Mutations in foot and mouth disease virus types A and O isolated from vaccinated animals

This paper (No. 12112019-00149-EN) has been peer-reviewed, accepted, edited, and corrected by authors. It has not yet been formatted for printing. It will be published in December 2019 in issue **38** (3) of the *Scientific and Technical Review.*

D.A. Abdulrahman [(1)], A.H. El-Deeb [(2)], N.G. Shafik [(3)], M.A. Shaheen [(1)] & H.A. Hussein [(2)*]

(1) Department of Virology, Animal Health Research Institute (AHRI), Nady El Seid Street, Ad Doqi, Giza, Egypt

(2) Department of Virology, Faculty of Veterinary Medicine, Cairo University, El-Shaheed Gamal El-Deen Afify Street, Giza, Egypt

(3) Quality Control Research Department for Evaluation of Large Animal Viral Vaccines, The Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), El Sekka El Baydaa Street, Abbasseya, Egypt

*Corresponding author: husvirol@cu.edu.eg

Summary

Foot and mouth disease (FMD) is responsible for serious economic losses in Egypt. Although vaccination is practised as the main control strategy, failure of vaccination has been reported in many cases, which can be due to a number of factors. Selection of FMD antigenic variants under the immune pressure of partially immunised hosts has been previously recorded. This study was designed to isolate and characterise foot and mouth disease viruses (FMDVs) circulating in Egyptian vaccinated animals. Serotype O, A and South African Territories (SAT) 2 FMDVs were detected in different Egyptian governorates during 2015, 2016 and 2017. The successful isolation of 15 FMDVs of the three serotypes is reported herein. Phylogenetic analysis based on the viral protein (VP) 1 gene showed that all serotype O isolates had East

Africa (EA)-3 topotypes. There was variation in 15–17 amino acids between the serotype O isolates of 2015 and those of 2016 and 2017. The serotype A isolates belonged to the A-Iran-05 lineage, with the exception of one isolate of 2016 which clustered with the African strains of G-IV. Serotype SAT2 FMDV was detected in two samples of 2017 and both were of lineage Alx-12 of topotype VII. The virus neutralisation test (VNT) using sera raised against vaccine strains confirmed the serotyping of the isolates and determined the antigenic relatedness between the isolates and the currently used vaccine strains. A decrease in the neutralising antibody titre of some serotype O and A isolates could be attributed to mutation in critical amino acids in the neutralising antigenic sites. Hence, this work supports previous studies describing the significance of amino acid substitutions within the antigenic sites of the virus in antibody neutralisation and immune escape.

Keywords

Antigenic variation – Egypt – Foot and mouth disease – Molecular epidemiology – Phylogenetic analysis – Virus neutralisation test.

Introduction

Foot and mouth disease (FMD) is a contagious viral disease that affects cloven-hoofed animals (order: Artiodactyla), including domestic and wildlife species (1). It is one of the diseases listed by the World Organisation for Animal Health (OIE) and causes severe economic losses. The major impact of FMD is related to the loss in productivity of adult animals and high mortality of young stock, in addition to animal trade restriction and the excessive costs of control measures in endemic countries (2).

Foot and mouth disease virus (FMDV) is the prototype of genus *Aphthovirus* within the family Picornaviridae. It has a single-stranded, positive-sense ribonucleic acid (RNA) genome of about 8,500 nucleotides. The viral capsid is non-enveloped, icosahedral in shape and consists of 60 copies each of four structural proteins: viral protein (VP) 1, VP2, VP3 and VP4 (3). The first three structural proteins are exposed

on the virus surface and contain five antigenic sites, three of which are located within VP1 (4).

The high genetic and, consequently, antigenic variability of FMDV is reflected in the presence of seven distinct serotypes (A, C, O, Asia1, South African Territories [SAT] 1, SAT2 and SAT3). Each serotype is further divided into topotypes, which have different geographical distributions, on the basis of analysis of the VP1 sequence, the most variable region (5). Serotypes A and O are the most prevalent, being reported in Asia, Africa and South America, while serotypes SAT1, 2 and 3 currently occur only in Africa and serotype Asia1 exists on the Asian continent (6). Serotype C has not caused any outbreaks since it was reported in Kenya and Brazil in 2004 (7).

Foot and mouth disease virus is characterised by a high rate of mutation that ranges from 10^{-3} to 10^{-5} per nucleotide site per replication. This is mainly attributed to the lack of RNA polymerase proofreading ability and results in a quasi-species structure of the FMD viral population (8). Genetic variation in the capsid encoding region is of extreme importance because it may cause changes in the viral epitopes and the development of new antigenic variants, which may compromise vaccination efficiency (9). This, in turn, necessitates regular study of the antigenic relationship between the field and vaccinal strains (10).

Foot and mouth disease has been reported to be endemic in Egypt since 1950, with the introduction of serotype SAT2. The SAT2 virus was again recorded in 2012 and appeared in two lineages, Alx-12 and Ghb-12, which both belong to topotype VII (11). Serotype O FMDV is the most prevalent and was responsible for outbreaks in 1987, 1989, 1990, 1991 and 1993 (12). It was the only serotype reported in Egypt between 1964 and 2005, with the exception of an outbreak involving serotype A in 1972 (13). However, serotype A was introduced again in 2006 through animal importation, causing the economic loss of about 1 million cattle (14). The three serotypes O, A and SAT2 have been co-circulating in Egypt since 2013 (15, 16, 17).

The aim of this work was to characterise the circulating FMDV strains in vaccinated animals in Egypt during 2015, 2016 and 2017 and to study the effect of immune pressure on their genetic and antigenic structure.

Materials and methods

Samples

A total of 94 clinical samples were collected from cattle (*Bos taurus*), buffalo (*Bubalus bubalis*) and sheep (*Ovis aries*) showing signs consistent with FMD, from different Egyptian governorates during 2015, 2016 and 2017. The infected animals had been vaccinated with FMD vaccine that was produced locally by the Veterinary Serum and Vaccine Research Institute (VSVRI) within six months of the onset of the clinical signs (Tables I, II and III). The FMD vaccine used in the vaccination programme was inactivated by 2-bromoethylamine hydrobromide (BEA) and adjuvanted with Oil Instruction Set Architecture (ISA) 206. It contained three FMDV strains, each of a different serotype: O PanAsia of the 2011 outbreak, A-Iran-05 of the 2011 outbreak and SAT2/VII/Ghb-12 of the 2012 outbreak, and contained three to six 50% cattle protective doses (PD50). Cattle and buffaloes were vaccinated with a dose of 3 ml whereas sheep and goats were vaccinated using 1.5 ml, with a booster dose administered to all species three to four weeks later (18, 19). The samples included oral swabs, epithelium and myocardial tissue and were prepared according to the OIE recommended protocols (20) (Table IV).

Table I

List of the dates of vaccination and sample collection during 2015

	Samples collected during 2015	
	Collection date	Vaccination date
Serotype A	January 2015	October 2014
	March 2015	December 2014
	April 2015	January 2015
	April 2015	February 2015
	June 2015	February 2015
Serotype O	April 2015	December 2014
	April 2015	February 2015
	July 2015	April 2015
	July 2015	May 2015
	July 2015	May 2015
	October 2015	August 2015
Negative	January 2015	October 2014
	January 2015	December 2014
	January 2015	December 2014
	February 2015	September 2014
	February 2015	December 2014
	February 2015	January 2015
	February 2015	January 2015
	March 2015	January 2015
	March 2015	February 2015
	April 2015	November 2014
	April 2015	November 2014
	April 2015	November 2014
	April 2015	December 2014
	April 2015	February 2015
	April 2015	February 2015
	April 2015	March 2015
	May 2015	February 2015
	July 2015	February 2015
	July 2015	April 2015
	July 2015	April 2015

The highlighted samples are the foot and mouth disease isolates studied

Table II

List of the dates of vaccination and sample collection during 2016

	Samples collected during 2016	
	Collection date	Vaccination date
Serotype A	January 2016	October 2015
	March 2016	December 2015
	August 2016	May 2016
	September 2016	July 2016
Serotype O	January 2016	September 2015
	January 2016	December 2015
	February 2016	November 2015
	November 2016	September 2016
	December 2016	October 2016
Negative	January 2016	August 2015
	January 2016	August 2015
	January 2016	August 2015
	January 2016	August 2015
	January 2016	August 2015
	January 2016	August 2015
	January 2016	August 2015
	January 2016	October 2015
	January 2016	November 2015
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	May 2016	January 2016
	June 2016	March 2016
	July 2016	March 2016
	July 2016	May 2016
	July 2016	May 2016
	July 2016	May 2016
	August 2016	June 2016
	September 2016	June 2016
	December 2016	October 2016
	December 2016	October 2016

The highlighted samples are the foot and mouth disease isolates studied

Table III

List of the dates of vaccination and sample collection during 2017

	Samples collected during 2017	
	Collection date	Vaccination date
Serotype O	January 2017	November 2016
	January 2017	November 2016
	January 2017	December 2016
	January 2017	December 2016
	February 2017	November 2016
	February 2017	November 2016
	February 2017	November 2016
	February 2017	December 2016
Serotype SAT2	January 2017	November 2016
	January 2017	November 2016
Negative	January 2017	September 2016
	January 2017	September 2016
	January 2017	October 2016
	January 2017	November 2016
	January 2017	November 2016
	January 2017	December 2016
	January 2017	December 2016
	January 2017	December 2016
	February 2017	November 2016
	February 2017	November 2016
	February 2017	November 2016
	February 2017	December 2016
	February 2017	December 2016
	February 2017	January 2017
	February 2017	January 2017
	February 2017	January 2017
	February 2017	January 2017

The highlighted samples are the foot and mouth disease isolates studied

Table IV

List of samples collected from vaccinated animals showing signs of foot and mouth disease infection

Year	Epithelium	Myocardial tissue	Swab	Total*	Cattle	Buffalo	Sheep
2015	30	1	–	**31**	22	9	–
2016	33	2	1	**36**	24	11	1
2017	20	4	3	**27**	17	10	–
Total*	**83**	**7**	**4**	**94**	**63**	**30**	**1**

* Of the total of 94 samples, 2 samples were from Alexandria, 3 from Asyut, 32 from Beheira, 8 from Beni Suef, 9 from Dakhlia, 2 from Damietta, 7 from Faiyum, 1 from Gharbia, 1 from Giza, 2 from Kafr El Sheikh, 1 from Luxor, 3 from Monufia, 1 from New Valley, 6 from Port Said, 14 from Qalyubia and 2 from Suez

Foot and mouth disease virus antigen detection

Serotype O, A, SAT1 and SAT2 FMDV antigens were detected in the prepared clinical samples using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna [IZSLER], Brescia, Italy) according to the manufacturer's instructions.

Foot and mouth disease virus isolation on tissue culture

The samples testing FMD-positive by ELISA were inoculated on a low-passage baby hamster kidney cell (BHK-21) monolayer as described by the OIE (20). The cultures were examined microscopically for the development of a cytopathic effect (CPE) through three successive passages. The specificity of the CPE for FMDV was confirmed by the aforementioned antigen-detection sandwich ELISA kit.

The virus titre in the third passage of each isolate was determined by performing 10-fold serial dilutions, with each dilution represented in five wells of a 96-well tissue culture plate. The TCID50/ml (50% tissue culture infective dose per millilitre) was calculated according to the Reed and Muench equation (21).

Foot and mouth disease virus neutralisation test

The serotype identity of the isolated viruses was confirmed by virus neutralisation test (VNT), according to the OIE methods (20). Polyclonal sera against each of the serotype O, A and SAT2 vaccine strains, kindly provided by the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), were used in the β procedure, in which constant virus and varying serum dilutions were used, neutralisation test utilising 100 TCID50 of each virus. The end-point titres were calculated as the logarithm (base 10) of the reciprocal of the last serum dilution that could neutralise 100 TCID50 of FMDV in 50% of the wells (22).

Ribonucleic acid extraction

The total RNA was extracted from the tissue culture harvests using a Patho Gene-Spin^{TM} deoxyribonucleic acid (DNA)/RNA extraction kit according to the manufacturer's instructions. The extracted RNA was eluted in 30 µl of the kit elution buffer and stored at –70°C for further use.

One-step reverse transcription polymerase chain reaction

Amplification of the full (for serotype O and A FMDVs) or partial (for serotype SAT2 viruses) VP1 coding region was performed using three primer sets and a Thermo Scientific Verso 1-Step reverse transcription polymerase chain reaction (RT-PCR) Reddy Mix Kit. The cycling parameters were 50°C for 30 min and 95°C for 15 min, then 35 cycles of denaturation at 95°C for 60 s, annealing at the suitable temperature for each primer set for 30 s and elongation at 72°C for 120 s, followed by a final extension at 72°C for 5 min (Table V).

Table V

List of primers used for reverse transcription polymerase chain reaction

FMD serotype	Primer ID	Sequences, 5'–3'	Annealing temperature	Location	Amplified product	Ref.
O	O-1C244F	GCAGCAAAACACATGTCAAACACCTT	60°C	VP3	1165 bp	(23)
	EUR-2B52R	GACATGTCCTCCTGCATCTGGTTGAT		2B		
A	A-1C612F	TAGCGCCGGCAAAGACTTTGA	55°C	VP3	814 bp	(23)
	EUR-2B52R	GACATGTCCTCCTGCATCTGGTTGAT		2B		
SAT2	1D209F	CCACATACTACTTTTGTGACCTGGA	58°C	1D	715–730 bp	(24)
	2B208R	ACAGCGGCCATGCACGACAG		2B		

bp: base pairs
F: forward
FMD: foot and mouth disease
ID: identification number
R: reverse
SAT: South African Territories
VP: viral protein

The PCR products were analysed by electrophoresis on a 1.5% agarose–tris–acetate–ethylenediaminetetraacetic acid (EDTA) gel containing ethidium bromide for nucleic acid staining. A 100 base-pair (bp) DNA ladder, manufactured by GeneDireX, was run alongside the products to confirm the expected size of the bands.

Viral protein 1 gene sequencing

The RT-PCR products were purified using a Thermo Scientific GeneJET Gel Extraction Kit according to the manufacturer's instructions and eluted in 50 µl elution buffer. Sequencing of the PCR products was performed using a BigDye™ Terminator V3.1 Cycle Sequencing Kit and the same forward and reverse primers as those used in the RT-PCR (23, 24).

The VP1 sequences generated were subjected to multiple sequence alignments using the CLUSTAL W 1.4 tool (25) implemented in BioEdit v7.2.5 (26). Neighbour-joining phylogenetic trees were constructed using MEGA 7 and the reliability of each tree branch was estimated by performing 1,000 bootstrap replicates (27).

Results

Serotyping enzyme-linked immunosorbent assay of the clinical samples

Of the total of 94 samples collected during three successive years (2015, 2016 and 2017) from vaccinated animals, 30 samples tested positive for serotypes A, O and/or SAT2 FMDV by antigen detection ELISA. Serotype O was predominant, with 19 out of the 30 samples, followed by serotype A with 9 samples and finally serotype SAT2 with only 2 samples.

Isolation and propagation of foot and mouth disease viruses

Propagation of the ELISA-positive samples (n = 30) on the BHK-21 cell line resulted in 15 FMDV isolates. The FMDVs from six serotype

O-, seven serotype A- and two serotype SAT2-positive samples were adapted on the BHK-21 cell line and showed the characteristic CPE for three successive passages 24–48 h post-inoculation (Table VI).

Table VI

Details of the positive samples and isolates for the foot and mouth disease enzyme-linked immunosorbent assay

FMDV serotype	ELISA-positive samples				FMD isolates			
	2015	2016	2017	Total	2015	2016	2017	Total
Serotype O	6	5	8	**19**	2	1	3	**6**
Serotype A	5	4	–	**9**	4	3	–	**7**
Serotype SAT2	–	–	2	**2**	–	–	2	**2**
Total	**11**	**9**	**10**	**30**	**6**	**4**	**5**	**15**

ELISA: enzyme-linked immunosorbent assay
FMD: foot and mouth disease
FMDV: foot and mouth disease virus
SAT: South African Territories

Testing of the harvested tissue culture supernatants by serotyping ELISA confirmed the successful isolation of the FMDVs. Each of the isolates gave positive result for the respective serotype with no cross-reaction with other serotypes.

Molecular characterisation of foot and mouth disease viruses by reverse transcription polymerase chain reaction

The RT-PCR revealed the true positivity of the 15 isolated viruses with bands of the expected size: 1165 bp, 814 bp and 715–730 bp for serotypes O, A and SAT2, respectively.

Phylogenetic analysis

Sequences of the complete or partial VP1-coding region of the 15 FMDV isolates were submitted to Genbank under the accession numbers listed in Table VII.

Table VII

Designations and accession numbers of the foot and mouth disease isolates

FMD isolate designation	Accession number
A/Egy/Beheira/2015	MG552837
A/Egy/Damietta/2015	MG552838
A/Egy/Monufia/2015	MG552839
A/Egy/Qalyubia/2015	MG552840
A/Egy/Beni Suef/2016	MG552841
A/Egy/Damietta/2016	MG552842
A/Egy/Faiyum/2016	MG552843
O/Egy/Beni Suef/2015	MG552844
O/Egy/Faiyum/2015	MG552845
O/Egy/Port Said/2016	MG552846
O/Egy/Beheira/2017	MG552847
O/Egy/Dakahlia/2017	MG552848
O/Egy/Qalyubia/2017	MG552849
SAT2/Egy/Alexandria/2017	MG552850
SAT2/Egy/Dakahlia/2017	MG552851

FMD: foot and mouth disease

Phylogenetic trees were constructed on the basis of the nucleotide sequence alignment of the VP1 gene of the isolates from this study together with other Egyptian isolates, reference prototypes and the strains currently used in the vaccine formulation.

Although all FMDV isolates of serotype O clustered with East Africa (EA)-3 prototypes (Fig. 1), the two serotype O isolates of 2015 (O/Egy/Beni Suef/2015 and O/Egy/Faiyum/2015) showed a higher degree of identity to each other (99%) than to the other serotype O

isolates of 2016 and 2017 (86–87%). The 2016 and 2017 isolates (O/Egy/Port Said/2016, O/Egy/Beheira/2017, O/Egy/Dakahlia/2017 and O/Egy/Qalyubia/2017) were almost identical, with a percentage identity that ranged between 94 and 99%.

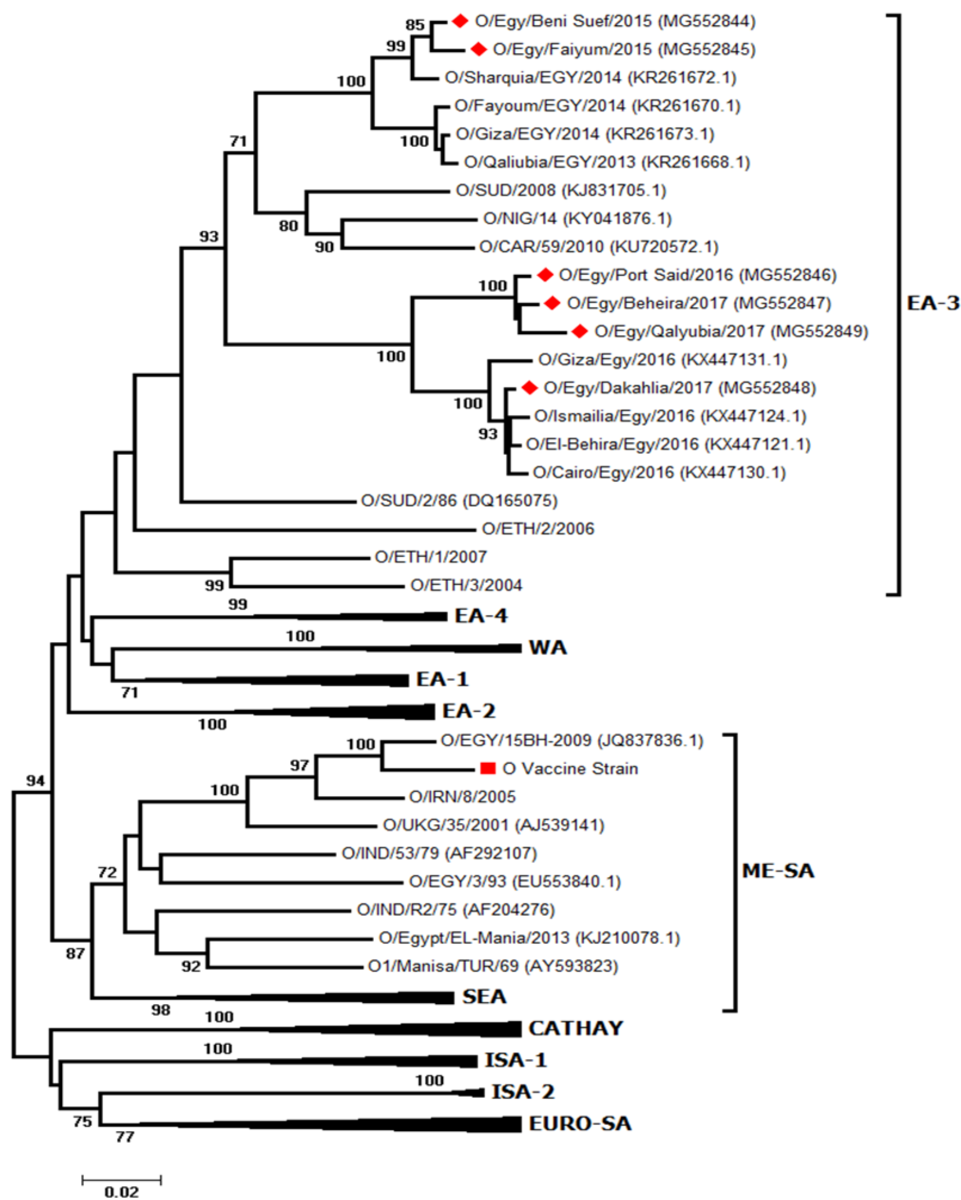

Fig. 1

Phylogenetic tree of serotype O isolates

Neighbour-joining phylogenetic analysis based on the VP1 coding sequence showing the relationships among serotype O FMD isolates of this study together with other contemporary and reference viruses. The six serotype O isolates of this study are indicated by red diamonds (♦) while the vaccine strain is indicated by a red square (■). Bootstrap values over 70% are shown above the branches

FMD: foot and mouth disease
VP: viral protein

In addition, the serotype O isolates of 2015 were closely related to the reported FMDVs O/SUD/2008 (KJ831705.1) and O/Sharquia/EGY/2014 (KR261672.1), with percentage identities of 91–92% and 98%, respectively. In contrast, the serotype O isolates of 2016 and 2017 were related only by 89–90% to O/SUD/2008 (KJ831705.1) and by 87–88% to O/Sharquia/EGY/2014 (KR261672.1); they were more closely related to O/Ismailia/EGY/2016 (KX447124.1), with a percentage identity that ranged between 94 and 99% (Table VIII).

Table VIII

Percentage nucleotide identity between serotype O isolates of this study and other isolates from Egypt and other countries

	Sequence ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	O/Egy/Beni Suef/2015 (MG552844)		99%	87%	87%	87%	86%	87%	98%	85%	83%	84%	92%	89%	82%
2	O/Egy/Faiyum/2015 (MG552845)	99%		87%	87%	87%	86%	87%	98%	85%	83%	84%	91%	89%	82%
3	O/Egy/Port Said/2016 (MG552846)	87%	87%		99%	95%	98%	95%	87%	84%	82%	82%	90%	88%	81%
4	O/Egy/Beheira/2017 (MG552847)	87%	87%	99%		95%	98%	95%	87%	85%	82%	81%	90%	88%	81%
5	O/Egy/Dakahlia/2017 (MG552848)	87%	87%	95%	95%		94%	99%	88%	85%	83%	83%	89%	88%	82%
6	O/Egy/Qalyubia/2017 (MG552849)	86%	86%	98%	98%	94%		94%	87%	84%	81%	81%	89%	87%	80%
7	O/Ismailia/Egy/2016 (KX447124.1)	87%	87%	95%	95%	99%	94%		88%	84%	83%	82%	89%	88%	82%
8	O/Sharquia/EGY/2014 (KR261672.1)	98%	98%	87%	87%	88%	87%	88%		86%	83%	84%	92%	89%	82%
9	O/Egypt/EL-Mania/2013 (KJ210078.1)	85%	85%	84%	85%	85%	84%	84%	86%		87%	88%	85%	83%	86%
10	O/EGY/15BH-2009 (JQ837836.1)	83%	83%	82%	82%	83%	81%	83%	83%	87%		87%	83%	82%	97%
11	O/EGY/3/93 (EU553840.1)	84%	84%	82%	81%	83%	81%	82%	84%	88%	87%		83%	82%	87%
12	O/SUD/2008 (KJ831705.1)	92%	91%	90%	90%	89%	89%	89%	92%	85%	83%	83%		92%	82%
13	O/NIG/14 (KY041876.1)	89%	89%	88%	88%	88%	87%	88%	89%	83%	82%	82%	92%		81%
14	O vaccinal strain	82%	82%	81%	81%	82%	80%	82%	82%	86%	97%	87%	82%	81%	

Unlike the O isolates of this study, the vaccine strain, together with other Egyptian strains O/Egypt/EL-Mania/2013 (KJ210078.1), O/EGY/15BH-2009 (JQ837836.1) and O/EGY/3/93 (EU553840.1), clustered with the Middle East–South Asia (ME-SA) prototypes (Fig. 1). There was a variation between serotype O isolates and the vaccine strain of 18–20% in the nucleotide sequence and 18–21% in 213 amino acids of VP1 (Fig. 2).

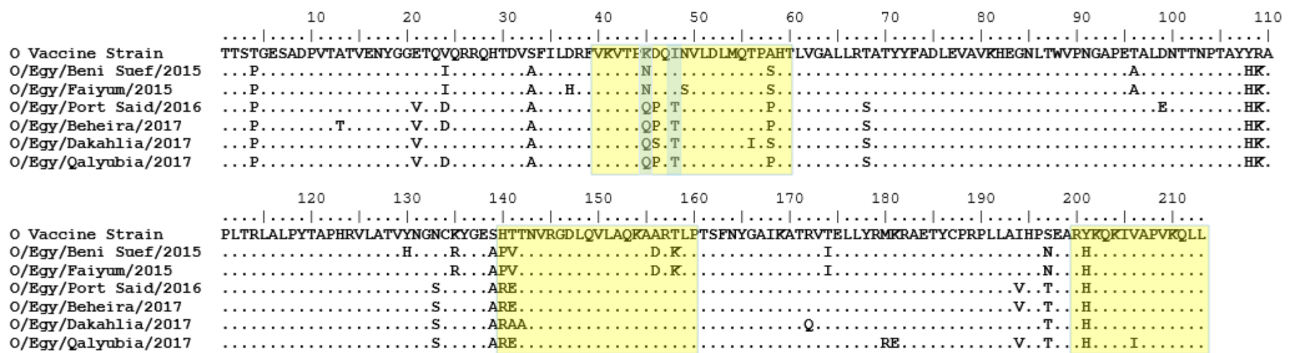

Fig. 2

Alignment of viral protein 1 deduced amino acid sequences of the serotype O isolates of this study and the currently used vaccine strain

The light-yellow shading indicates the neutralising antigenic sites while the light-blue shading indicates the mutated critical amino acids

Six of the seven serotype A isolates from this study showed high relatedness to each other (96–100%). However, they had much lower percentages of identity (74–76%) with the seventh isolate (A/Egy/Faiyum/2016). The first six isolates showed high identity (96–99%) with the Egyptian isolates of 2013, A/Cairo/EGY/2013 (KR092701.1) and A/Egypt/Al-Fayoum/2013 (KJ210071.1), and 94–96% identity with isolates from Libya and Iraq, A/LIB/14/2009 (KF112913.1) and A/IRQ/09 (JN099701.1). These, together with the vaccine strain, were grouped with serotype A Asian strains, whereas A/Egy/Faiyum/2016 showed 99% identity with A/Ismailia/Egy/2016 (KX446997.1) and was grouped alone with G-IV of the African A strains (Table IX and Fig. 3).

Table IX

Percentage nucleotide identity between serotype A isolates of this study and other isolates from Egypt and other countries

	Sequence ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	A/Egy/Beheira/2015 (MG552837)		97%	97%	96%	96%	97%	74%	78%	77%	96%	96%	74%	94%	94%	94%
2	A/Egy/Damietta/2015 (MG552838)	97%		99%	99%	99%	100%	76%	79%	78%	99%	98%	76%	96%	96%	96%
3	A/Egy/Monufia/2015 (MG552839)	97%	99%		98%	98%	99%	75%	79%	78%	98%	98%	75%	96%	96%	96%
4	A/Egy/Qalyubia/2015 (MG552840)	96%	99%	98%		98%	99%	75%	79%	78%	98%	98%	75%	96%	96%	95%
5	A/Egy/Beni Suef/2016 (MG552841)	96%	99%	98%	98%		99%	75%	79%	78%	98%	97%	75%	96%	96%	95%
6	A/Egy/Damietta/2016 (MG552842)	97%	100%	99%	99%	99%		76%	79%	78%	99%	98%	76%	96%	96%	96%
7	A/Egy/Faiyum/2016 (MG552843)	74%	76%	75%	75%	75%	76%		82%	80%	75%	76%	99%	76%	76%	76%
8	A/EGY/1/72 (EF208756)	78%	79%	79%	79%	79%	79%	82%		83%	79%	79%	82%	79%	80%	79%
9	A/EGY/1/2006 (EF208757.1)	77%	78%	78%	78%	78%	78%	80%	83%		79%	79%	80%	79%	79%	79%
10	A/Cairo/EGY/2013 (KR092701.1)	96%	99%	98%	98%	98%	99%	75%	79%	79%		98%	75%	96%	96%	96%
11	A/Egypt/Al-Fayoum/2013 (KJ210071.1)	96%	98%	98%	98%	97%	98%	76%	79%	79%	98%		76%	97%	97%	96%
12	A/Ismailia/Egy/2016 (KX446996.1)	74%	76%	75%	75%	75%	76%	99%	82%	80%	75%	76%		76%	76%	76%
13	A/LIB/2009 (KF112913.1)	94%	96%	96%	96%	96%	96%	76%	79%	79%	96%	97%	76%		100%	99%
14	A/Iraq/2009 (JN099701.1)	94%	96%	96%	96%	96%	96%	76%	80%	79%	96%	97%	76%	100%		99%
15	A vaccinal strain	94%	96%	96%	95%	95%	96%	76%	79%	79%	96%	96%	76%	99%	99%	

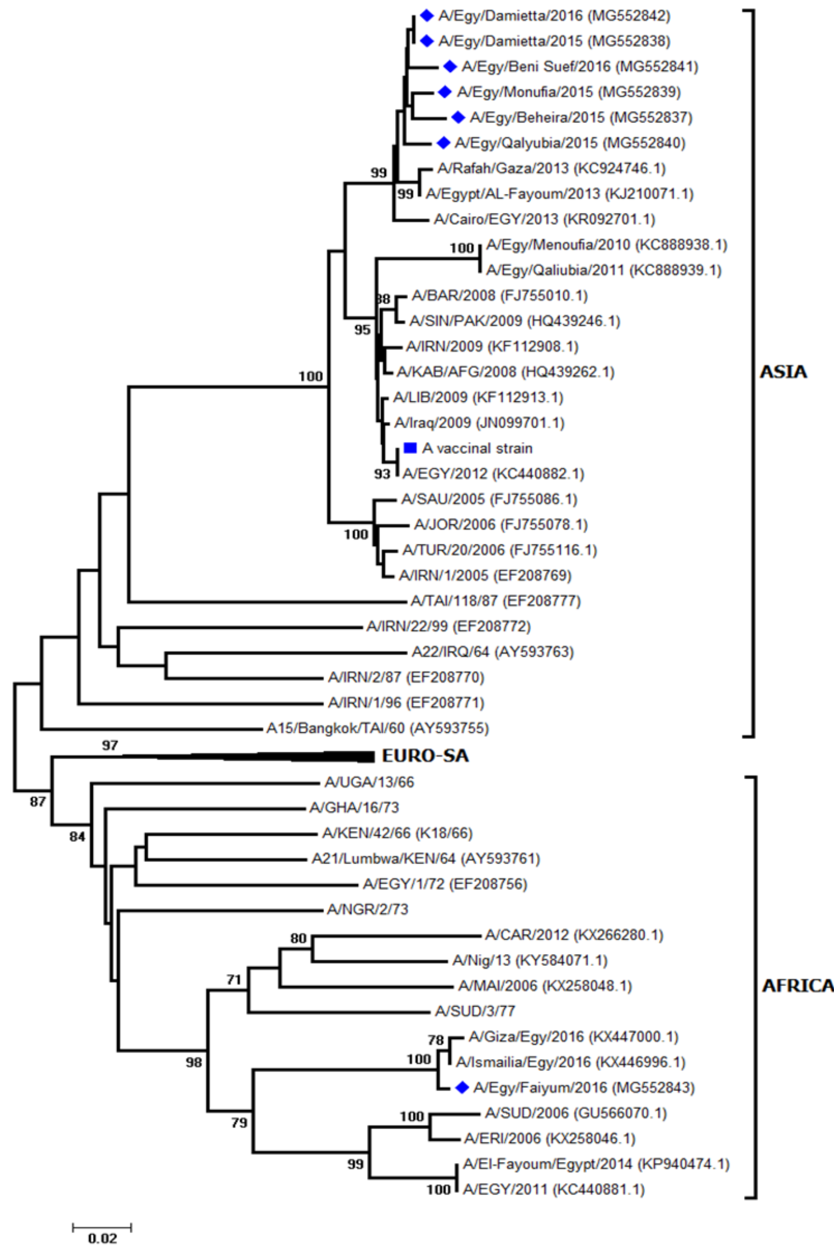

Fig. 3

Phylogenetic tree of serotype A isolates

Neighbour-joining phylogenetic analysis based on the VP1 coding sequence showing the relationships among the serotype A FMD isolates of this study together with other contemporary and reference viruses. The seven serotype A isolates of this study are indicated by blue diamonds (◆) while the vaccine strain is indicated by a blue square (■). Bootstrap values over 70% are shown above the branches

FMD: foot and mouth disease
VP: viral protein

The Asian strains of this study were closely related to the currently used vaccine strain (94–96%), which belongs to the A-Iran-05 lineage. However, the African serotype A isolate showed a percentage identity of only 76% with the vaccine strain. The alignment of VP1 amino acids showed that the serotype A Asian isolates had a variation of 9–19 amino acids when compared with the vaccine strain, while the serotype A African isolate differed from the vaccine strain in 33 amino acids (Fig. 4).

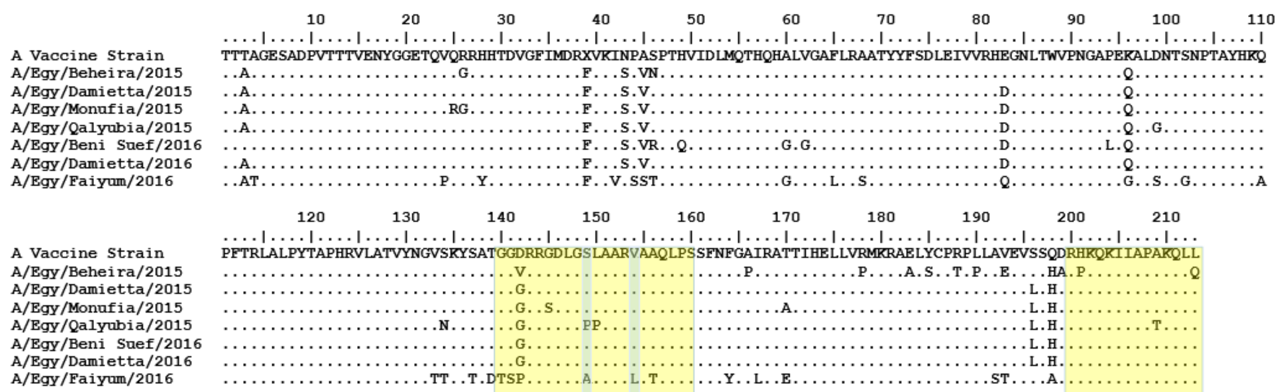

Fig. 4

Alignment of viral protein 1 deduced amino acid sequences of the serotype A isolates of this study and the currently used vaccine strain

The light-yellow shading indicates the neutralising antigenic sites while the light-blue shading indicates the mutated critical amino acids

Sequencing of the two SAT2 isolates of this study (SAT2/Egy/Alexandria/2017 and SAT2/Egy/Dakahlia/2017) revealed that they had 2% divergence. Both isolates were related to the Egyptian isolates SAT2/EGY/2/2012 (JX570617.1) and SAT2/EGY/24/2014 (KY825720.1), with a sequence identity of 92% and 95%, respectively. These were also related to other SAT2 isolates from Sudan, SAT2/SUD/4/2010 (KF112968.1) (94%), and Ethiopia, SAT2/ETH/2/2015 (KX258071.1) (93%) (Table X).

Table X

Percentage nucleotide identity between serotype SAT2 isolates of this study and other isolates from Egypt and other countries

	Sequence ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	SAT2/Egy/Alexandria/2017 (MG552850)		98%	92%	87%	88%	88%	88%	95%	86%	87%	86%	94%	93%	87%
2	SAT2/Egy/Dakahlia/2017 (MG552851)	98%		92%	88%	89%	89%	89%	95%	86%	88%	86%	94%	93%	88%
3	SAT2/EGY/2/2012 (JX570617.1)	92%	92%		91%	91%	91%	91%	94%	89%	91%	89%	96%	93%	90%
4	SAT2/EGY/4/2012 (JX570619.1)	87%	88%	91%		100%	100%	100%	89%	98%	100%	91%	89%	88%	99%
5	SAT2/EGY/9/2012 (JX570622.1)	88%	89%	91%	100%		100%	100%	89%	98%	100%	91%	89%	89%	99%
6	SAT2/EGY/H1Fay/2012 (KF055861.1)	88%	89%	91%	100%	100%		100%	89%	98%	100%	91%	89%	89%	99%
7	SAT2/EGY/H1Ghb/2012 (KF055860.1)	88%	89%	91%	100%	100%	100%		89%	98%	100%	91%	89%	89%	99%
8	SAT2/EGY/24/2014 (KY825720.1)	95%	95%	94%	89%	89%	89%	89%		87%	89%	88%	96%	96%	89%
9	SAT2/Qaliubia/2015 (KU897094.1)	86%	86%	89%	98%	98%	98%	98%	87%		98%	89%	87%	87%	98%
10	SAT2/PAT/1/2012 (JX014256.1)	87%	88%	91%	100%	100%	100%	100%	89%	98%		91%	89%	89%	100%
11	SAT2/LIB/1/2003 (JX570631.1)	86%	86%	89%	91%	91%	91%	91%	88%	89%	91%		89%	88%	90%
12	SAT2/SUD/4/2010 (KF112968.1)	94%	94%	96%	89%	89%	89%	89%	96%	87%	89%	89%		95%	89%
13	SAT2/ETH/2/2015 (KX258071.1)	93%	93%	93%	88%	89%	89%	89%	96%	87%	89%	88%	95%		88%
14	SAT2 vaccinal strain	87%	88%	90%	99%	99%	99%	99%	89%	98%	100%	90%	89%	88%	

Phylogenetic analysis placed the two SAT2 isolates in one cluster with the strains of SAT2/VII/Alx-12 lineage. The currently used SAT2 vaccine strain, however, clustered with other Egyptian SAT2 strains, SAT2/EGY/H1Ghb/2012 (KF055860.1) and SAT2/Qaliubia/2015 (KU897094.1), which belong to the SAT2/VII/Ghb-12 lineage (Fig. 5). The SAT2 isolates studied showed identities of 87% and 88% with the vaccine strain. Deduced amino acids from the partial VP1 sequence of the two SAT2 isolates varied in 13 amino acids when compared with the SAT2 vaccine strain (Fig. 6).

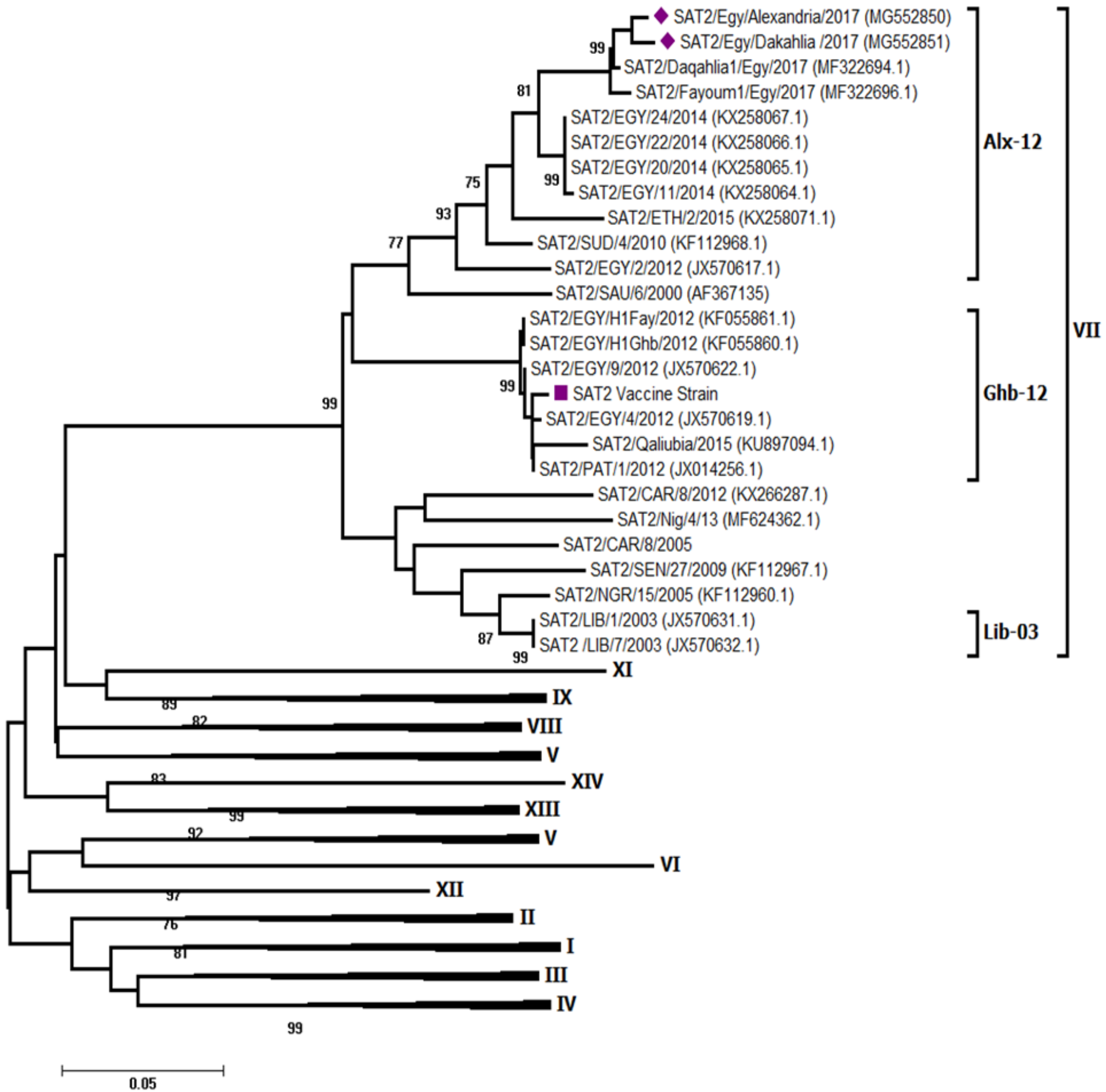

Fig. 5

Phylogenetic tree of serotype SAT2 isolates

Neighbour-joining phylogenetic analysis based on the VP1 coding sequence showing the relationships among serotype SAT2 FMD isolates of this study together with other contemporary and reference viruses. The two serotype SAT2 isolates of this study are indicated by purple diamonds (◆) while the vaccine strain is indicated by a purple square (■). Bootstrap values over 70% are shown above the branches

FMD: foot and mouth disease
SAT: South African Territories
VP: viral protein

```
                            70        80        90       100       110       120       130       140       150       160       170
                           .|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SAT2 Vaccine Strain        STYYFCDLEIACVGDHTRVFWQPNGAPRTTQLGDNPMVFAKGGVTRFAIPFTAPHRLLSTVYNGECVYTKAAAAIRGDRAALAAKYADTNHTLPPTFNFGYVTVDKP
SAT2/Egy/Alexandria/2017   A.................A.................................................KETPT..............G......S.....F......
SAT2/Egy/Dakahlia/2017     ..................A.........A.......................................KETPT..............G......S.....F......

                              180       190       200       210
                           ....|....|....|....|....|....|....|....|.
SAT2 Vaccine Strain        VDVYYRMKRAELYCPRPLLPAYKHTDRDRFDAPIGVERQTL
SAT2/Egy/Alexandria/2017   ......................E.AS...............
SAT2/Egy/Dakahlia/2017     ......................E.AS...............
```

Fig. 6

Alignment of viral protein 1 deduced amino acid sequences of the serotype SAT2 isolates of this study and the currently used vaccine strain

The light-yellow shading indicates the neutralising antigenic sites

SAT: South African Territories

Virus neutralisation test

Table XI shows the neutralising antibody titre, which reflects the antigenic relationship between isolates from this study and the strains used for vaccination.

Table XI

Results of titration and virus neutralisation testing of foot and mouth disease isolates from this study

FMD isolate	FMD virus titre (TCID50/ml) (log10)	Neutralising antibody titre (log2)
O/Egy/Beni Suef/2015	4	5
O/Egy/Faiyum/2015	4.5	5
O/Egy/Port Said/2016	4.2	4.5
O/Egy/Beheira/2017	5	4
O/Egy/Dakahlia/2017	4	4
O/Egy/Qalyubia/2017	4.5	4
A/Egy/Beheira/2015	4	7
A/Egy/Damietta/2015	4.8	7
A/Egy/Monufia/2015	5	7
A/Egy/Qalyubia/2015	4.5	6.5
A/Egy/Beni Suef/2016	4	7
A/Egy/Damietta/2016	5	7
A/Egy/Faiyum/2016	4	4
SAT2/Egy/Alexandria/2017	4.4	6
SAT2/Egy/Dakahlia/2017	4	6

FMD: foot and mouth disease
TCID: tissue culture infective dose

Discussion

As a developing country, Egypt depends on importation of meat and live animals to narrow the gap between production and consumption. This increases the chance of introducing new FMDV strains by the movement

of infected animals across international borders. Both Asian and African FMDV strains have been introduced into Egypt, and this is facilitated by its unique transcontinental location (15).

Control of FMD in Egypt relies mainly on quarantine and mass vaccination. Obligatory vaccination of all ruminants every six months and of dairy cows every four months is performed using a trivalent vaccine which is prepared from local isolates and contains strains of serotypes A, O and SAT2 (18, 28).

The high mutation rate of FMDV, caused by its error-prone replication, results in a population with extensive genetic diversity (8). Nucleotide substitutions in the viral capsid region may lead to the emergence of antigenic variants that could be selected for replication under the immune pressure of the partially immunised host. Animals in FMD-endemic areas where there is a shortage in vaccination coverage are most likely to experience partial immunity (29). This necessitates constant surveillance of the circulating strains by recognising the clinical signs and implementing laboratory tests to detect any variants (30).

Antigen detection ELISA is the preferred procedure for FMD diagnosis, as stated by Ferris and Donaldson (31), and revealed the co-circulation of serotypes A and O in Egypt during 2015 and 2016. Viruses of serotypes O and SAT2, but not serotype A, were detected in samples collected at the beginning of 2017. The absence of serotype A FMDV in 2017 in this study may be due to the limited number of samples tested and is, therefore, inconclusive.

The BHK-21 cell line was chosen for the isolation trials in this study for its ability to support FMDV growth and develop a CPE within 72 h, which is manifested by cell rounding, granulation and detachment (32). Although the BHK-21 cell line is 10 times less sensitive than primary cultures of bovine thyroid and porcine or ovine kidney in detecting low amounts of infectivity, it is more susceptible and develops a higher virus titre than the Instituto Biologico-Rim Suino-2 (IBRS-2) and Mengeling-Vaughn porcine

kidney (MVPK) cell lines. Moreover, the production of a consistent quality of primary cell cultures is time-consuming and costly (33). All viruses underwent a maximum of three passages on BHK-21 cells to preclude passage-induced mutations (34).

Viral protein 1 is the most variable region of the virus capsid, with nucleotide substitutions of up to 1% per year (35). It has a significant role in the host humoral immune response as the G-H loop (residues 140–160) together with the carboxy terminus (residues 200–213) comprise a major immunodominant neutralising epitope. The G-H loop also has a conserved Arg-Gly-Asp (RGD) sequence in its central region which serves as an integrin-binding motif (36). In this study, the VP1 gene was chosen for amplification because of its significance in the genetic characterisation of FMDV strains. Phylogenetic analysis based on VP1 sequences can help in understanding the disease epidemiology and deducing the source of outbreak-causing strains. Moreover, the VP1 gene sequence serves in predicting the antigenic structure of the FMDV epitopes (37).

Although all serotype O isolates in this study clustered with the EA-3 prototypes, they formed two distinct clusters, reflecting a variation of 14% in the VP1 gene. This implies the probability of two separate introductions of type O FMDV into Egypt and confirms the findings of other researchers who previously reported the same genotype (16). Also, the World Reference Laboratory for FMD (WRLFMD) reports multiple EA-3 outbreaks recorded in Egypt since its introduction in 2012 (38).

Phylogenetic analysis of the serotype A isolates of 2015 and 2016 revealed the co-circulation of African and Asian type A strains. All the Asian strains in this study belonged to the A-Iran-05 lineage which was first reported in Iran in 2003 and had spread throughout the Middle East by 2009 (39). Moreover, genotype IV of the African A topotype was also detected in one sample isolated in Faiyum governorate during 2016. This genotype was responsible for the Egyptian FMD outbreak in February 2012 and is genetically linked to the sub-Saharan African strains (40).

Serotype SAT2 FMDV was introduced to Egypt in 2012 and detected in two lineages, SAT2/VII/Ghb-12 and SAT2/VII/Alx-12, which both belong to topotype VII of SAT2 virus and differ by approximately 10% (11). Viruses of the two lineages were isolated from different Egyptian governorates during the succeeding years (15, 17). The SAT2 isolates of this study belong to the SAT2/VII/Alx-12 lineage, with only 2% divergence between them. The SAT2/VII/Ghb-12 lineage, however, was not detected in any of the isolated viruses, which may be due to the limited number of samples.

The virus neutralisation test, as the 'gold standard', was used for serotype identification and assessment of the isolates' antigenic variation (1). The neutralising antibody titre differed among the isolates of the same serotype, possibly reflecting variations in their neutralising antigenic sites. Out of five antigenic sites that have been identified on the type O FMDV capsid surface, three are located within VP1. Antigenic site 1 consists of residues 140–160 within the βG–βH loop along with the C-terminus residues. The βB–βC residues (residues 42–51) comprise antigenic site 3, while antigenic site 5 is formed by residue 149 of VP1 (41). Equivalent neutralising antigenic sites have been identified for the other serotypes (4).

All the serotype O isolates studied belonged to a different genotype from the vaccine strain and, consequently, antigenic variation is not unexpected. The serotype O isolates of 2015 showed a higher neutralising antibody titre in comparison with the 2016 and 2017 isolates. The reduction in the neutralisation titre may be attributed to the mutation (isoleucine→threonine) at position 48 of VP1 which has been reported to be a critical residue of antigenic site 3 (41, 42). This substitution from a hydrophobic to a neutral residue may be responsible for changing the overall confirmation of the antigenic site. In addition, a mutation at residue 45 of all the studied isolates, (lysine→asparagine) in 2015 isolates and (lysine→glutamine) in 2016 and 2017 isolates, may be responsible for the general reduction in the neutralising titre. Interestingly, a mutation at the same position (lysine→threonine) was reported in a previous study, when

the viruses were grown under vaccinal immune pressure, as an attempt to escape from antibodies to the epitopes that have either lysine or glutamate at this position (41).

The serotype A isolate designated as A/Egy/Faiyum/2016 showed a much lower neutralising titre than the other serotype A isolates, which can be explained by this isolate belonging to a different genotype from the vaccine strain. Its low reactivity with the anti-vaccinal sera can be attributed to multiple amino acid substitutions, at residues 83 (glutamate→glutamine), 149 (serine→alanine) and 154 (valine→leucine). An additional substitution (phenylalanine→leucine) was observed at residue 65, which, although the residue is completely buried in the virus, may change the surface structure (43). Also, the isolate A/Egy/Qalyubia/2015 showed a lower antibody titre and one antigenically critical amino acid mutation at residue 149 (serine→proline) (43). Residue 149 has been previously shown to be a strong predictor of the antigenic match to the vaccine strain (44). Nonetheless, the role of these amino acid substitutions in FMDV antigenic variation cannot be confirmed without further investigation of the other antigenic sites.

Conclusions

The results obtained in the present study document the circulation of FMDV serotypes O, A and SAT2 among the vaccinated animal population in Egypt, raising the need to investigate the possibility of vaccination failure. Multiple introductions of new FMDVs and their poor antigenic matching to the vaccine strain are considered major possible causes for the vaccination failure seen in Egypt. Additionally, it is theorised that the constant evolution of FMDV, particularly under vaccinal pressure, could be responsible for the observed immune escape. Nevertheless, other potential causes should be considered, such as the lack of consistent vaccine quality control and potency, the breakdown of the vaccine cold chain and its improper storage, the variation in the vaccination technique including decreased vaccine dose and faulty administration, as well as undetected infection of animals at the time of vaccination. Therefore, this

study endorses prevention of transboundary introduction of FMDV by the application of efficient quarantine measures. The authors also recommend optimisation of the vaccine-based control programme by selecting the appropriate vaccine strain, ensuring vaccine quality, and improving vaccination coverage and the assessment of population immunity post vaccination. In addition, it is imperative constantly to monitor the antigenic changes in the immunogenic sites of the virus in order to detect the circulating variants and update the vaccine accordingly.

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