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Co-infection with different serotypes of FMDV in vaccinated cattle in Southern Egypt

Amira Adel Al-Hosary[1] · Ahmed Kandeil[2] · Ahmed Nageh El-Taweel[2] · Ann Nordengrahn[3] · Malik Merza[3] · Rebecca Badra[5] · Ghazi Kayali[4,5] · Mohamed A. Ali[2]

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Abstract

During 2015–2016 period, an outbreak of foot-and-mouth disease virus (FMDV) was observed in cattle in four governorates of the upper of Egypt. The infection was extended to the vaccinated cattle. A total of 54 mouth swabs and serum samples were collected from vaccinated cattle for serological and virological investigation. The typical clinical signs of FMDV infection were observed in all cattle under investigation. All samples were positive for FMDV using molecular methods, while the serological method showed 85% positive of tested samples. Typing of FMDV-positive samples using serotype-specific primers showed that 51.8% of samples were serotype O, 9.2% were serotype A, and 18.5% were SAT 2. Surprisingly, co-infections of serotypes A/SAT 2 (12.9%) and O/SAT 2 (7.4%) were also detected. By geographical location, the 3 serotypes A, O, and SAT2 were detected in all four governorates. The phylogenetic assessment of the detected viruses showed that two distinct groups of FMDV serotype O of East Africa-3 (EA-3) topotype were most closely related to circulating viruses in Sudan, as well as FMDV strains belonging to the topotype VII of serotype SAT 2. The detected SAT 2 strains clustered in separate clades in topotype VII, indicating new incursions. The VP1 signatures and protein sequences of some characterized viruses were analyzed. Multiple mutations were detected in VP1. Therefore, to enhance the control of FMD in Egypt, we recommend establishing an active surveillance system to characterize newly emerging virus strains/serotypes and subsequently updating vaccine strains.

Keywords Foot-and-mouth disease virus · Co-infection · Vaccination · Egypt

Introduction

Foot-and-mouth disease virus (FMDV) is a highly contagious virus infecting cloven-hoofed mammals leading to huge economic losses in livestock production worldwide and

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Amira Adel Al-Hosary and Ahmed Kandeil have contributed equally as first authors.

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✉ Ahmed Kandeil
Kandeil_a@hotmail.com

✉ Ghazi Kayali
ghazi@human-link.org

✉ Mohamed A. Ali
mohamedahmedali2004@yahoo.com

[1] Department of Animal Medicine (Infectious Diseases), Faculty of Veterinary Medicine, Assiut University, Po. Box 71526, Assiut, Egypt

[2] Center of Scientific Excellence for Influenza Viruses, Water Pollution Research Department, Environmental Research Division, National Research Centre (NRC), Egypt, Dokki, Giza 12622, Egypt

[3] Boehringer Ingelheim Svanova, Uppsala Business Park, Po. Box 1545, Uppsala, Sweden

[4] Department of Epidemiology, Human Genetics, and Environmental Sciences, University of Texas Health Sciences Center, Houston, TX, USA

[5] Human Link, Hazmieh, Lebanon

especially in Asia, South America, and Africa [1]. FMDV belongs to the *Aphthovirus* genus and has seven distinct serotypes (A, O, C, Asia, Southern African Territories (SAT) 1, SAT2, and SAT3) [2]. There are no cross-protections among the seven serotypes of FMDVs nor between variant viruses of the same serotype [3].

Serotypes A, O, and SAT2, were previously detected during outbreak waves in Egypt [4]. Serotype O was the most prevalent since 1951 [4]. In 2006, serotype A appeared in Egypt and rapidly spread throughout the ruminant population, causing severe economic losses [5]. Since 2006, serotypes A and O were continuously circulating in Egypt [4]. In 2012, Egypt reported FMD SAT2 outbreaks in cattle and buffaloes in several governorates causing massive losses among infected animals [6, 7]. An estimated 6.3 million heads of buffalo and cattle and 7.5 million heads of sheep and goats are at risk of infection [8]. Animal movement across international borders predisposes Egypt to new emerging FMDV serotypes [9]. Furthermore, trade of live animals complicates eradication efforts especially in areas where the virus is enzootic [10–12]. There are limited data about FMDV evolution in Egypt and about the efficacy of the used vaccines. Genetic and antigenic relatedness of commercially used inactivated FMDV vaccine strains and the Egyptian-circulating viruses is unknown. Immunity induced by vaccination could be limited even when the vaccine strain matches the circulating serotype. For this reason, it is important to characterize circulating viruses in order to control the disease by vaccination [13]. Accordingly, FMDV vaccine seed strains should be continuously updated based on antigenic and genetic analyses to offer the best level of protection. This study was carried out to describe FMDV serotypes responsible for the outbreaks during 2015 and 2016 in different governorates in upper Egypt in vaccinated FMDV cattle.

Materials and methods

Sample collection

A total of 54 mouth swabs and serum samples were collected from 1–5-year-old cattle with clinical signs of FMD during 2015–2016. The cattle came from four governorates in Southern Egypt (El-Fayoum, Assiut, El-Minya, and Sohag) (Fig. 1), and included 5 females and 49 males. Swabs were sampled in transport DMEM medium with 2% antibiotic–antimycotic (BioWhittaker, Walkersville, USA) and transported to the laboratory on ice. All collected samples

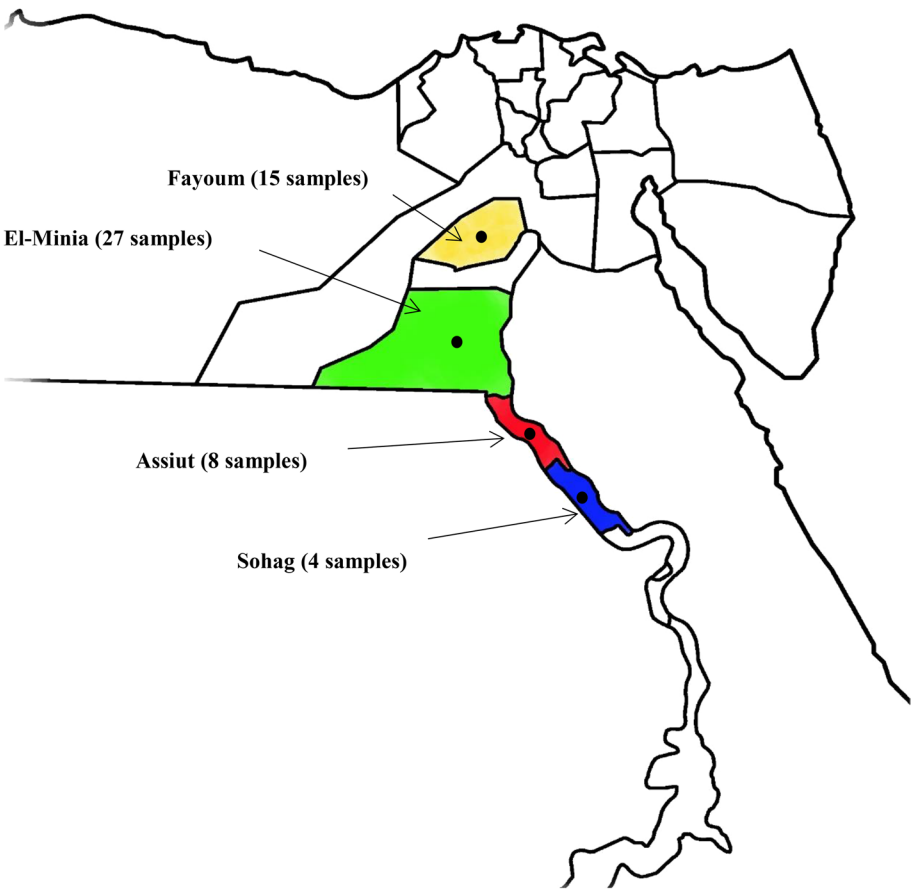

Fig. 1 Map of Egypt showing the geographical sampling governorates sites

were obtained from animals previously vaccinated with a locally produced, inactivated, oil vaccine against serotypes O1, A, and SAT2. This vaccine was provided to the owners by the veterinary authority during a mass vaccination campaign.

Detection, serotyping, and sequencing of FMDV

Viral RNA was extracted from 140 µl of each collected sample using QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RT-PCR was performed for FMDV detection first using QIAGEN One-Step RT-PCR and universal primers 1F/1R (10 pM of each) [14] in a 25-µl reaction mixture [5 µl of 5X reaction buffer, 1 µl dNTPs (10 mM), 1 µl enzyme mix, 1 µl (10 µM) forward primer, 1 µl (10 µM) reverse primer, 10 µl ddH_2O, and 5 µl of RNA sample]. The RT-PCR cycling conditions were an initial 30-min incubation at 50 °C, DNA polymerase activation at 95 °C for 15 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, and a final 10-min extension at 72 °C. The PCR products were then analyzed on gel electrophoresis for the presence of a 328-bp band. For serotype detection, samples that tested positive for FMDV underwent a second RT-PCR with the same conditions as described above using serotype-specific primers as previously described (Table S1) [14]. The obtained results were confirmed in two different labs using the same protocol. The final PCR products were gel purified and both strands sequenced using the same primers at the Macrogen sequencing facility (Macrogen, Seoul, South Korea). The contiguous sequence was assembled using SeqMan in Lasergene 7. The obtained sequences (8 assembled sequences for serotype O from 4 governorates and 4 assembled sequences for Serotype SAT2 from El-Minya governorate) were deposited into the GenBank under accession numbers from MG925044 to MG925055 (Table S2). Positive serotype A samples could not be sequenced due to low yield in the RT-PCR products.

Related FMDV VP1 sequences were obtained from GenBank by BLASTN analysis and FMD prototype strain sequences were obtained from WRIFMD (http://www.wrlfmd.org/fmd-prototype-strains/download-fmd-prototype-strain-sequences). Sequence alignments were performed using BioEdit 7.0 software. The phylogenetic tree was constructed using MEGA7 program by applying the Maximum Likelihood method with Kimura's two-parameter substitution model and 1000 bootstrap replicates.

Serological testing

FMD NS ELISA kit (SVANOVIR® FMDV 3ABC-Ab) was used for the detection of all FMDV serotypes in cattle sera following kit instructions. Briefly, a volume of 50 µl of pre-diluted serum sample was added to wells coated with FMDV viral antigen. After incubation at 37 °C for 30 min, the plate was washed 3 times using washing buffer (PBS-Tween Buffer). A volume of 50 µl of horseradish peroxidase conjugate was added to treated wells, and then the plate was sealed and incubated at 37 °C for 30 min. The plate was washed 3 times. Substrate solution (50 µl) was added to treated wells. The plate was incubated for 30 min at room temperature in the dark. Stop solution (50 µl) was then added to treated wells and the optical density (OD) was measured at 405 nm by a microplate photometer.

Samples with Percent Positivity Value [PPV = (Test sample /Positive control)*100] results < 48 were considered negative and those with a PPV ≥ 48 were considered positive.

Results

Molecular characterization of FMDV in collected samples

Collected samples were obtained from animals with typical symptoms of FMDV infection including high fever, blisters inside the mouth, excessive salivation, blisters on the feet, lameness, udder and teat vesicles, and decreased food consumption. All the 54 collected samples were positive for FMDV by RT-PCR using the universal primers that generated 328 bp PCR products. Typing of FMDV-positive samples using serotype-specific primers identified serotypes O (51.8%), A (9.2%), SAT2 (18.5%), co-infection A/SAT2 (12.9%), and co-infection O/SAT2 (7.4%) (Fig. 2a).

By geographical location, serotype O was the most prevalent in El-Fayoum (66.6%) followed by El-Minya (51.8%), Assiut (37.5%), and Sohag (25%). SAT2 infection was detected in all four governorates with the highest detection rate in Sohag (50%), followed by Assiut (25%), El-Minya (18.5%), and El-Fayoum (6.6%). Co-infection with O and SAT2 serotypes was detected in El-Minya (2 of 27 samples) and Assiut (2 of 8 samples). Co-infection with A and SAT 2 serotypes was detected in all four governorates, while co-infection with serotype O and A was not detected in any of the collected samples.

Serological characterization of FMDV in infected cattle

A total of 46 sera samples, equivalent to 85%, tested positive. Overall, the tested-positive cattle sera from El-Fayoum and Sohag showed higher percentages of positivity (100%) than those from Assiut (87%) and El-Minya (74%) (Fig. 2b).

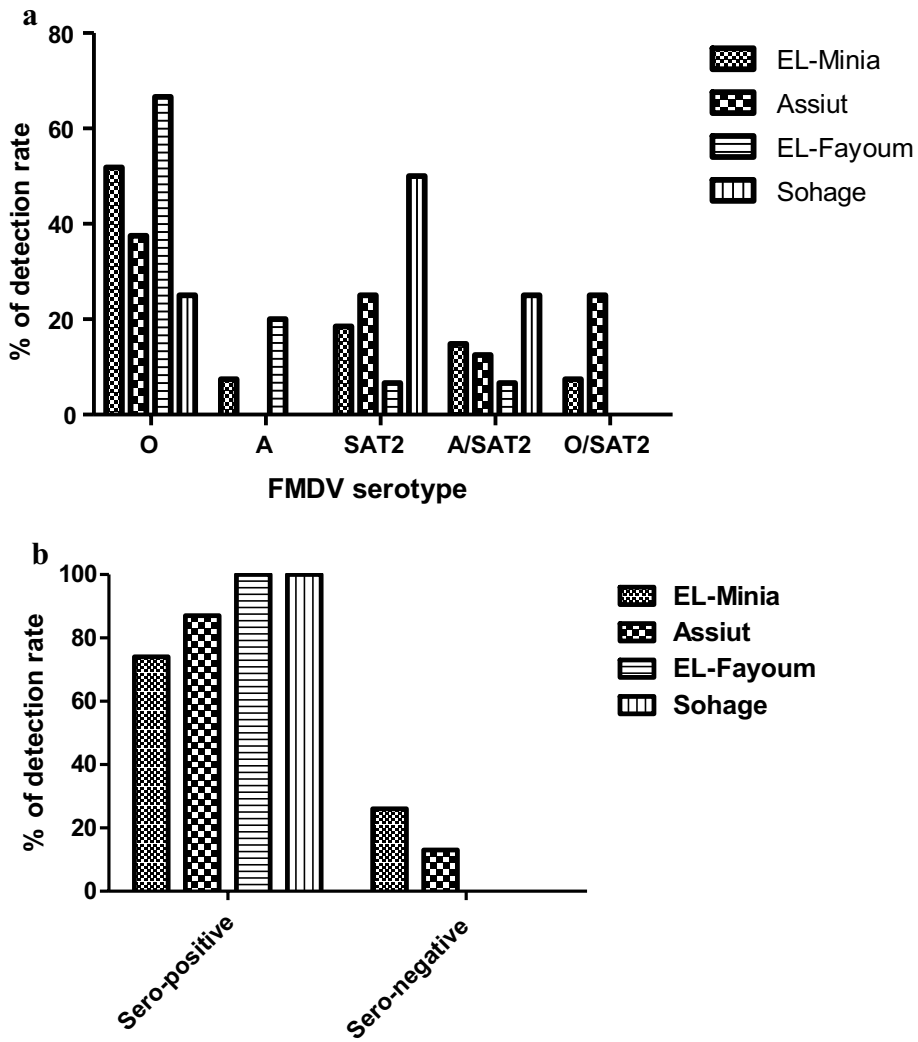

Fig. 2 Detection rates of FMDV in the collected animal samples from four governorates: **a** Molecular serotyping of FMDV in the collected animal samples from four governorates **b** Serological detection rate of FMDV in collected sera by location

Comparison of amino acid sequences and phylogenetic analysis of the VP1 proteins of SAT2

Amino acid sequences of VP1 proteins of four SAT2 samples from four different governorates were generated from nucleotide sequences of the original field sample material. Comparison of the sequences showed that the VP1 proteins of those specimens were 100% identical. By BLASTN analysis, the Egyptian SAT2 FMDV viruses from 2016 were more related to Sudanese SAT2 viruses (96%) than to 2012 Egyptian SAT2 viruses (90%). Phylogenetic analysis showed that all characterized SAT2 FMDV from Egypt are related to topotype VII (Fig. 3). The SAT2 sequences from this work were closer to Sudanese sequences from 2007/2010 than to Egyptian 2012 SAT 2 FMDV sequences.

Comparison of the amino acid sequences of the G-H loop region in VP1 revealed several differences between the Egyptian SAT2 FMDV from 2012 and the currently characterized SAT2 FMDVs. (Fig. 4). No substitutions were observed in the viral RGD tri-peptide (144–146) involved in the attachment of FMDV to the cells [15]. We also observed the presence of the RGD motif, characterized by the presence of a positively charged arginine (R) residue at site 147, associated with the SAT2 serotype [16].

For the bordering residues of the RGD region, there were no amino acid substitutions at sites RGD − 1, + 1 compared to previously detected viruses in Egypt. Analysis of three previously known neutralization sites: (+ 2, + 3) site, (+ 10) site, and (+ 12) site [17] showed the identity of residues at + 2 (A), + 3 (A), + 10 (G), and + 12 (N). At neutralization site (+ 10), substitution D156G was observed. Also, the 2015 viruses were characterized by TPT at − 3 to − 5 of the RGD in G-H loop compared to AAA in the 2012 SAT 2 viruses. Furthermore, residue at site − 7 of these viruses was characterized with a residue K at site 137.

The carboxyl-terminal region, another important antigenic site (at amino acid positions 193–215) of VP1,

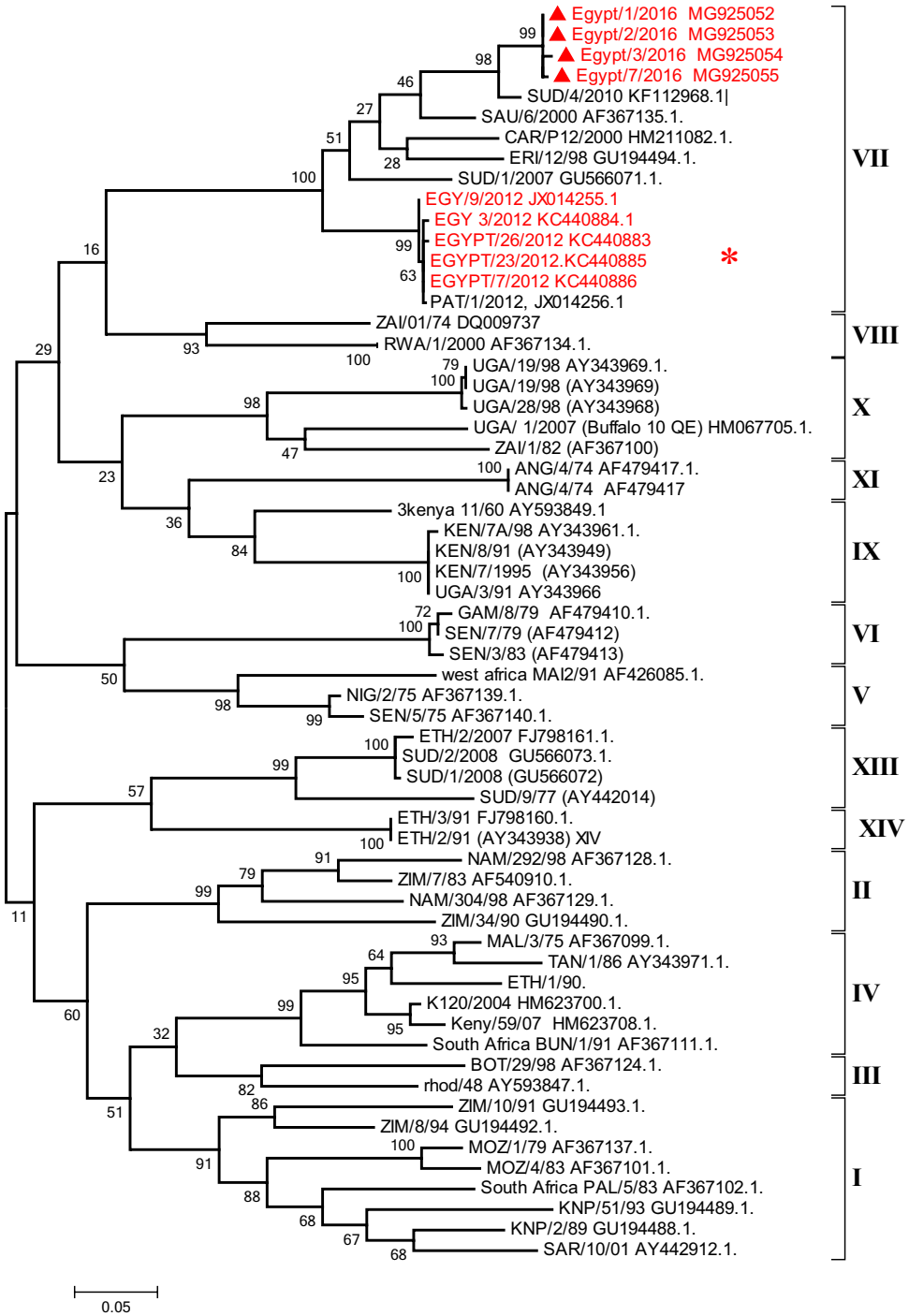

Fig. 3 Maximum Likelihood tree based on the VP1 coding sequence showing the relationship between the Egyptian SAT2 FMDVs detected in upper Egypt in 2015 and other contemporary topotypes of SAT 2 FMDVs. Bootstrap values are shown above the branches. The tree was generated using MEGA7 with bootstrap method and Kimura 2-parameter model. Sequences obtained from this study were marked with red triangles. Asterisk indicates the lineage of vaccine seed strains used in Egypt

exhibited homogeneity among characterized viruses in this study. Three differences between the FMDVs were detected in this study and viruses from 2012 were observed at amino acid positions K198E, T200A, and D201G in the carboxyl-terminal region.

Comparison of amino acid sequences and phylogenetic analysis of the VP1 of serotype O

Amino acid sequences of the VP1 proteins of serotype O samples were generated from nucleotide sequences. The

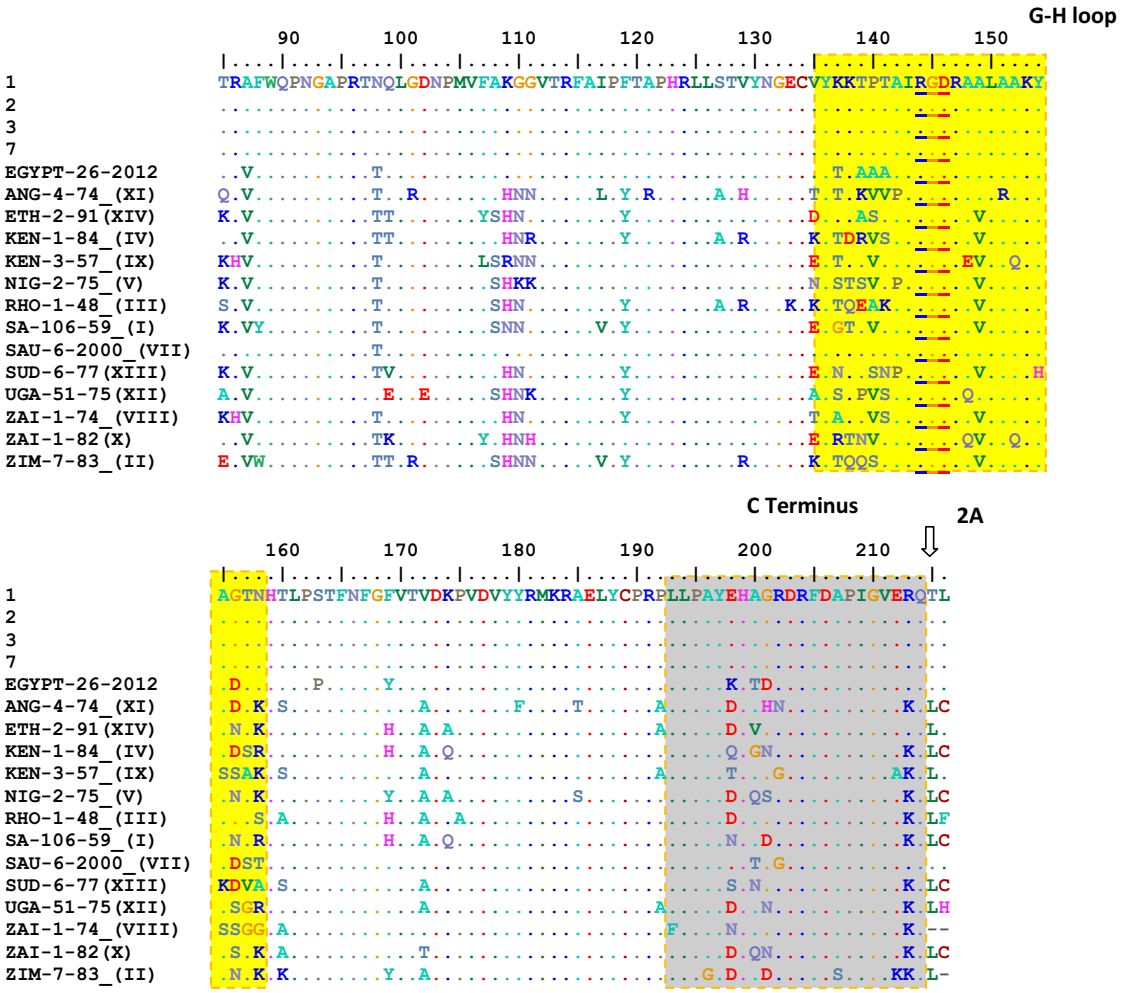

Fig. 4 Alignment of the deduced amino acid sequences of VP1 surface proteins of FMDV isolates in Egypt compared with different SAT 2 FMDV topotypes. A dot (.) indicates identity with the Egyptian 2015 amino acid sequences. The G–H loop and carboxyl-terminus region are shaded yellow and gray, respectively

sequence comparison of the Egyptian isolates from 2006 till 2016 showed variability among detected viruses. Phylogenetic analysis showed that all previously detected Egyptian FMDV of serotype O were related to topotype East Africa (EA)-3 and ME-SA (Middle East–South Asia) (Fig. 5). All the obtained sequences in this study fell into a single topotype, East Africa (EA)-3. The detected serotype O strains clustered into two main distinct groups. One cluster included samples that were collected from cattle in El-Minya and Fayoum that were related to viruses from Northern Egypt forming a group which we named group (1). Six viruses from 4 governorates (≈88% nucleotide identity) were similar to previously detected viruses from 2013 to 2014 forming a cluster which we named group (2). The sequence homology between the 2 groups was 88%.

By comparing the amino acid sequences of the G-H loop region, results revealed that the FMDV serotype O samples in this study differed from previous Egyptian viruses. Group 1 is characterized by amino acid substitutions V25I, K46S/N, T97A, and ^{135}CRYGNVA^{141}. Group 2 is distinguished with amino acid substitutions E21V, K45Q, D46S, I48T, T56I, N134S, R134S, and R172Q (Fig. 6). The amino acid sequence comparison showed that the group 1 O FMDVs from 2016 were almost identical to O/Sharquia/EGY/2014 which was part of the group 1 O FMDVs from 2013 to 2014, while the group 2 O FMDVs had 14 aa differences from the group 1 2013–2014 O FMDVs, indicating that these viruses were from different origins.

Discussion

FMD is one of the most detrimental viral diseases of livestock causing huge economic losses worldwide. In Egypt, it is an enzootic disease and usually causes outbreaks at varying time intervals. Despite using different types of

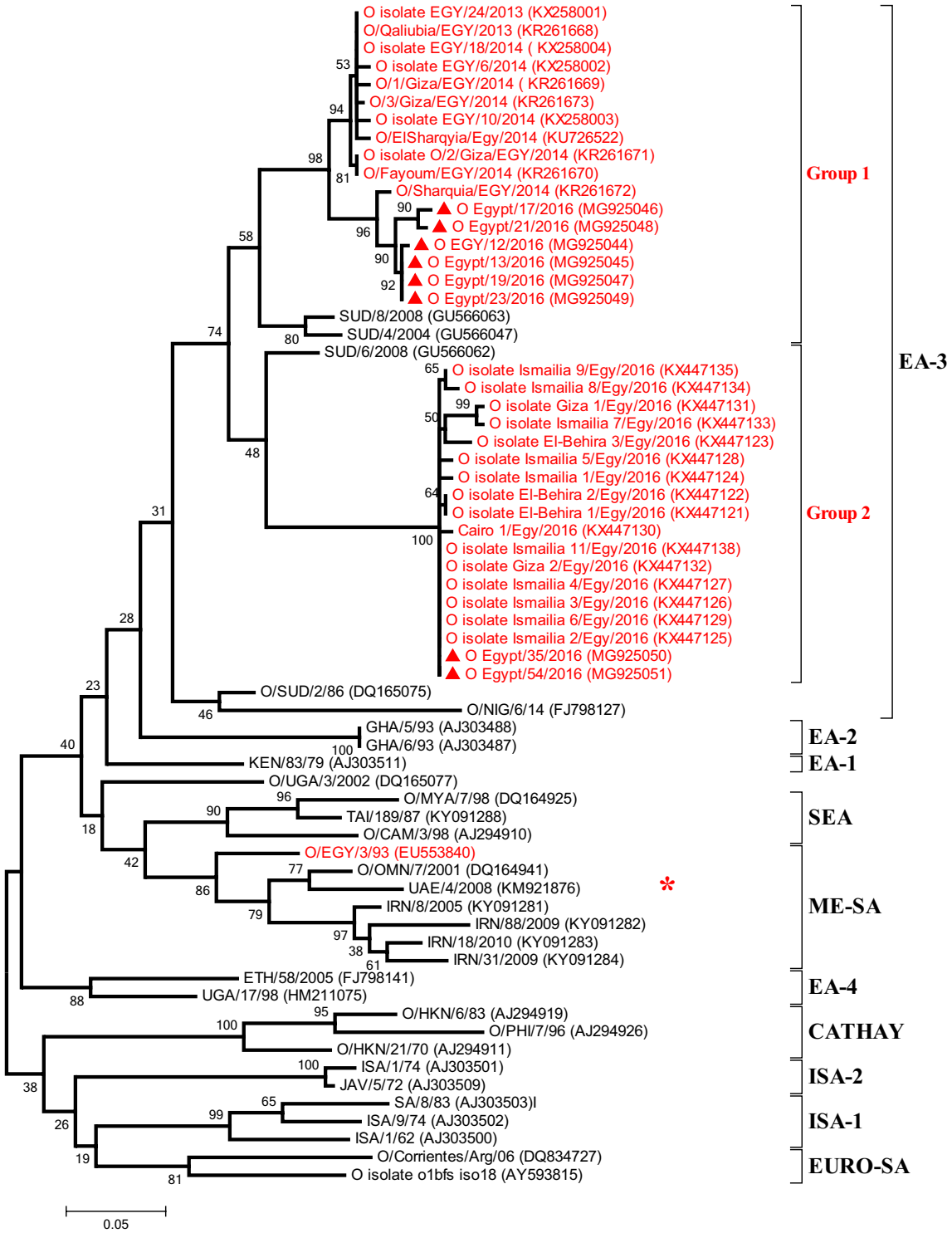

Fig. 5 Maximum Likelihood phylogenetic tree of the VP1 of FMDV serotype O. The tree was generated using MEGA7 with bootstrap method and Kimura 2-parameter model. All Egyptian sequences are shown in red and the obtained sequences in this study are marked with red triangles. Asterisk indicates the linage of vaccine seed strains used in Egypt. The O serotype is divided into eleven topotypes: East Africa 1 to 4 (EA-1 to -4), Southeast Asia (SEA), Europe-South America (EURO-SA), Indonesia-1 and −2 (ISA-1 and −2), CATHAY, West Africa (WA), and Middle East-South Asia (ME-SA)

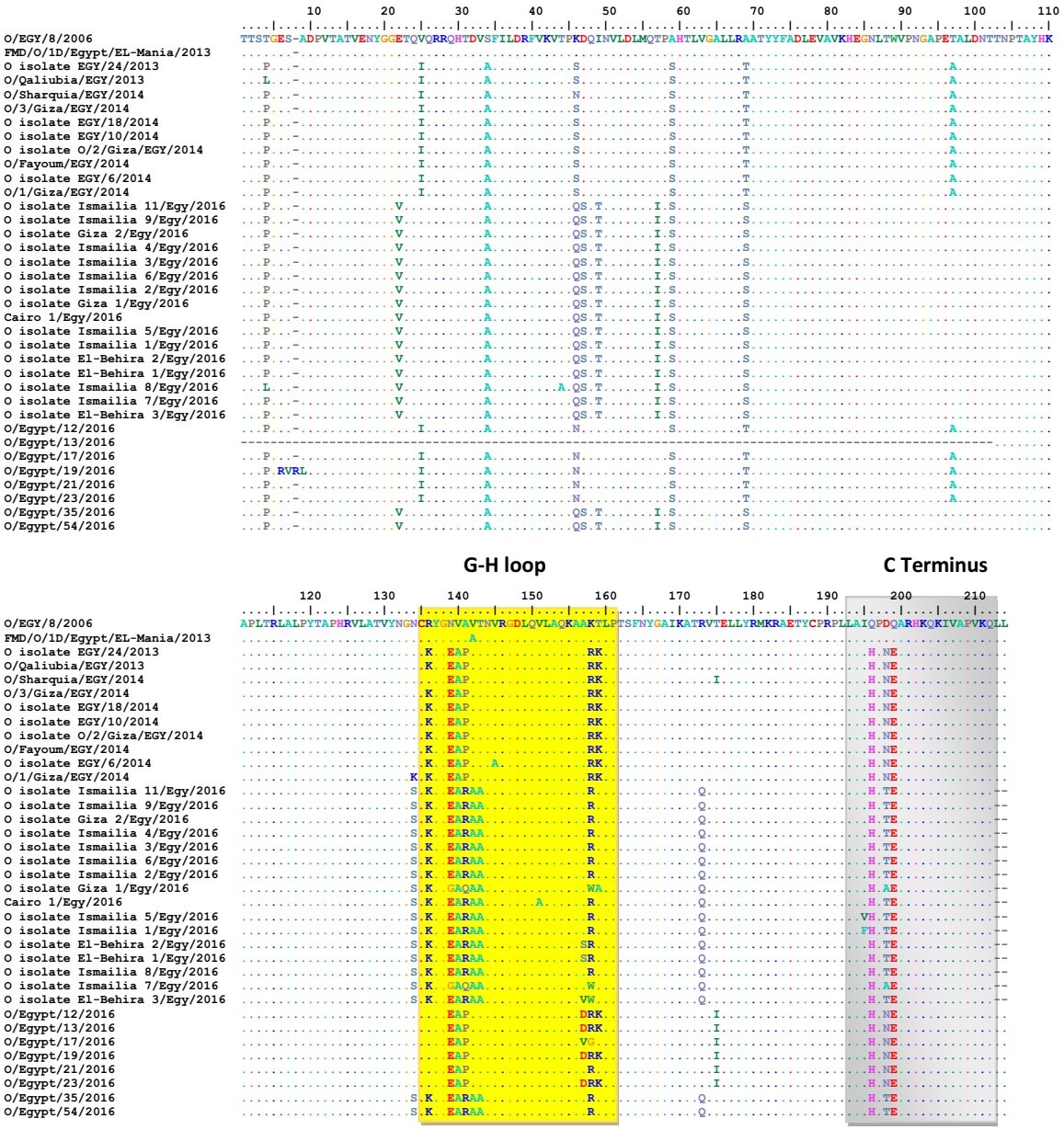

Fig. 6 Alignment of the deduced amino acid sequences of VP1 surface proteins of FMDV isolates in Egypt compared to different O FMDV serotypes. '.' indicates an amino acid site identical to Egyptian isolates of 2006. The major immunodominant sites of the protein (the G–H loop and carboxyl-terminus region) are shaded in yellow and gray

commercial vaccines in Egypt, FMDV outbreaks were annually reported [4]. The most commonly used vaccine in Egypt is a locally developed trivalent vaccine against serotypes A, O, and SAT2. This vaccine contains virus of FMD types "O" (Panasia/outbreak or O/EGY/3/93), "A" (Iran O5/outbreaks 2011), and "SAT2" (Egypt topotype VII outbreak 2012). All commercially used vaccines in Egypt are evaluated by the Central Laboratory for Evaluation of Veterinary Biologicals (CLEVB) before being approved and released to the market. A previous study demonstrated the efficacy of this vaccine against serotypes O and A using challenge experiment [18].

The level of antibody titers raised against commercial vaccines is the reference criterion to evaluate vaccine potency as it is correlated with protection rate. However, this level is affected by several factors such as vaccine seed strains, vaccine antigen content, vaccine formulation, virus challenge dose, and route of infection [19]. A previous study showed that animals challenged with heterologous viruses lacking reactivity with vaccine seed strains required revaccination to introduce protective antibody titers [20]. Additionally, under the field conditions in which a vaccine strain is poorly matched to circulating strains, FMD viruses could

genetically diversify [21]. Thus updating or inclusion of new variant strains based on surveillance studies in the vaccine formulations is highly recommended.

Although 85% of animals sampled during our study were seropositive for FMDV, all were positive for the FMDV by PCR despite the fact that all animals were vaccinated. These findings, suggesting vaccination failure, might be related to the antigenic mismatch between the vaccine seed strains and circulating viruses. Genetic mutations may lead to a new antigenic variant capable of escaping the animal immune system, especially with the absence of cross protection between the different serotypes of FMDV [22]. Therefore, further molecular identification and characterization studies are needed in the future to detect any mutations in the isolated strains.

In fact, multiple FMDV serotype combinations were observed in the SPCE which is similar to previous reports. Serotype O of FMDV was the most common serotype isolated during our study clustered into two main distinct groups. All of those new strains were closely related to topotype EA-3 previously characterized in Egypt from 2014 to 2016 [23]. The currently characterized serotype O viruses of group 1 and 2 had only 86% and 85% nucleotide identity, respectively, with the previously characterized FMD/O/Dakahlia/Egypt/2014 strain (accession number KP940473) isolated from Dakahlia government (Nile Delta region) of Egypt [24] suggesting the introduction of new viruses into the country.

Simultaneous circulation of the three serotypes of FMDV in Egypt increases the probability that animals are co-infected with different serotypes. In the present study, some samples were found to contain more than one FMDV serotype. The observed mixed serotypes infections with A/SAT2 and O/SAT2 were similar to previous findings [25–28]. Previous studies showed that the recombination found in the genome of FMDVs has been associated with co-infection of an animal with two or more FMDV serotypes [29, 30].

The phylogenetic tree of VP1 of SAT 2 showed that the detected SAT2 FMDVs in 2015 were genetically distinct from the previously characterized SAT2 from 2012 in Egypt and Libya [7, 31] and were closely related to SAT2/SUD/4/2010 (accession number KF112968) isolated from Sudan, suggesting that the source of this emerging virus might be related to importation of live animals from Sudan or through uncontrolled boundaries. One of the interesting results of this study was the detection of two distinct groups of serotype O FMDVs in EA-3 topotype, responsible for the 2015–2016 outbreaks which differ from vaccine strains of the ME-SA topotype. Co-circulation of multiple serotypes with multiple genetic variants indicates the necessity of continuous monitoring of the genetic changes in endemic FMDVs in Egypt. Conducting vaccines-strains efficacy matching in Egypt would be of interest to check if the protection acquired from induced antibodies is sufficient or there is a need to develop a new vaccine strain. It is also important to investigate the correct handling of the vaccines (maintenance of cold chain, disposal of seal-broken vaccine, etc.) to figure out if the lack of protection is due to vaccine breaches or low competence of the induced antibodies. FMDV vaccine candidates from local strains that reacted well with the circulating strains should be used and continuously updated based on antigenic and genetic analyses of local strains.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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