Research Article

Molecular Detection of FMDV Serotype A Isolated from the Egyptian Delta During 2019-2020

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Abstract | Egypt is one of the countries threaten with foot and mouth disease virus (FMDV) that attacks animals every year. The introduction of new topotypes/lineages of the existing FMD viruses represents a constant risk for cattle production. Serotype A is one of FMDV that is already existing and incriminated lastly in serval problems in cattle population. So, this study aimed at molecular characterization of serotype A FMDV that has been involved in the latest FMD outbreaks in Egypt. Thirty-six samples (26 blood and 10 oral epithelial tissue samples) were obtained from suspected cattle in three Egyptian governorates during 2019-2020. The samples were screened for FMDV by means of real-time RT-PCR that showed nearly 86% (n=31) of the examined samples to be FMDV positive. Virus isolation was carried out on ten of the FMDV positive samples that were selected according to their cycle threshold (C_t) values. Moreover, the serotype A amplicons were subjected to sequencing of the VP1 gene. Through phylogenic analysis of three FMDV serotype A isolates, it has been deduced that two isolates were clustered to Asian Iran-05 topotype with close similarities (99.42-99.57%) to the local vaccine strain, while the other one belonged to African topotype genotype IV (GIV) and was genetically different from the local vaccine strain with 85.65% identity. These findings support the suggestion of adding the African topotype GIV as a supplementary vaccine to the currently used one.

Keywords | FMDV, Serotype A, Genotype IV, VP1gene, phylogenic analysis.

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INTRODUCTION

Foot and mouth disease (FMD) is one of the most threatening infectious vesicular viral diseases of domestic and wild cloven-hoofed animals, mostly cattle, sheep, goats, and swine (Knight-Jones et al., 2016; Shanafelt and Perrings, 2017). Further, FMD is a cross border disease that rapidly spread between countries causing severe economic and social impact in accordance to the World Organization for Animal Health (OIE) (OIE, 2020a). Foot and mouth disease virus (FMDV) is a positive sense RNA virus that belongs to genus *Aphthovirus* of family Picornaviridae. There are 7 genetically and antigenically different serotypes: A, O, C, SAT1, SAT2, SAT3, and Asia 1 (Rodríguez et al., 2020). Each serotype has several topotypes which are further divided into lineages with diverse genotypes (Saduakassovaa et al., 2018). The genome of each serotype is about 8.2 Kb and is surrounded by a protein capsid (Martinez-Salas and Belsham, 2017). The capsid includes 60 copies of four structural proteins (VP1–4). VP1, 2 and 3 are exposed on

the surface, whereas VP4 is internalised (Racaniello et al., 2001). The crystallographic configuration of the viral protein coat indicated that immunological determinants are often encountered on surface-oriented interlinking loops among structural components (Fry et al., 1999). The evolutionary preserved Arg-Gly-Asp (RGD) amino acid element inside the G-H loop in VP1 gene is important for viral attachment to host cells (Carrillo et al., 2005) and stimulation of host protective immune responses (Marrero et al., 2015; Fernandez-Sainz et al., 2019). The RNA polymerase's low fidelity results in error-prone viral replication and genetic alterations (Herod et al., 2017). The involvement of immunological epitopes can result in the emergence of new virus variants. Immunity to one serotype does not confer protection contrary to other serotypes or to variants in the same serotype (Fernandez-Sainz et al., 2019). Furthermore, phylogenetic analyses using multiple alignments of the VP1 gene sequences aid in identifying the variety, genetic correlation, and geographical distribution of various FMDV serotypes (Cottam et al., 2006; Pybus et al., 2009). VP1 also contains serotype-specific amino acid residues that allow different serotypes to be distinguished (Carrillo et al., 2005).

Egypt is considered one of FMD enzootic countries since 1950s, causing a dangerous threat to animal wealth (Ahmed et al., 2012). Although there were minor outbreaks caused by FMDV serotype A in Egypt since 1953, serious FMDV serotype A outbreak causing huge economic damages has been described at the beginning of 2006, this might have been related with the importation of live animals from African republics (Abd El-Rhman et al., 2020). In the past 11 years, Asian and African topotypes of serotype A were identified in Egypt (Sobhy et al., 2018). In 2020, four FMD outbreaks were detected, two confirmed as SAT2 serotype topotype VII and the others were confirmed as serotype A lineage AFRICA/IV (WRLFMD, 2020). The strategy for FMD outbreaks prevention in Egypt is mainly dependent on vaccination every 6 months using locally produced trivalent inactivated vaccine (O Panasia-2/A Iran-05/SAT2/EGY-A-2012) (El-Bagoury et al., 2014). As a result of the outbreak reported in October 2020, the vaccine was recently changed by adding serotype A lineage (A/AFRICA/G-IV) to it (WRLFMD, 2021). The importation of animals from FMDV endemic zones and the application of incompatible vaccines are the main causes of vaccination failure and could hinder the control of FMD in endemic countries that depend on inactivated FMDV vaccination (Paton et al., 2009; FAO, 2016; Singh et al., 2019). Globally where FMDV exist, serotype A is present (Parida, 2009). The continuous follow up of new FMDV variants will aid in appropriate vaccine matching in order to ensure proper and up-to-date preventive actions. Thus, the goal of this work was to sequence the full VP1 gene to determine the molecular and genetic characteristics of FMDV serotype A that was circulating in Egypt in 2019-2020.

MATERIAL AND METHODS

Clinical samples

Thirty-six samples (26 blood and 10 oral epithelial samples) were collected from clinically infected cattle from Qalubia (n =12), Sharkia (n =16) and Gharbia (n =8) provinces during 2019-2020. The collected oral epithelial samples were maintained in transport medium comprising penicillin (1000 IU), neomycin sulphate (100 IU), polymyxin B sulphate (50 IU), and mycostatin (100 IU) in equal volumes of glycerol and phosphate buffer saline (PBS) (Sigma, USA). The ultimate pH of the glycerol/buffer mixture was between 7.2 and 7.6 and was stored at -80°C until use according to protocols proposed by (OIE, 2018). The samples were transported to the Animal Health Research institute (AHRI), Dokki, Giza.

Samples preparation

Suspensions were ready by grinding 2 grams of oral epithelial samples with sterile sand in a sterile mortar with 2-3 ml PBS and antibiotic. The suspensions were centrifuged at 3000 rpm for 10 minutes, the supernatants were collected and filtered through a 0.22 Millipore filter and stored at -80°C till virus isolation on BHK21 cells (OIE, 2021).

Virus isolation

Continuous cell line of Baby hamster kidney cells (BHK21, clone13) was used for virus isolation from oral epithelial samples. The prepared virus suspension (0.3ml) was inoculated to cell culture. The inoculated cell cultures were incubated at 37 °C and 5% CO_2 for 72 h with daily examination till CPE development (OIE, 2021).

Extraction of Viral RNA

The total RNA was extracted from the collected samples using QIAamp Viral RNA Mini Kit (Qiagen) cat. no. 52904 as stated by the manufacturer's commands. The extracted RNA was stored at -20°C for further analysis.

Real-time RT-PCR (qRT-PCR)

The extracted RNA from the collected samples (26 blood and 10 oral epithelial samples) were tested for the existence of FMDV genomic RNA by primers and probes targeted directly to the conserved 3D gene as shown in Table 1. Primers and probe synthesized by Invitrogen; USA were used in accordance with the manufacturer's guidelines for 3D gene amplification. The reaction was run on StepOne Real Time PCR instrument (Applied Biosystems, USA) using the subsequent thermal profile: reverse transcription at 50°C for 15 minutes then initial denaturation at 95°C for 5 minutes, 45 cycles at 95°C for 15 seconds and an

Table 1: Primers of real-time RT-PCR targeting 3D gene and one-step RT-PCR targeting 1D gene of FMDV.

Primer	Sequence 5'-3'	Amplicon size	References
3D-F	ACT GGG TTT TAC AAA CCT GTGA	106 bp	(Callahan et al., 2002)
3D-R	GCG AGT CCT GCC ACGGA		
3D-Probe	FAM-TCC TTT GCA CGC CGT GGG AC- TAMRA		
A-Egy-F	GGAATCWGCAGACCCTGTC	750 bp	(Shehata et al., 2016)
O-Egy-F	CCTCCTTCAAYTACGGT	283 bp	(Bachanek-Bankowska et l., 2016)
SAT2-Egy-F	TGAYCGCAGTACACAYGTYC	666 bp	(Shehata et al., 2016)
Reverse primer Nk61	GACATGTCCTCCTGCATCTG		(Knowles et al., 2005)

Table 2: Results of FMDV detection by qRT-PCR.

	Sharkia		Qalubia		Gharbia		Total
	Blood	Oral epi.	Blood	Oral epi.	Blood	Oral epi.	
FMDV positive	11	3	5	5	5	2	31
FMDV negative	2	-	2	-	1	-	5

nealing and extension step at 60˚C for 1 minutes at which the fluorescence data were collected in each cycle. Samples that had cycle threshold (Ct) less than 30 were checked using conventional one-step RT-PCR.

Conventional one-step RT-PCR

The extracted RNA from the 10 oral epithelial samples was subjected to RT-PCR by Thermo scientific verso 1-step RT-PCR Ready Mix Kit (Thermoscientific, USA) according to the manufacturer's instructions. The RT-PCR reaction was performed in a final volume of 25 µl consisting of 12.5 µl of 2X 1-step PCR Ready Mix, 0.5 µl of Verso Enzyme Mix, 1.25 µl RT-Enhancer, 1 µl of specific forward and reverse primer for serotypes A, O and SAT2 as shown in Table 1, and 5 µl of viral RNA extract. The cycling conditions were cDNA synthesis at 50 ˚C for 30 minutes and Verso inactivation at 95 ˚C for 2 minutes, then 35 cycles of denaturation at 95 ˚C for 60 seconds, annealing at 60 ˚C for 30 seconds and elongation at 72 ˚C for 1minute, followed by a final extension at 72˚C for 5 minutes. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel (Roskams and Rodgers, 2002).

Sequencing and phylogenetic analysis

The PCR products were refined by QIAquick Gel Extraction Kit (Qiagen, Germany) in accordance to manufacturer's guidelines. Sequencing of the PCR products was operated using BigDye™ Terminator V3.1 Cycle Sequencing Kit by the previously used forward and reverse primers for serotype A as in Table 1. The attained nucleotide sequences for viral serotype A were computationally and bioinformatically analyzed to identify the substitution rates and construct a phylogenetic tree (Saitou and Nei, 1987; Kumar et al., 2018). Multiple sequence alignment (MSA) was performed via ClustalW/BioEdit software- v7.2.5 (Hall, 1999). Results of MSA were clipped and exposed from columns comprising gaps and phylogenetic tree was created by MEGA software version 6 using the neighbor-joining method with bootstrapping over 1000 replicates (Felsenstein, 1985; Kumar et al., 2018).

RESULTS

FMDV detection by qRT-PCR

The 36 samples were primarily screened for FMDV by qRT-PCR. Thirty-one (21 blood and 10 oral epithelium) (86%) samples were positive using pan-FMDV 3D primers-probe set (Table 2, Figure 1).

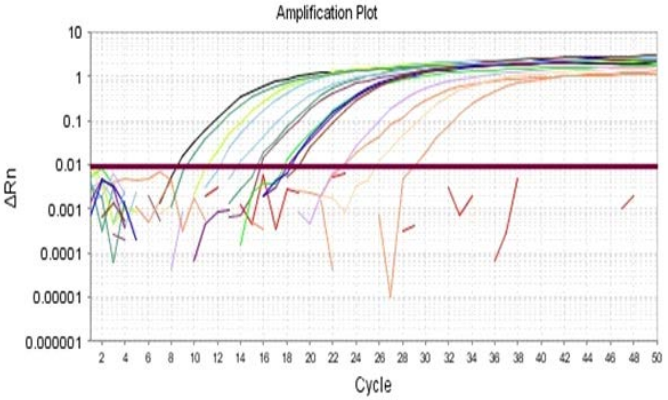

Figure 1: The amplification plots of Pan-serotypic (3D) qRT-PCR assay of collected field samples.

Isolation of FMDV on BHK-21 cell line

Virus isolation was carried out using the ten oral epithelium samples positive for FMDV that were selected ac

Table 3: FMDV isolates used for sequencing and phylogenetic analysis of serotype A.

ID	Accession #	FMD Topotype	Sample material	Province
FMD/A/EGY/AHRI/G1-2020	OL769313	Asian topotype (Iran-05 lineage)	Oral epithelium	Sharkia
FMD/A/EGY/AHRI/G2-2019	OL769314	Asian topotype (Iran-05 lineage)	Oral epithelium	Qalubia
FMD/A/EGY/AHRI/G3-2020	OL769315	African topotype (genotype IV)	Oral epithelium	Gharbia

Table 4: The amino acid differences in the main antigenic sites of VP1 in the new isolate of serotype A (African genotype IV) and vaccinal strain in Egypt.

No.	Residue number	Location	Vaccine strain A/EGY 1/2012	FMD/A/EGY/AHRI/G1-2020(OL769313)
1	43	βB-βC loop	N	T
2	44	βB-βC loop	P	S
3	137	βG-βH loop	S	T
4	139	βG-βH loop	T	G
5	140	βG-βH loop	G	T
6	141	βG-βH loop	G	S
7	142	βG-βH loop	D	P
8	144	βG-βH loop	R	Q
9	149	βG-βH loop	S	A
10	198	carboxy terminus	Q	G
11	201	carboxy terminus	H	Y
12	202	carboxy terminus	K	Q
13	204	carboxy terminus	K	R

cording to their Ct value (lower than 30). The selected viral samples produced characteristic cytopathic effect (CPE) on BHK-21 cells after 24-48 hours from inoculation, cell rounding, degeneration, and cytoplasmic granularity are observed, followed by total cell lysis as shown in Figure 2.

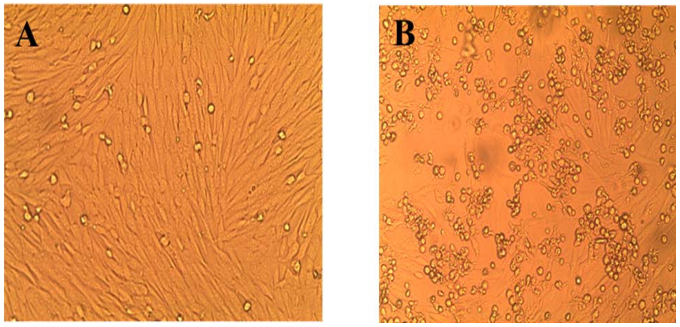

Figure 2: Pictures showing the FMDV's cytopathogenic effect on inoculated BHK-21 cells. Panel (A) control BHK 21 cell line, Panel. (B) BHK21 Cells showing characteristic CPE.

Amplification of VP1gene by one step RT-PCR

The ten oral epithelial samples isolated on BHK-21 cells and detected by qRT-PCR having Ct lower than 30 were further tested by specific primers of serotypes A, O, and SAT2. The RT-PCR for FMDV isolates revealed that serotypes A, O and SAT2 were identified in 5, 3 and 2 isolates, respectively (Figure 3).

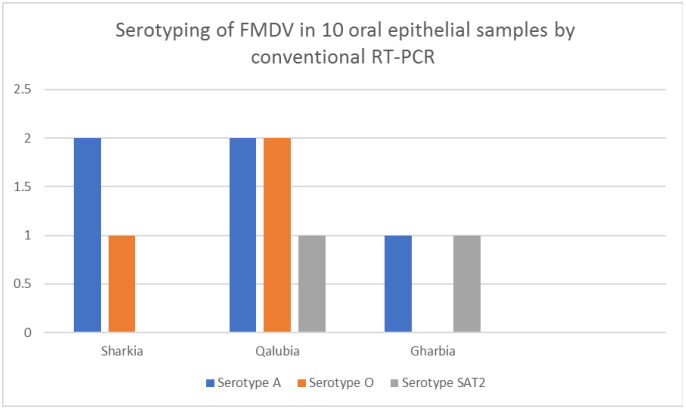

Figure 3: Serotyping of FMDV in 10 oral epithelial samples by conventional RT-PCR.

Phylogenetic analysis and relationships among some global and circulating FMDV serotype A

VP1 Full-length gene sequences of three FMDV serotype A isolates from oral epithelium obtained in this study were submitted to GenBank under accession numbers OL769313, OL769314 and OL769315 (Table 3).

Phylogenetic analysis and multiple sequence alignment

shown that two of the newly circulating FMDV strains FMD/A/EGY/AHRI/G2-2019 (OL769314) and FMD/A/EGY/AHRI/G3-2020 (OL769315) belong to Iran-05 lineage of Asian topotype and were closely related to the vaccinal strain A/EGY 1/2012 (KC440882) with high nucleotide similarity 99.42 and 99.57%, respectively as shown in Figure 4.

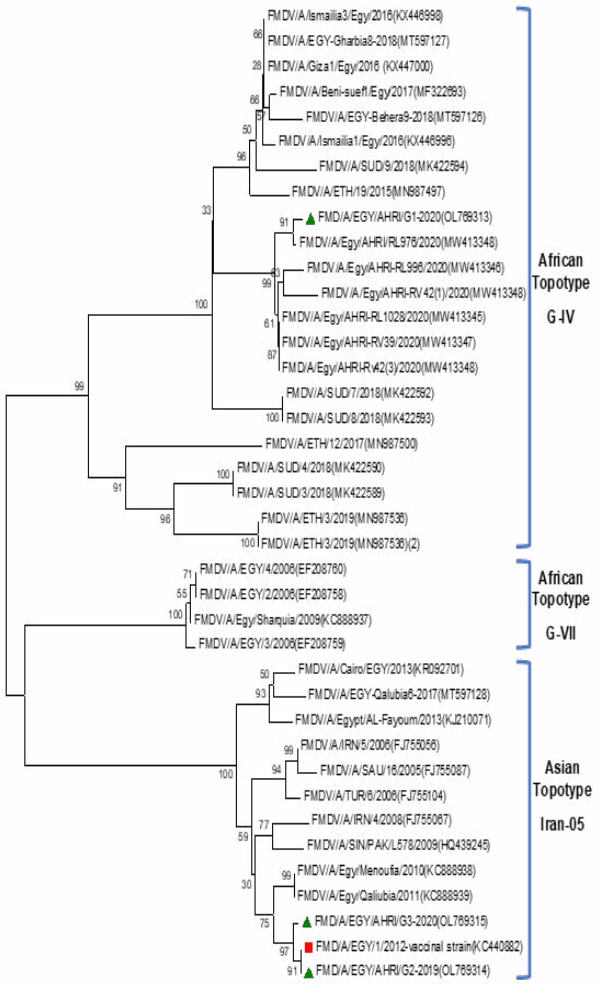

Figure 4: Phylogenetic tree constructed using the neighbor joining method depending on the VP1 gene sequences of the three FMDV isolates identified in this study and 36 additional FMDV serotype A sequences obtained via the GenBank (https://www.ncbi.nlm.nih.gov/genbank) dataset. The bootstrap probabilities are signified by numbers at the internal nodes (1000 replicates). Red square refers to vaccinal strain, green triangles refer to isolates of present study.

The isolate FMD/A/EGY/AHRI/G1-2020 (OL769313) belonged to genotype IV of African topotype) and was closely related to the Egyptian FMD viruses recently isolated in 2020 (MW413348, MW413351, MW413347, MW413345, MW413346, MW413349 and MW413350) with identity ranging from 97.31 to 99.68%, whereas it had a lower identity percentage (91.25 to 93.68%) with strains isolated between late 2015 to 2018 (MT597127, KX446998, KX446996, MF322693, MT597126, KX447000). This isolate has external relation to Ethiopian strain ETH/19/2015 (MN987497) and Sudanese strain A/SUD/9/2018 (MK422594) with identity of 93.38 and 92.11%, respectively as shown in Figure 4. The acquired VP1's deduced amino acid sequences of FMD/A/EGY/AHRI/G1-2020 (OL769313) as illustrated, showed amino acid variations in the major antigenic sites (Table 4, Figure 5).

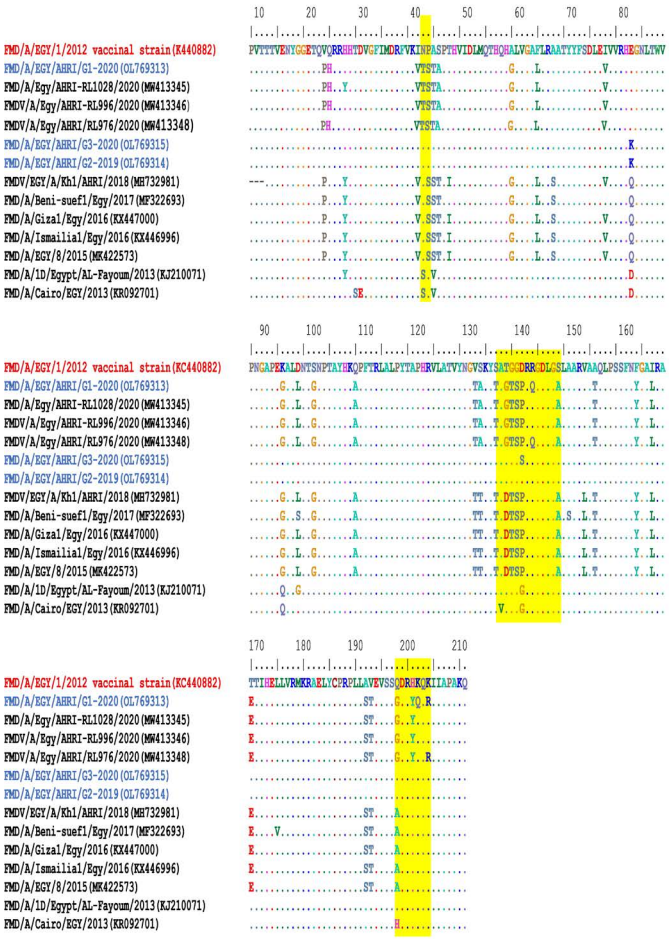

Figure 5: Deduced amino acid sequences within the VP1 gene of the new FMDV isolates in this study were compared to existing FMDV type A isolates over the years. The highlighted regions refer to the significant variable antigenic sites chosen in the comparison.

DISCUSSION

FMD is a major universal concern. It seriously affects cattle production and blocks the global trade. Egypt's exhaustive FMD control system, which includes vaccinating animals with a locally inactivated polyvalent vaccine containing serotypes O pan Asian II (EGY/2010), A Iran 05 (A/EGY/1/2012), SAT2 (EGY/Gharbia/2012), and SAT2 (LIB/2018). Although the vaccination program was successful in lowering FMD incidence, uncontrolled animal movement results in circulation of FMDV strains. There

have been at least eight variants of circulating FMDV serotypes A, O, and SAT2 isolated in the last decade (El-mayet et al., 2020). These variants included A-Iran 05, A-African genotype IV, A-African genotype VII, O-Pan Asia II, O East Africa, SAT2 Ghb-12, SAT2 Lib-12, and SAT2 Alx12. Furthermore, Egypt's distinct geographical position increases the risk of circulating variants from East African and Asian countries infiltrating the country (El-mayet et al., 2020).

In general, the variable severity of FMD occurrence is attributed to either absence of vaccination because of reluctance of the farmers to vaccinate their cattle against FMD due to the fear of drop in milk yield after vaccination or vaccination failure in vaccinated herds/areas mainly due to unmatchable vaccinal strain with the circulating virus (Singh et al., 2019). Because of the high mutation rate of FMDV, new FMDV strains emerges (Fernandez-Sainz et al., 2019). So, there is a crucial requirement for continuous screening, genetic and antigenic characterization of the circulating FMDV strains and revealing their relationship with the vaccinal strains to assist in FMD control (Knowles et al., 2016).

Depending on the above, the current study focused on molecular characterization of the circulating FMDV (serotype A) during 2019-2020 by collecting 36 clinical samples from different localities in three Delta governorates (Sharkia, Qalubia and Gharbia). In accordance to (Ahmed et al., 2012) who stated that Delta Governorates are considered due to their great density of animal population, so the primary struck of infection was firstly noted in the Delta region as in FMDV outbreak of genotype SAT2. The collected samples were blood and oral epithelium as the virus could be detectable in different samples depending on the stage of infection. If animals are clinically affected, the preferred samples would be materials from the lesions. For animals not showing obvious clinical signs, blood may be useful for virus isolation (OIE, 2020b).

According to qRT-PCR results, the highest incidence of the disease was 87.5% in governorates Sharqia (14/16) and Gharbia (7/8). While in Qalubia, the incidence was 83.3% (10/12) as shown in Table 2. In contrast to the disease incidence in 2017-2018, Qalyobia was the highest governorate in incidence with percent of 70% followed by Sharqia (40%) (Abd El-Rhman et al., 2021). On the other hand, conventional RT-PCR revealed that the three FMDV serotypes A, O and SAT2 were detected in Qalubia, serotypes A and O were detected in Sharquia. While in Gharbia, serotypes A and SAT2 were identified as shown in Figure 3. In 2016-2019, the three FMDV serotypes A, O and SAT2 were detected in Qalubia and Sharquia. While in Gharbia, serotypes O and SAT2 were identified (Shahen et al., 2020). Every year, there is a dominant circulating strain with alternative displacement and winter peak of 2 to 3 circulating strains (El-mayet et al., 2020).

VP1 sequencing and analysis of clinical samples collected from different localities helps in construction of phylogenetic tree to understand the molecular epidemiology of FMDV and tracing the source of newly emerging strains (Knowles et al., 2016). The VP1 sequencing analysis revealed that two viral isolates of serotype A FMD/A/EGY/AHRI/G2-2019 (OL769314) and FMD/A/EGY/AHRI/G3-2020 (OL769315) are cladded with A-Iran 05 lineage of Asia topotype, which was firstly recorded in Egypt during 2010 (Sobhy et al., 2018; WRLFMD, 2018) and the last report was during 2017 (Shahen et al., 2020), indicating a clear difference from EGY/2/2006 (EF208758) and EGY/4/2006 (EF208760) as they were related to Africa topotype genotype VII that were circulating previously, and were incriminated in outbreaks during 2006. They were Clustered in the same group of the vaccine strain A/EGY 1/2012 (KC440882) with identity 99.42 and 99.57%, respectively. While the 3rd isolate of serotype A FMD/A/EGY/AHRI/G1-2020 (OL769313) was genotype IV of the African topotype which was first recorded during 2012 and was recorded in some occasions during 2016 and 2018 (Sobhy et al., 2018; WRLFMD, 2018), indicating clear difference from the vaccine strain A/EGY 1/2012 (KC440882) with identity 85.65%.

According to numerous publications, serotype A (Asia topotype) was detected during the last 11 years (Sobhy et al., 2018). Apart from that, serotype A genotype IV (Africa topotype) was identified in 2016-2019 (Soltan et al., 2019, Shahen et al., 2020). The new strain FMD/A/EGY/AHRI/G1-2020 (OL769313) presented similarity to previously described strains from Sudan in 2018 (92.74%–92.11) and Ethiopia in 2015 (93.38%). These findings suggest the possibility of genotype-IV entrance to Egyptian territories directly from Sudan or Ethiopia as was previously described (Duchatel et al., 2019).

Moreover, the deduced amino acid sequences of currently circulating serotype A of genotype Iran-05 were genetically related to the vaccination strain, with only minor differences in some amino acids. While the genotype VII was closely related to strains recently isolated in 2020 by (Hassan et al., 2022) but deviated from the previously published Egyptian sequences either African genotype-VII or Asian genotype-Iran-05 as shown in Figure 4. Furthermore, Multiple amino acid substitutions were observed in VP1 gene in the major antigenic regions in comparison to the local vaccinal strain. The amino acid constitution among the circulating FMDV serotype A African genotype VII and vaccinal strain A/ EGY/1/2012 exhibited significant

amino acid variation in the FMDV antigenic sites. These areas range from 43-44, 136–151, 149 and 198–204 of VP1 amino acids (Table 4, Figure 5). In those positions, the majority of the amino acid changes were conserved. Some residues, on the other hand, were identified as surface or significant antigenic sites (Reeve et al., 2016). As previously reported by the WRLFMD (2016), the vaccine strain of origin (Iran-05) demonstrated no protection against G-IV strains in a vaccine matching challenge test. So, our results assured that the locally produced trivalent inactivated vaccine should be changed to include the serotype A lineage. (A/AFRICA/G-IV) to the vaccine in accordance with WRLFMD, (2021).

CONCLUSION

The recent study shows that the recently circulating FMDV serotype A in 2019-2020 in Egypt belongs to Asian Iran-05 and Africa GIV topotypes. The circulating serotype A isolates were Asian Iran-05 that are closely related to the vaccinal strain while the circulating Africa GIV one exhibited remarkable genetic differences and its inclusion in the locally polyvalent vaccine is a timely decision to reduce the risk of future FMD outbreaks.

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CONFLICTS OF INTEREST

There are no conflicts of interest stated by the authors.

AUTHORS CONTRIBUTION

All authors contributed equally.

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