal and reference strains with accession number of each.

Table (3) : Nucleotide divergence (%) between type O isolated during 2012 and other closely related, reference and vaccinal strains using Maximum composite likelihood method of MEGA 4 software

Isolate's name	Nucleotide divergence (%)																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1-FMDV type O identified in 2012																														
2-O1/Manisa/Turkey/69 (AJ251477)	16																													
3-O/HKN/6/83 (AJ294919)	25	26																												
4-O/PHI/5/95 (DQ164946)	25	27	10																											
5-O/ISR/2/88 (DQ164899)	16	16	26	27																										
6-O/India/R2/75 (AF204276)	16	9	24	26	17																									
7-O/TAW/2/99 (AJ539136)	10	10	24	26	15	11																								
8-O1/Sharquia/EGY/72 (DQ164871)	19	6	26	27	19	9	11																							
9-O/EGY/8KFS-2009 (JQ837834)	4	13	26	26	16	13	9	16																						
10-O/EGY/15BH-2009 (JQ837836)	4	13	25	25	17	13	09	16	0																					
11-O/EGY/2MNF-2009 (JQ837835)	4	13	25	25	17	13	9	16	0	0																				
12-O/EGY/MNF-2009 (JQ837833)	4	14	25	26	17	13	10	16	1	0	0																			
13-O/EGY/3/93 (EU553840)	15	11	27	27	16	10	12	12	15	15	15	15																		
14-O/PAK04/2006 (EF494500)	5	12	26	27	15	10	7	14	2	2	2	3	13																	
15-O/Israel/ 07-6380 (FJ175662)	6	12	27	27	15	11	8	13	3	3	3	4	13	1																
16-O/BHU/27/2004 (HQ268524)	6	12	26	28	16	11	6	14	5	5	5	6	12	3	4															
17-O/MAY/1/2004 (HQ632770)	6	12	27	27	17	11	5	13	5	5	5	5	13	3	4	1														
18-O/Akterek/02/2012 (JQ765584)	8	14	29	29	16	15	11	16	8	8	8	8	15	6	7	8	8													
19-O/Tynali/05/2011 (JQ765583)	7	14	27	28	17	13	10	15	7	7	7	8	15	6	6	7	7	2												
20-O/PAK/45/2008 (GU384683)	9	11	26	26	17	13	7	12	7	7	7	8	13	6	6	5	5	8	7											
21-O/Korday/02/2012 (JQ765585)	8	14	29	29	16	15	11	16	8	8	8	8	15	6	7	8	8	0	2	8										
22-O/Korday/2010 (JQ765586)	5	15	29	30	16	14	9	17	5	5	5	6	14	3	4	4	5	5	5	8	5									
23-O/Iran/1/2010 (HQ663879)	9	16	28	29	17	14	12	17	6	6	6	6	16	5	6	7	7	10	9	10	10	6								
24-O/NEP/002/2003 (HQ268519)	6	12	27	26	16	10	5	13	5	5	5	5	13	3	4	1	1	8	7	5	8	5	7							
25-OKAB/AFG/L2826/2009 (HQ439238)	5	14	25	27	15	12	9	15	4	4	4	4	13	2	4	5	5	8	7	8	8	4	6	5						
26-O/PAK/53/2007 (FJ798179)	5	12	26	27	14	11	8	14	2	2	2	3	14	1	2	4	4	6	6	6	6	4	5	4	2					
27-O/UKG/4998/2001(EF552694)	12	11	27	28	17	14	2	14	12	12	12	13	13	10	10	8	8	12	12	9	12	10	13	8	11	11				
28-O/JOR/7/2006 (FJ561318)	5	13	27	27	15	11	8	14	2	2	2	3	13	0	1	4	3	7	6	6	7	3	6	3	3	1	10			
29-O/MAY/3/2000 (HQ632768)	13	12	25	28	17	13	2	13	12	12	12	12	14	9	10	8	7	14	13	9	14	12	13	7	11	10	4	10		
30-O/MAY/3/2006 (HQ116206)	5	12	26	27	15	10	6	14	4	4	4	5	12	2	04	2	2	8	7	5	8	4	6	2	4	3	9	3	9	
31-O/MAY/7/2005 (HQ116201)	5	12	26	27	15	10	6	14	4	4	4	5	12	2	4	2	2	8	7	5	8	4	6	2	4	3	9	3	9	0

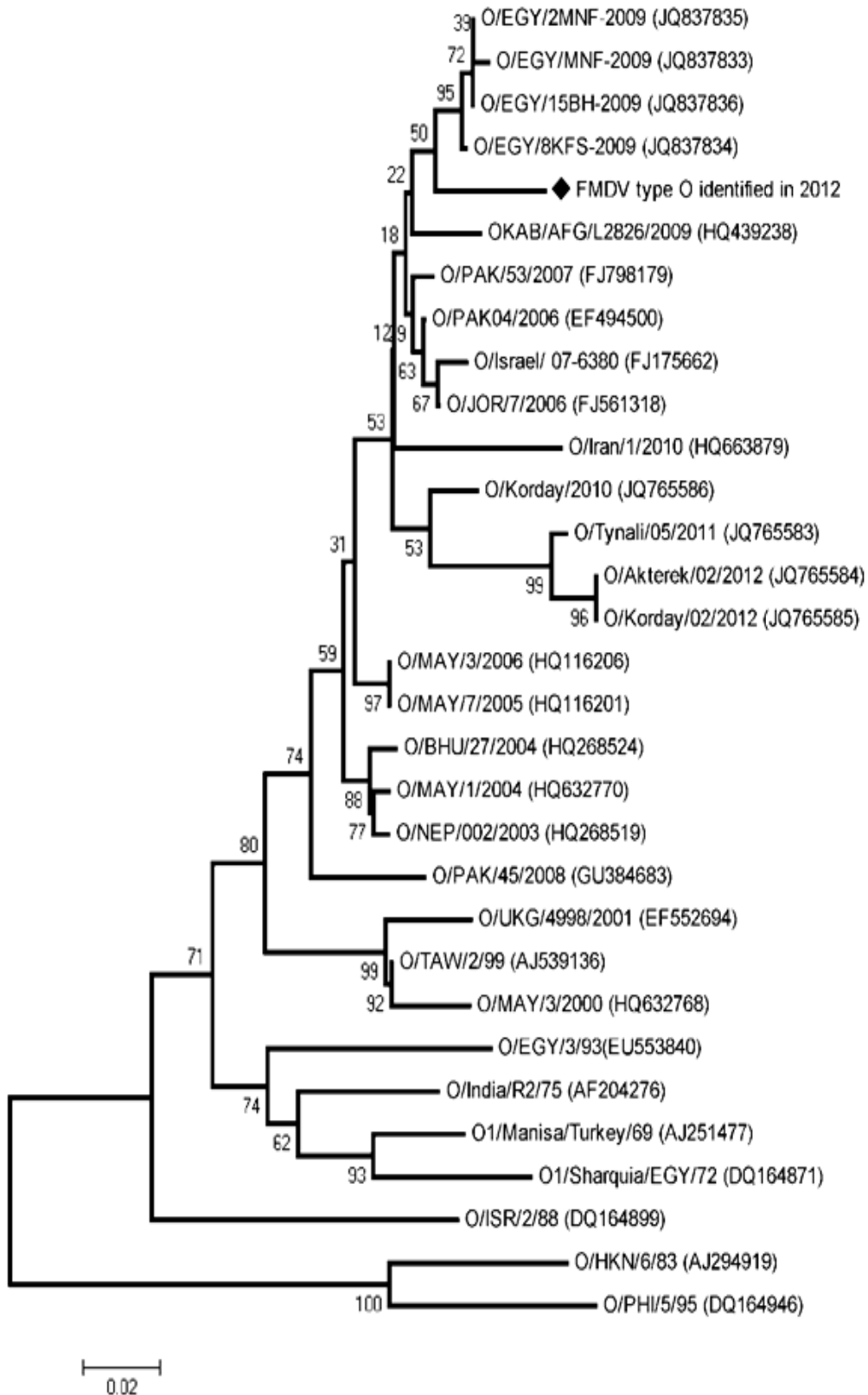


Figure (3) : Illustrates rooted neighbor-joining (NJ) tree of type O isolated in 2012 (marked) with other closely related, vaccinal and reference strains with accession number of each.

Table (4) : Nucleotide divergence (%) between type A isolated during 2011 and other closely related, reference and vaccinal strains using Maximum composite likelihood method of MEGA 4 software

Isolate's name	Nucleotide divergence (%)																																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38			
1-FMDV type A identified in 2011																																									
2-A/IND/7/82(FJ755014)	11																																								
3-A/TAI/2/97 (EF208778)	12	12																																							
4-A/IRN/22/99 (EF208772)	17	12	16																																						
5-A/IRN/1/96 (EF208771)	16	10	18	15																																					
6-A/SAU/23/86(EU414536)	13	8	14	8	10																																				
7-A/SAU/41/91 (FJ755085)	15	8	14	12	14	9																																			
8-A/IRQ/24/64(AJ251474)	23	15	19	15	13	17	18																																		
9-A24/Cruzeiro/Brazil/55^	17	17	20	21	17	18	21	18																																	
10-A/TAI/118/87 (EF208777)	15	8	5	13	15	10	11	16	21																																
11-A/IRN/2/87 (EF208770)	17	10	14	14	14	9	4	15	20	11																															
12-A/SAU/16/95(EU553874)	13	11	14	14	11	9	13	21	21	13	14																														
13-A/IRN/1/2005 (EF208769)	5	8	11	14	13	10	9	17	16	12	12	10																													
14-A/EGY/2/2006 (EF208758)	24	22	20	24	23	26	22	16	21	21	20	26	21																												
15-A/EGY/4/2006 (EF208760)	22	21	19	22	22	25	21	15	20	20	18	25	20	1																											
16-A/EGY/3/2006 (EF208759)	22	23	21	25	24	27	23	18	22	22	21	28	22	3	2																										
17-A/EGY/5/2006 (EF208761)	24	25	23	26	26	29	25	19	24	24	22	28	23	4	3	1																									
18-A/Egy/2006/1sm (EF159977)	22	21	19	22	22	25	21	15	20	20	18	25	20	1	0	02	3																								
19-A/EGY/IS/2011(JX645709)	2	10	12	15	14	13	15	20	15	15	15	13	5	21	20	20	21	20																							
20-A/EGY/KFS/2011(JX645708)	3	9	11	16	13	12	14	19	16	14	16	10	4	21	20	22	24	20	3																						
21-A/EGY/GZ/2011(JX645707)	4	10	12	15	14	13	14	19	15	15	14	13	4	20	19	21	22	19	2	3																					
22-A/EGY/1/72(EF208756)	22	20	24	24	18	22	25	21	22	25	24	21	16	15	17	19	15	20	19	20																					
23-A isolate 4258(JN099702)	3	9	11	16	15	12	13	20	16	14	15	12	3	21	20	22	24	20	3	2	1	21																			
24-A/SAR/AFG/L1434/2009*	3	9	11	16	15	12	14	21	16	14	16	12	4	22	21	23	25	21	3	2	3	21	2																		
25-A/PAK5/2006 (EF494488)	5	7	11	14	13	10	11	18	16	12	13	10	1	21	20	22	24	20	5	4	3	21	2	4																	
26-A/IRN/7/2005 (JF749847)	5	8	11	14	13	10	9	17	16	12	12	10	0	21	20	22	23	20	5	4	4	21	3	4	1																
27-A/AFG186/2005 (JF749845)	5	8	11	14	13	10	9	17	16	12	12	10	0	21	20	22	23	20	5	4	4	21	3	4	1	0															
28-A/Turkey/2006 (JF749841)	5	8	11	14	13	10	9	17	16	12	12	10	0	21	20	22	23	20	5	4	4	21	3	4	1	0	0														
29-A/KAB/AFG/L1163/2008"	3	9	11	16	15	12	13	20	16	14	15	12	3	21	20	22	24	20	3	2	1	21	0	2	2	3	3	3													
30-A/BAR/7/2008(FJ755011)	3	9	11	16	15	12	13	20	16	14	15	12	3	21	20	22	24	20	3	2	1	21	0	2	2	3	3	3	0												
31-A/AFG/44/2007 (FJ755009)	4	8	12	15	14	11	12	19	17	13	14	11	2	22	21	23	25	21	4	3	2	22	1	3	1	2	2	2	1	1											
32-A/TAI/003/2001(HQ268508)	11	13	5	16	13	15	15	18	17	7	13	13	10	18	17	19	20	17	8	9	8	19	9	9	9	10	10	10	9	9	10										
33-A/TUR/25/2007(FJ755125)	5	8	11	14	13	10	9	17	16	12	12	10	0	21	20	22	23	20	5	4	4	21	3	4	1	0	0	0	3	3	2	10									
34-A/TUR/24/2006(FJ755117)	5	8	11	14	13	10	9	17	16	12	12	10	0	21	20	22	23	20	5	4	4	21	3	4	1	0	0	0	3	3	2	10	0								
35-A/TUR/12/2005(FJ755100)	5	8	11	14	13	10	9	17	16	12	12	10	0	21	20	22	23	20	5	4	4	21	3	4	1	0	0	0	3	3	2	10	0	0							
36-A/SAU/16/2005 (FJ755087)	5	8	11	14	13	10	9	17	16	12	12	10	0	21	20	22	23	20	5	4	4	21	3	4	1	0	0	0	3	3	2	10	0	0	0						
37-A/IRN/1/2008 (FJ755066)	5	7	11	12	13	8	10	19	16	10	12	8	2	22	21	23	25	21	5	4	5	21	4	4	2	2	2	2	4	4	3	9	2	2	2	2	2	2			
38-A/IRN/15/2007 (FJ755061)	5	7	11	12	13	8	10	19	16	10	12	8	2	22	21	23	25	21	5	4	5	21	4	4	2	2	2	2	4	4	3	9	2	2	2	2	2	0			
39-A/IRN/7/2006(FJ755057)	5	8	11	14	13	10	9	17	16	12	12	10	0	21	20	22	23	20	5	4	4	21	3	4	1	0	0	0	3	3	2	10	0	0	0	0	0	2	2		

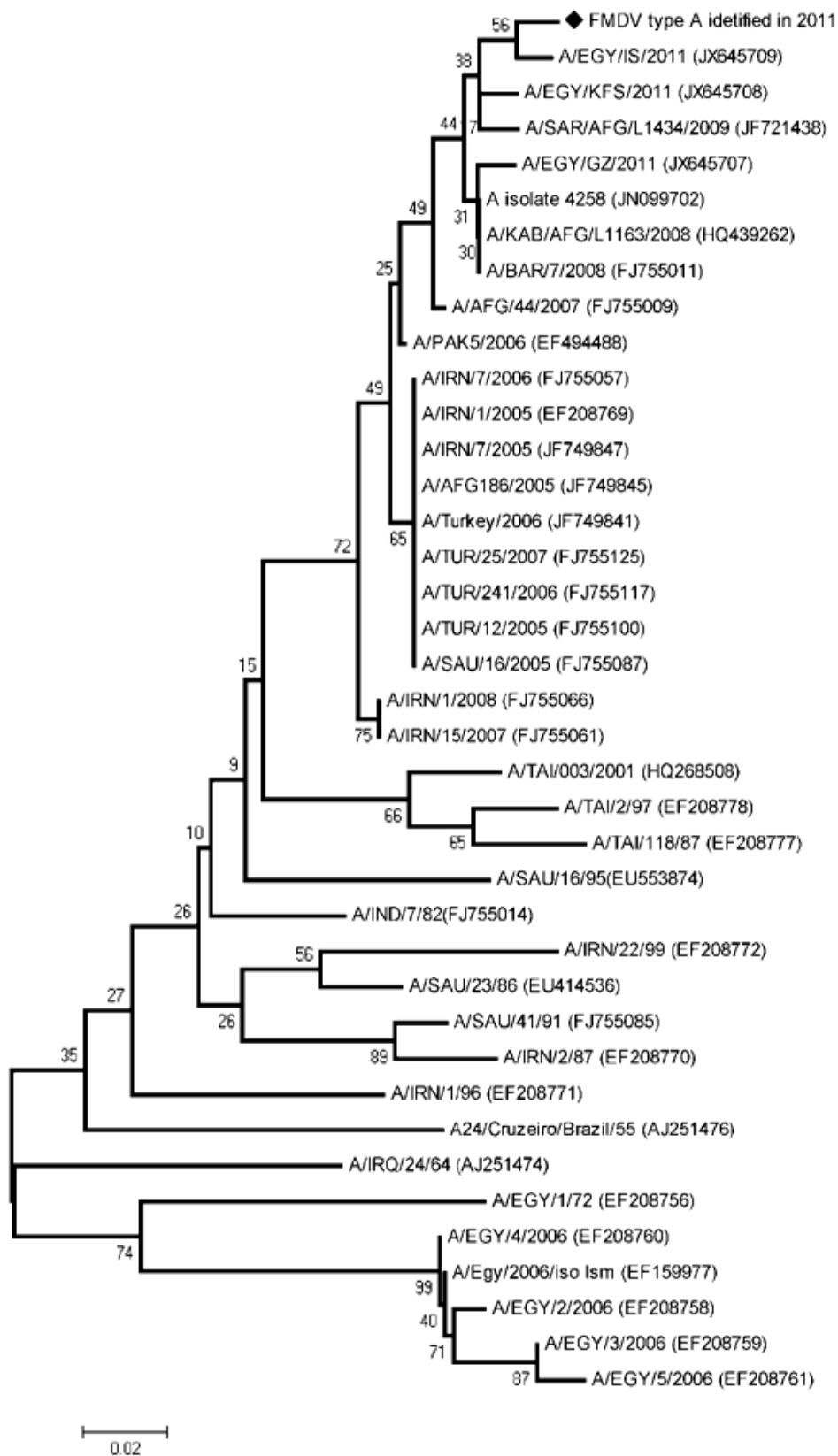


Figure (4) : Illustrates rooted neighbor-joining (NJ) tree of type A isolated in 2011 (marked) with other closely related, vaccinal and reference strains with accession number of each.

Table (5) Nucleotide divergence (%) between type A isolated during 2012 and other closely related, reference and vaccinal strains using Maximum composite likelihood method of MEGA 4 software

Isolate's name	Nucleotide divergence (%)																																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37		
1-FMDV type A identified in 2012																																							
2-A/IND/7/82(FJ755014)	19																																						
3-A/TAI/2/97 (EF208778)	18	19																																					
4-A/IRN/22/99 (EF208772)	20	21	23																																				
5-A/IRN/1/96 (EF208771)	18	18	21	19																																			
6-A/SAU/41/91 (FJ755085)	16	17	18	14	18																																		
7-A/IRQ/24/64 (AJ251474)	26	21	24	23	17	20																																	
8-A24/Cruzeiro/Brazil/55 (AJ251476)	22	26	25	28	21	23	20																																
9-A/IRN/2/87 (EF208770)	19	18	17	15	15	5	17	23																															
10-A/TAI/118/87(EF208777)	19	16	7	22	17	18	20	24	18																														
11-A/SAU/16/95 (EU553874)	18	18	19	17	11	18	16	21	16	16																													
12-A/IRN/1/2005 (EF208769)	8	19	19	17	14	22	23	16	20	16																													
13-A/EGY/2/2006 (EF208758)	30	32	29	32	26	30	25	24	27	31	30	30																											
14-A/EGY/4/2006 (EF208760)	30	31	29	32	25	29	24	23	27	30	28	29	1																										
15-A/EGY/3/2006 (EF208759)	29	32	29	32	25	29	25	24	27	32	29	30	2	1																									
16-A/EGY/5/2006 (EF208761)	29	33	29	33	26	30	25	24	27	32	29	31	2	1	0																								
17-A/Egy/2006/iso 1sm (EF159977)	29	31	28	31	25	29	24	23	26	30	29	29	1	0	1	1																							
18-A/EGY/KFS/2011 (JX645708)	8	24	23	26	22	21	31	27	24	25	23	11	35	34	35	36	34																						
19-A/EGY/GZ/2011 (JX645707)	8	24	24	24	22	22	31	27	23	25	23	11	35	34	35	36	33	2																					
20-A/EGY/IS/2011	3	23	21	22	20	20	28	25	21	23	20	11	33	32	31	32	32	5	4																				
21-A/EGY/1/72 (EF208756)	28	29	27	29	23	27	25	24	27	28	25	30	20	20	20	21	19	30	32	30																			
22-A_isolate_4234 (JN099689)	3	18	18	19	17	16	25	22	19	19	17	6	28	28	29	29	27	6	5	5	26																		
23-A/PAK5/2006 (EF494488)	7	17	18	16	16	15	23	23	17	19	16	1	30	30	31	31	29	10	10	9	29	5																	
24-A/KAB/AFG/L1163/2008 (HQ439262)	3	18	19	19	17	16	26	23	19	19	18	6	29	28	29	30	28	6	5	5	27	0	5																
25-A/SAR/AFG/L1434/2009 (HQ439273)	3	19	18	20	17	16	26	23	18	21	17	8	29	29	29	30	29	8	8	5	27	2	7	3															
26-A/SIN/PAK/L758/2009 (HQ439250)	4	19	20	20	17	17	26	23	19	19	18	7	29	28	29	30	28	6	6	6	26	1	6	1	3														
27-A/BAR/6/2008 (FJ755010)	3	18	19	19	17	16	26	23	19	19	18	7	28	28	29	29	27	6	6	6	26	1	5	1	3	0													
28-A/SIN/PAK/L4/2008 (JN006722)	8	18	17	18	17	15	23	24	16	17	17	4	31	30	31	32	30	11	11	11	28	6	3	6	8	7	6												
29-A/AFG/44/2007 (FJ755009)	7	17	18	17	16	15	23	23	17	19	16	2	30	29	30	31	29	10	9	9	29	4	1	5	6	5	5	2											
30-A/AFG/160/2005 (EF457982)	7	18	19	17	16	14	22	23	16	20	16	1	30	29	30	31	29	11	11	10	30	5	1	6	7	6	6	4	2										
31-A/IRN/4/2008 (FJ755067)	7	19	19	17	18	16	24	24	18	19	18	4	30	30	31	31	29	10	9	9	28	4	2	5	6	5	5	3	1	3									
32-A/SAU/16/2005 (FJ755087)	7	18	19	16	16	14	22	23	17	19	16	1	30	30	31	31	29	10	10	9	30	5	0	5	7	6	6	3	1	0	3								
33-A/IRN/3/2007 (FJ755060)	8	18	19	16	16	16	23	24	18	20	16	3	31	31	32	32	30	12	11	11	28	5	1	6	8	6	6	4	2	2	4	2							
34-A/IRN/5/2006 (FJ755056)	8	18	20	18	18	15	23	25	17	20	16	2	32	32	32	33	31	11	11	11	31	6	2	6	8	7	7	5	3	1	5	2	3						
35-A/TUR/8/2005 (FJ755096)	8	19	20	18	17	15	22	24	17	20	17	1	30	30	31	31	29	11	11	11	31	6	1	6	8	7	7	4	2	1	4	1	3	2					
36-A/TUR/13/2008 (FJ755141)	9	20	19	19	18	14	22	23	15	20	17	3	28	28	29	29	28	13	13	11	29	7	4	7	9	8	8	5	4	3	6	3	5	5	4				
37-A/TUR/20/2006 (FJ755116)	8	18	19	17	17	14	22	23	16	19	16	0	29	29	30	30	28	11	11	10	30	6	1	6	8	7	6	3	2	0	3	1	2	2	1	3			
38-A/TUR/1018/2007 (FJ755131)	9	20	19	19	18	15	22	23	16	21	18	4	29	29	30	30	28	13	13	11	29	7	4	7	9	8	8	4	4	4	6	4	5	5	5	1	3		

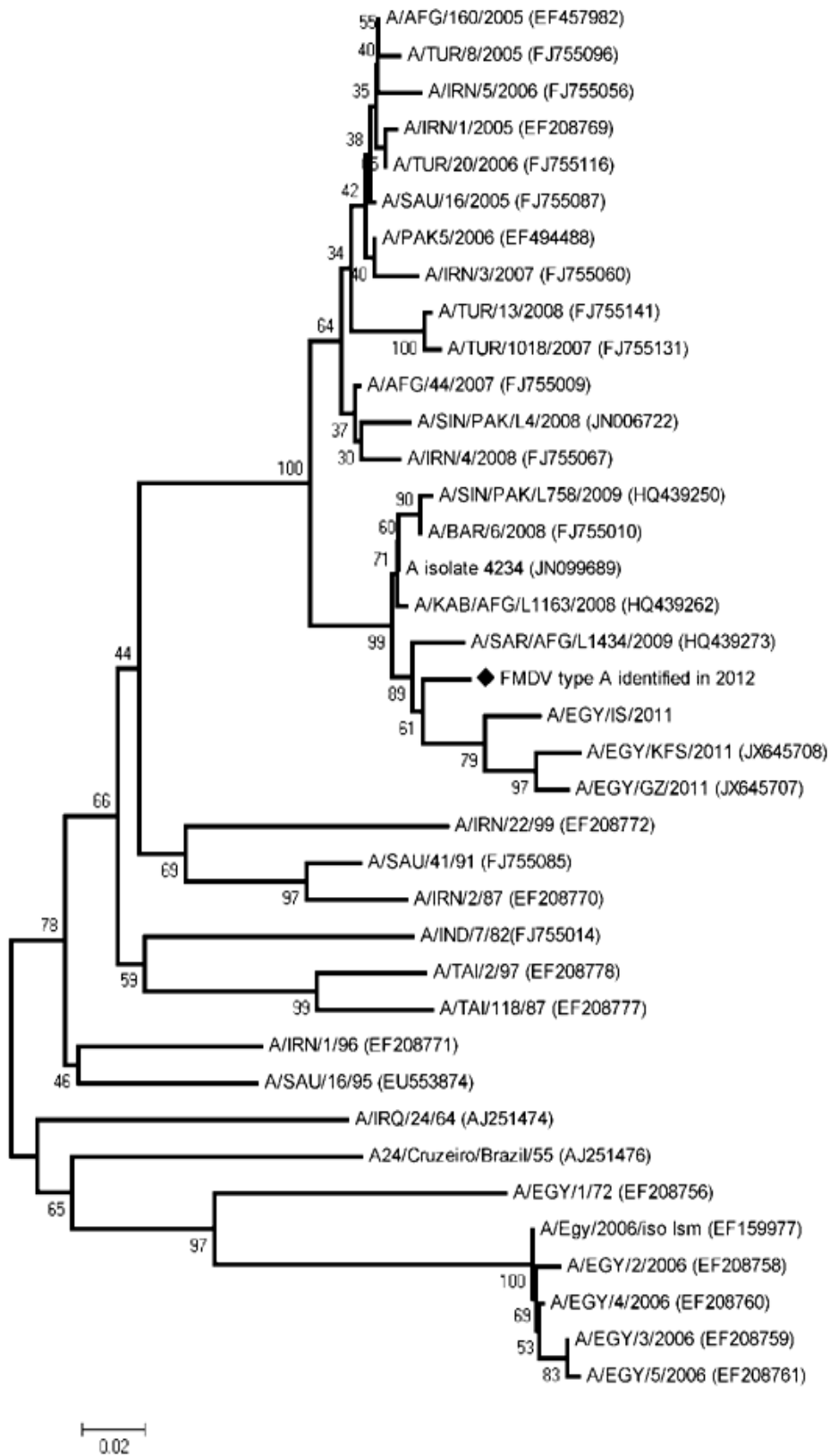


Figure (5) illustrates rooted neighbor-joining (NJ) tree of type A isolated in 2012 (marked) with other closely related, vaccinal and reference strains with accession number of each.

Table (6) Nucleotide divergence (%) between type SAT2 isolated during 2012 and other closely related, reference and vaccinal strains using Maximum composite likelihood method of MEGA 4 software

Isolate's name	Nucleotide divergence (%)																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1-FMDV type SAT2																													
2-SAT2/ZAI/1/82(AF367100)	36																												
3-SAT2/UGA/51/75(AY343963)	34	31																											
4-SAT2/UGA/19/98(AY343969)	33	23	27																										
5-SAT2/ETH/2/91(FJ798159)	36	36	34	34																									
6-SAT2/KEN/2/84(AY343941)	32	29	30	27	30																								
7-SAT2/EGY/9/2012(JX014255)	2	34	33	32	35	31																							
8-SAT2/EGY/7/2012(JX013978)	3	34	33	33	35	31	0																						
9-SAT2/EGY/11/2012(JX570624)	2	34	33	32	35	31	0	0																					
10-SAT2/EGY/13/2012(JX570625)	3	34	33	33	35	31	0	0	0																				
11-SAT2/EGY/5/2012(JX570620)	3	35	34	33	36	32	0	0	0	0																			
12-SAT2/EGY/4/2012(JX570619)	3	35	34	33	36	32	0	0	0	0	0																		
13-SAT2/EGY/3/2012(JX570618)	3	34	33	33	35	31	0	0	0	0	0	0																	
14-SAT2/EGY/26/2012(JX013979)	3	34	34	33	35	32	0	0	0	0	0	0	0																
15-SAT2/EGY/23/2012(JX013980)	3	35	33	33	35	31	0	0	0	0	0	0	0	0															
16-SAT2/EGY/2/2012(JX570617)	12	35	33	34	33	34	10	10	10	10	10	10	10	10	10														
17-SAT2/EGY/14/2012(JX570626)	3	34	33	33	35	31	0	0	0	0	0	0	0	0	0	10													
18-SAT2/EGY/15/2012(JX570627)	3	34	33	33	35	31	0	0	0	0	0	0	0	0	0	10	0												
19-SAT2/EGY/10/2012(JX570623)	3	34	33	33	35	31	0	0	0	0	0	0	0	0	0	10	0	0											
20-SAT2/EGY/6/2012(JX570621)	3	34	33	33	35	31	0	0	0	0	0	0	0	0	0	10	0	0	0										
21-SAT2/LIB/7/2003(JX570632)	12	35	34	34	36	33	10	10	10	10	10	10	11	10	11	12	10	10	10	10									
22-SAT2/PAT/1/2012(JX014256)	3	34	33	33	35	31	0	0	0	0	0	0	0	0	0	10	0	0	0	0	10								
23-SAT2/CAR/8/2005(JX570616)	13	35	34	37	36	34	11	11	11	11	12	12	12	12	12	14	11	11	11	11	7	11							
24-SAT2/ERI/1/98(AY343933)	13	35	31	34	34	34	12	12	12	12	12	12	12	12	12	13	12	12	12	12	10	12	10						
25-SAT2/SUD/1/2007(GU566071)	14	36	33	36	36	35	12	12	12	13	13	13	12	13	13	13	12	12	12	12	8	12	8	11					
26-SAT2/SAU/6/00(AY297948)	14	34	33	34	34	35	12	12	12	12	12	12	12	12	12	9	12	12	12	12	10	12	10	11	12				
27-SAT2/NIG/2/2007(JX570636)	14	35	33	35	35	35	12	13	12	13	13	13	13	13	13	13	13	13	13	13	9	13	9	12	5	10			
28-SAT2/3kenya-21(AY593849)	30	29	28	31	30	17	29	29	29	30	30	30	29	30	29	31	29	29	29	29	32	29	31	30	32	30	32		
29-SAT2/KEN/3/57(AJ251473)	32	31	31	33	32	19	31	31	31	31	31	31	31	31	31	32	31	31	31	31	34	31	32	32	34	31	33	5	
30-SAT2/ZIM/5/83(JQ639289)	35	35	32	36	33	33	32	33	32	33	33	33	32	32	33	35	33	33	33	33	34	33	35	36	35	36	34	35	36

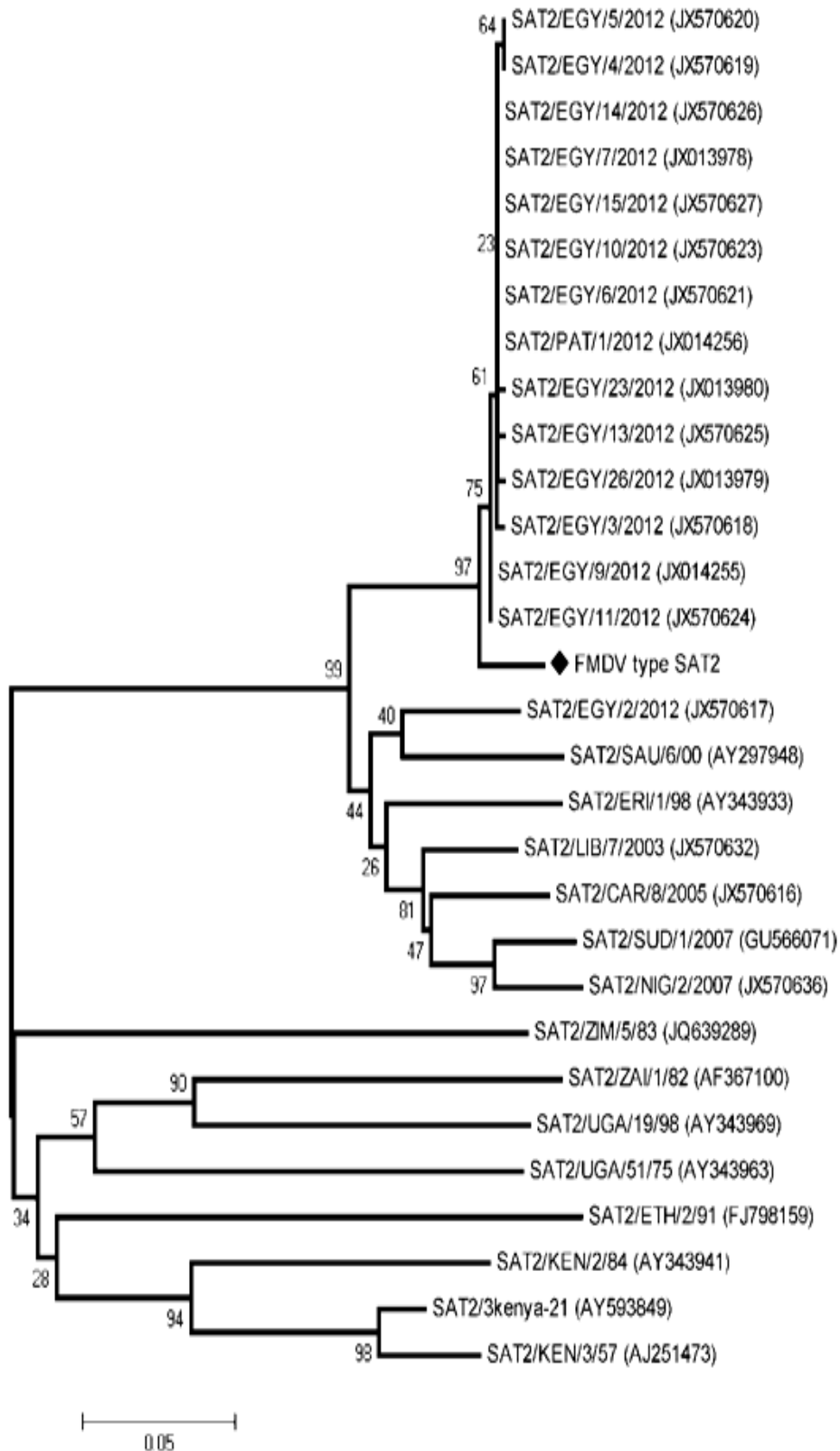


Figure (6) illustrates rooted neighbor-joining (NJ) tree of type SAT2 isolated in 2012 (marked) with other closely related, vaccinal and reference strains with accession number of each.

4. DISCUSSION

Many outbreaks of foot and mouth disease occur every year although the regular vaccination of cattle by the government with bivalent vaccine of FMD (type O and A) so it is important to determine the type and genetic characters of the virus causing these outbreaks and its relationship with the vaccinal strains. This study began in 2011 and during the study an outbreak of foot and mouth disease occurred (March, 2012), thousands of animals took infection and died during this outbreak, although many animals were vaccinated with bivalent vaccine of FMD (type O and A). It was important to determine whether this outbreak occurred due to type O and A which were reported in Egypt or due to a new type of FMD virus to which animals are naïve. In this study several samples were collected from cattle with different ages (during, 2011 and 2012) to determine the type of FMD virus which caused these outbreaks in order to determine the strain of the virus which should be used as a vaccine.

The present study indicated that foot and mouth disease is prevalent in Egypt with several outbreaks occur from time to time causing mild to severe lesions in cattle. The disease was caused by type A, O (which are endemic in Egypt) and SAT2 which was introduced for the first time since 1950 in 2012. The most prevalent clinical symptom found during clinical examination of animals from which samples were taken during, 2011 and 2012 was fever ranged from 39.5°C to 41°C. Severe oral and foot lesions were observed during, 2012 and mild lesions were observed during, 2011 which may return to the result obtained by viral isolation of FMDV type SAT2 during, 2012 to which animals are naïve. Animals which were infected early during March, 2012 suffered from severe clinical symptoms while animals infected late during March, 2012 suffered from mild clinical symptoms, which may be resulted from the adverse environmental condition (windy and cold weather) occurred early during March, 2012 which inhibited animals' immunity and led to secondary bacterial infection with *Pasteurella* and *clostridia* spp. Sudden death was observed in young as well as adult animals. Death in adult animals can be returned to secondary bacterial infection (*Pasteurella* and/or *Clostridia* spp., while in young animals is a result of myocarditis (Domingo *et al.*, 1990) and secondary bacterial infection. SAT 2 FMD infections played an important role in appearance of HS outbreak in buffalo and cattle in Alexandria governorate; Egypt causing high mortality rates especially in 6-15 months age buffalo and cattle calves respectively (Elshemey and Abd-Elrahman, 2013).

RT-PCR procedures have been evaluated at the world reference laboratory (WRL) for the routine diagnosis of FMD virus using universal primers for all seven serotype. Real-time RT-PCR is a powerful technique for reliable detection of FMDV (Reid *et al.*, 1998 and Paixão *et al.*, 2008).

The most prevalent type of FMDV during 2012 was SAT2 as it was isolated from 20.1% from all collected samples. It is the first outbreak of type SAT2 on Egypt since 1950 and the animals were not vaccinated against this type, which can explain the rapid spread and high prevalence of it among animals. Type A was not identified in Sharkia governorate during 2012, while it was identified in Behera governorate in 3.5% of total collected samples. Type O was identified from all localities subjected to this study with the highest percentage was detected in 11.4% of samples, this result is lower than the result obtained by Jamal *et al.* (2011) who identified serotype O in 28% from oral swab and epithelial samples collected from clinically suspected cases and the result obtained by EL-Shehawey *et al.* (2011) who detected type O on 42.1% from collected samples.

Understanding the epidemiology of a disease is essential for the formulation of the most effective control strategies. Determining the source of outbreaks is an important element of epidemiological investigations and for FMD this can be done by nucleotide sequencing (Samuel and Knowles, 2001). The main immunogenic site 1 of FMD viruses is located on the 1D region which makes the VP1 region the gene of choice to study variation and relationships between isolates (Sangare, 2005). Nucleotide sequence analysis of the G-H loop in the VP1 region of the viral genome is required to develop a specific vaccine against local strains of the virus (Hui, 2004).

Sequencing of 1D gene (VP1) of FMDV type O detected during 2011 and subsequent phylogenetic analysis with MEGA 4 software revealed that this isolate is closely related to Egyptian isolates of 2009 and isolates of Pakistan, 2006, 2007 and 2008 which belongs to ME-SA Topotype and PanAsia-2 Lineage. Nucleotide divergence with O/EGY/3/93 (which is used as a vaccinal strain of the vaccine produced by Serum and Vaccine Research Institute, Abbassia and belongs to Sharquia-72 lineage) is 15% (table 17) and amino-acid substitution is 32. Type O identified during 2012 is also closely related to OKAB/AFG/L2826/2009 which belongs to ME-SA topotype and PanAsia-2 lineage and has a high nucleotide divergence with O/EGY/3/93 (15%), (table 18). As Samuel and Knowles (2001) set a cut-off value for the division of FMDV topotypes at 15% and 20% nucleotide difference for the Eurasian and Southern African serotypes, respectively, this indicates that FMD type O vaccinal strain (O/EGY/93) has a high nucleotide divergence from the type O field strains which was isolated during 2011 and 2012 and because subtype do not induce total cross protection against other viruses of the same serotype (Brown, 2003 and Jamal *et al.*, 2011), it is advisable to include these isolates of the new lineage or the most closely related reference strain (O/TAW/2/99) in the vaccine production with O/EGY/93 to induce complete protection against circulating viruses. This result also indicates that the isolates of 2011 and 2012 belongs to the same topotype and lineage and that can be confirmed by the result obtained by Sangare *et al.* (2004) who reported that long-term maintenance of certain topotypes occurred

for periods of up to 24 years and appeared in more than one country.

Type A which was identified during 2011 and 2012 is closely related to the isolates of 2011(A/EGY/GZ/2011, A/EGY/IS/2011 and A/EGY/KFS/2011), also is closely related to the isolates of Asia topotype and Iran 05 lineage (A isolate 4234, A/SAR/AFG/L1434/2009 and A/KAB/AFG/L1163/2008) while it is highly divergent from A/EGY/2006 {> 20% nucleotide difference (table 19 and 20)} which belongs to another topotype (Africa) and which is used as a vaccinal strain of the vaccine produced by Serum and Vaccine Research Institute, Abbassia. From This result and as mentioned before we can conclude that vaccine should contain field strain of Asia topotype and Iran 05 lineage or the closely related reference strain (A/IRN/1/2005) with A/EGY/2006 (Africa topotype and G-VII^{KEN-05} lineage) to induce complete protection .

Phylogenetic analysis of SAT2 type which isolated during 2012 revealed that this isolate is closely related to other isolates of SAT2 type detected on Egypt (except SAT2/EGY/2/2012), (Figure 12) and share with them on the same origin (topotype VII and lineage Ghb-12). The most closely related foreign strains are SAT2/SAU/6/00 and SAT2/ERI/1/98 (topotype VII). Lineage Ghb-12 is distinct from a contemporary SAT 2 lineage of the same topotype of Libya (*Ahmed et al., 2012*), which indicates that disease source is not through the uncontrolled boundaries. It is the first FMD outbreaks due to this serotype in Egypt since 1950 so the animals were free from the antibody, which can illustrate the rapid spread of the disease. Vaccine should contain this new serotype and which was already done.

5. CONCLUSION

Molecular characterization of FMDV is essential for effective control strategies of the disease in Egypt and subsequently, FMD vaccines should be changed continuously due to continuous introduction and/or mutation of new serotypes of the virus.

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