j.Egypt.net.med.Assoc 81, no 1. 67 – 80 (2021)

MOLECULAR CHARACTERIZATION OF CIRCULATING FMD VIRUSES IN BOTH VACCINATED AND NON-VACCINATED CATTLES IN EGYPT

By

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ABSTRACT

Foot and mouth disease (FMD) is a cross-border epidemic transmissible animal disease affecting a wide range of animals. FMD is widespread globally. Egypt is under threat of infection from neighboring regions with a history of the disease from 1950 until now although a broad vaccination program was implemented. The research was planned to make molecular Surveillance of FMD viruses circulating in both vaccinated and non-vaccinated cattle in Egypt. Fourteen positive FMD antigen detection ELISA samples were serotyped using PCR specific primers and with trials for isolation on the BHK- 21cell line. Viral protein 1(VP1) gene of some representative viruses sequenced and phylogenetically analyzed to detect identity between detected viruses and other reference isolates. Three samples were positive for serotype O, three positive for serotype A and eight positives for serotype SAT2.VP1 encoding region sequencing and phylogenetic analysis revealed that all serotype O strain of the present studies are identical to each other with a percentage of 91% to 94% and found to be related to EA-3 topotype, all serotypes A strain were identical with 97% to 99% to each other and clustered to genotype G-IV of African strain and all serotype SAT2 were 99% identical to each other and were related to topotype VII, Lib-12 lineage that differs from the previously-reported Egyptian strains of Ghb-12 lineage of G-VII. The present study demonstrated the co-circulation of O, A, and SAT2 serotypes of FMD virus in Egypt in both vaccinated and non-vaccinated animals, and emerging of a new SAT2 subtype that guides us to increase attention to the borders to prevent the leakage of disease-carrying animals.

Keywords:

FMDV; ELISA; PCR; Phylogenetic analysis; vaccination failure.

INTRODUCTION

Foot and mouth disease (FMD) is a severe and highly contagious disease of cloven-hoofed domestic and wild animals with serious economic impacts globally **(Du *et al.*, 2011)** and characterized clinically by the development of vesicles in the mouth, nose, muzzle, and coronary bands of infected animals, as well as reducing or losing milk production in lactating animals and worse body conditions. **(Meyer and Knudsen, 2001)**. The disease is transmitted by direct and indirect exposure with both the discharge and excretion from acutely infected animals and contaminated fomites including air-born infection **(Alexandersen *et al.*, 2000)**. FMD is caused by FMDV of the genus *Aphthovirus*, of the *Picornaviridae* family. There are 7 immunologically different and distinct serotypes in the virus (O, A, C, SAT1, SAT2, SAT3, and Asia1) **(ICTV, 2000)**. Each serotype is further divided into topotypes, which have different geographical distributions **(Sangula *et al.*, 2011)**, In some cases, topotypes may be subdivided into sub-lineages and even sub-sub-lineages**(Di Nardo *et al.*, 2011)**.

Foot and mouth disease, first reported in Egypt in 1950, with SAT2 serotype infection. The SAT2 virus outbreaks were again reported in 2012 and appeared in two lineages, Alx-12 and Ghb- 12, which both belong to topotype VII **(Ahmed *et al.*, 2012)** and In 2018 outbreaks of SAT2, topotype VII, Lib-12 lineage were reported and might be introduced from Libya **(Soltan *et al.*, 2019)**.

Serotype O FMDV is the most prevalent type and was chargeable for outbreaks in 1987, 1989, 1990, 1991, and 1993 **(Samuel *et al.*, 1999)**. It was the only serotype pronounced in Egypt between 1964 and 2005, with the special case of an episode including serotype A in 1972. **(Rady *et al.*, 2014)** Nevertheless, serotype A has introduced once more in 2006 through animal importation, causing economic loss of about 1 million cattle **(Knowles *et al.*, 2007)**. Since 2013 Egypt was co-circulated with three serotypes, O, A and SAT2.**(Sobhy *et al.*, 2014)**. Egypt relies on quarantine and mass vaccination in the control program of FMD, mandatory vaccination is performed **(Aidaros, 2002)**. FMDV RNA polymerase lacks RNA polymerase proofreading ability that resulting in a high rate of mutation that ranges from 10^{-3} to 10^{-5} per nucleotide site per replication and resulting in quasispecies structures of the FMD viral assemblages. **(longjam and Tayo, 2011)** mutations in the capsid encoding region may result in evasion of the immune system and hence vaccination failure**(Haydon *et al.*, 2001b)**. So it is necessary to perform regular studies on the molecular makeup of the viral capsid encoding

genes(**Haydon *et al.*, 2001a**).

The present study aims to make molecular Surveillance of foot and mouth disease viruses detected in both vaccinated and unvaccinated animals in Egypt and try to find out the reasons for the vaccination failure among the animal population in Egypt and to know the causes of foot and mouth disease outbreak despite the application of a comprehensive vaccination in the disease control program.

MATERIAL AND METHODS

Samples:

Fourteen positive antigen detection ELISA samples were selected from vaccinated and non-vaccinated (depending on case history) FMD clinically infected cattle during 2016 and 2019 from different governorates in Egypt (Giza, Behera, Fayum, Dumyat, and Monufia). The affected animals showed high fever (40°C - 41°C), oral lesions (vesicles and ulcerations on the gum and tongue with excessive salivation), foot lesions (ulcerations on the inter-digital space with lameness), teat lesions (vesicles and ulcerations on the teat with difficulty on milking due to pain) and sudden death (occurred in young as well as old animals). Samples included 1 vesicular fluid, 11-tongue epithelium, and 2 myocardial tissues. The samples were collected and prepared as described by OIE(**OIE, 2019**).

Virus identification by indirect sandwich Enzyme Linked Immunosorbent Assay (ELISA):

Identification and serotyping of FMDV antigen performed using ELISA typing kits provided by the FMD World Reference Laboratory (IZSLER: Brescia, Italy) ELISA considered as the preferred procedures for the detection of FMD viral antigen and identification of viral serotypes **(Roeder and Le Blanc Smith, 1987; Ferris and Donaldson, 1992; OIE, 2019).**

Virus isolation:

Trials for FMD viral isolation were carried out on Baby Hamster Kidney-21 (BHK-21) cell line. It was carried out as described in OIE 2019 **(OIE, 2019)**.

Molecular detection by RT-PCR:

RNA was extracted using the QIAamp Viral RNA Mini Spin Kit (Qiagen, Hilden, Germany) manufacturer's protocol **(Hefnawy *et al.*, 2018).**

RT-PCR was done using primers for the most variable 1D region (VP1) genes to distinguish

between different FMDV serotypes. Primers used for serotypes O, A, and SAT-2 are shown in (Table 1).

the amplification protocol used a 25µL reaction mixture and the cycling condition was 50ºC for 30 min and 95ºC for 15 min in RT step; followed by 35 cycles consisting of 95ºC for 1 min for denaturation followed by annealing for 30 seconds at 60 ºC. Elongation was done at 72ºC for 1 min followed by a final extension cycle at 72ºC for 10 min.

Table (1): Primers used for typing of FMDV by RT-PCR.

Serotype	**Primer name**	**Primer sequence**	**location**	**Product size**	**reference**
Reverse (3serotypes)	**NK61**	**5'GACATGTCCTCCTGCATCTG-3'**	**2B**		**(Knowles *et al.*, 2005)**
A	**A-Egy-F**	**5'GGAATCWGCAGACCCTGTC-3'**	**1D**	**750 bp**	**(Shehata *et al.*, 2016)**
O	**O-EA-F**	**CCTCCTTCAAYTACGGT**	**1D**	**283 bp**	**(Bachanek-Bankowska *et al.*, 2016)**
SAT2	**SAT2-Egy-F**	**5'-TGAYCGCAGTACACAYGTYC-3'**	**1D**	**666 bp**	**(Shehata *et al.*, 2016)**

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

Sequencing of amplified RT-PCR products:

The RT-PCR products were purified using a Thermo Scientific Gene JET Gel Extraction Kit according to the manufacturer's instructions and eluted in 50 µl elution buffer. The 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 (**Reid *et al.*, 2000; Knowles *et al.*, 2016**).

The VP1 sequences generated were subjected to multiple sequence alignments using the CLUSTAL W 1.4 tool (**Thompson *et al.*, 1994**) implemented in BioEdit v7.2.5 (**Hall**

***et al.*, 2011**). Maximum Likelihood phylogenetic trees were constructed using MEGA 6. (**Tamura *et al.*, 2013**).

RESULTS

FMDV serotyping using antigen detection serotyping ELISA:

All the fourteen samples selected to be positive antigen detection ELISA, 3 samples were positive for serotype O, 3 positives for serotype A and 8 positives for serotype SAT2, also the harvested tissue culture supernatants examined by serotyping ELISA to confirm the successful isolation of the FMDVs and to avoid cross-reaction or even contamination between serotypes. Each of the isolates gave a positive result for the respective serotype.

Virus isolation and propagation on BHK-21 cell culture:

All samples were attempted to isolate but only all the samples of serotypes A and O gave a characteristic FMDV cytopathic effect (CPE), (rounding of cells, granularity of the cytoplasm, and complete cell lysis) for 3 successive passages on the BHK-21 cell line while all 8 SAT2 samples give no CPE on the BHK-21 cell line for 6 blind successive passages and results confirmed by ELISA and RT-PCR.

Molecular subtyping of FMDV using RT-PCR:

RT- PCR with serotype-specific primers was used to confirm the isolation of serotypes A and O and for amplification of VP1 of serotype SAT2 samples for the following sequencing the amplified products were analyzed by agarose gel electrophoresis, serotype (A) gave bands at 750bp and serotype (O) at 283bp and serotype (SAT2) at 666 bp compared with (M) marker.

Sequencing and phylogenetic analysis:

Nucleodite sequence of the partial VP1 coding region of the FMDVs were searched in the genbank database and submitted under the accession numbers listed in (Table 2)

Table (2): Designation and accession numbers of FMDV sequences of the recent study.

Sequence name	Accession numbers	serotype
FMDV/Fayoum/AHRI/2016 VP1 gene, partial cds.	**MN853330**	**A**
FMDV/Giza/AHRI/2016 VP1 gene, partial cds.	**MN853329**	**A**
FMDV/A/Giza/AHRI/2018 VP1 gene, partial cds.	**MN866309**	**A**
FMDV/SAT2/Munifia/AHRI/2019/ VP1 gene, partial cds	**MN853328**	**SAT2**
FMDV/Suze/AHRI/2018 VP1 gene, partial cds.	**MN853327**	**SAT2**
FMDV/O/behera/AHRI/2016 VP1 gene, partial cds	**MN866306**	**O**
FMDV/O/PortSaid/AHRI/2016 VP1 gene, partial cds.	**MN866307**	**O**
FMDV/O/Giza/AHRI/2018 VP1 gene, partial cds.	**MN866308**	**O**

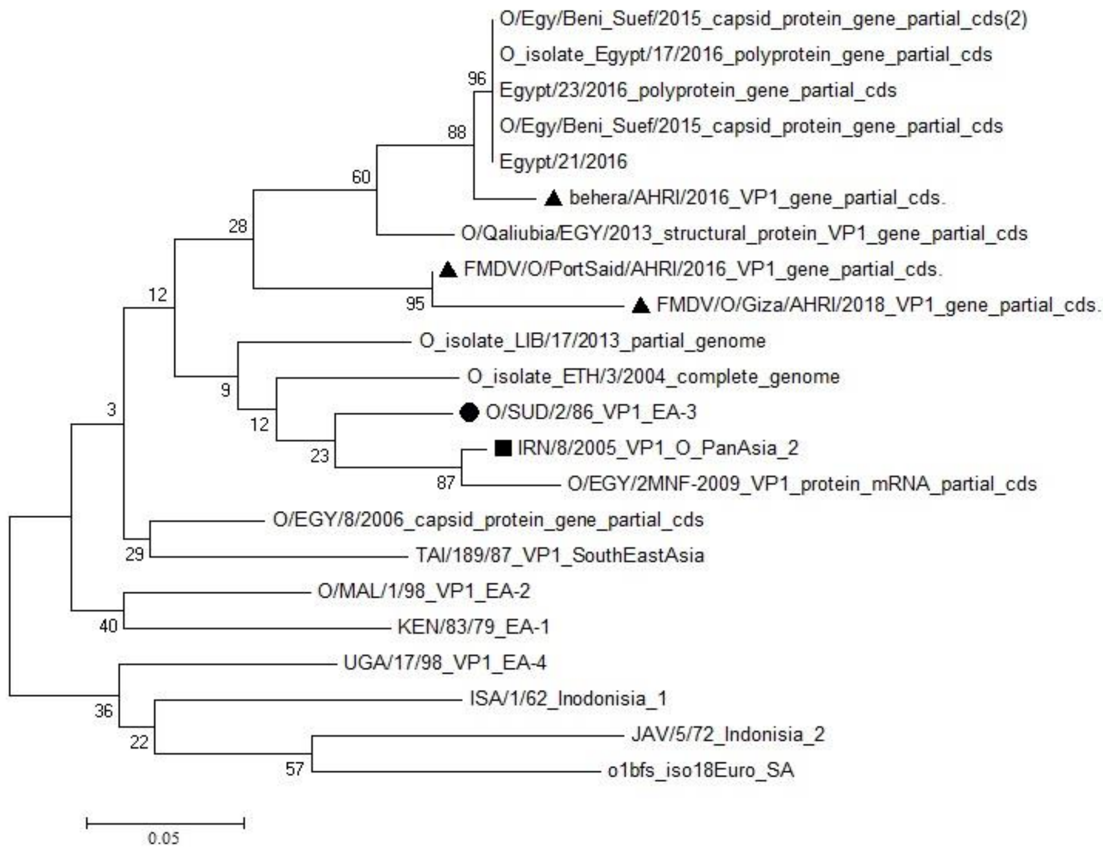

Fig. (1): Maxium Likelihood Phylogenetic analysis of our isolated Egyptian FMD virus serotype O compared with other closely related viruses. The three serotype O isolates of this study are indicated by a triangle (▲) while the vaccine strain is indicated by a square (■). While Prototype strain for the topotype related to our isolates is indicated by circle (●).

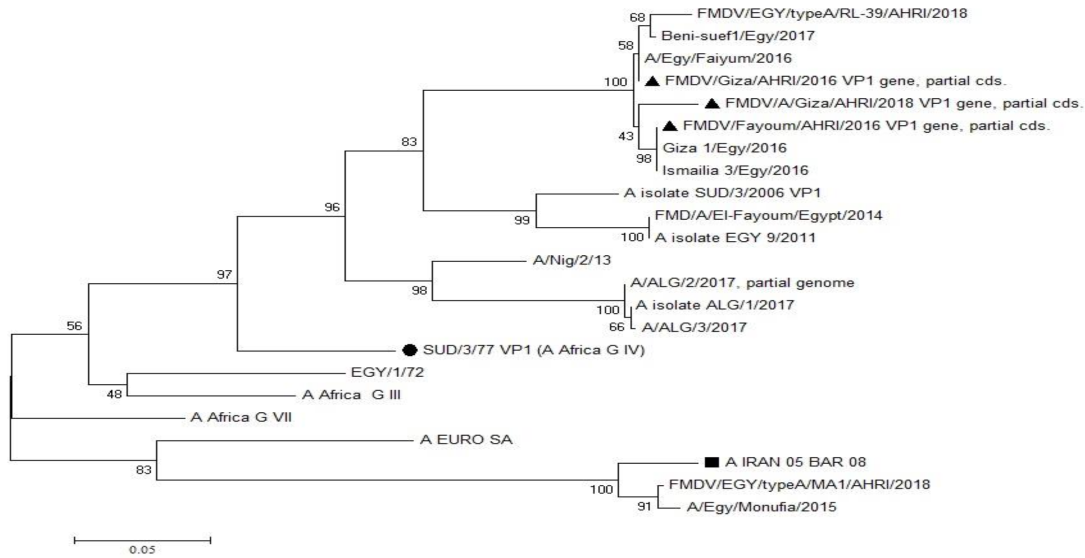

Fig. (2): Maxium Likelihood Phylogenetic analysis of our isolated Egyptian FMD virus serotype A compared with other closely related viruses. The three serotype A isolates of this study are indicated by a triangle (▲) while the vaccine strain is indicated by a square (■). While Prototype strain for the topotype related to our isolates is indicated by circle (●)

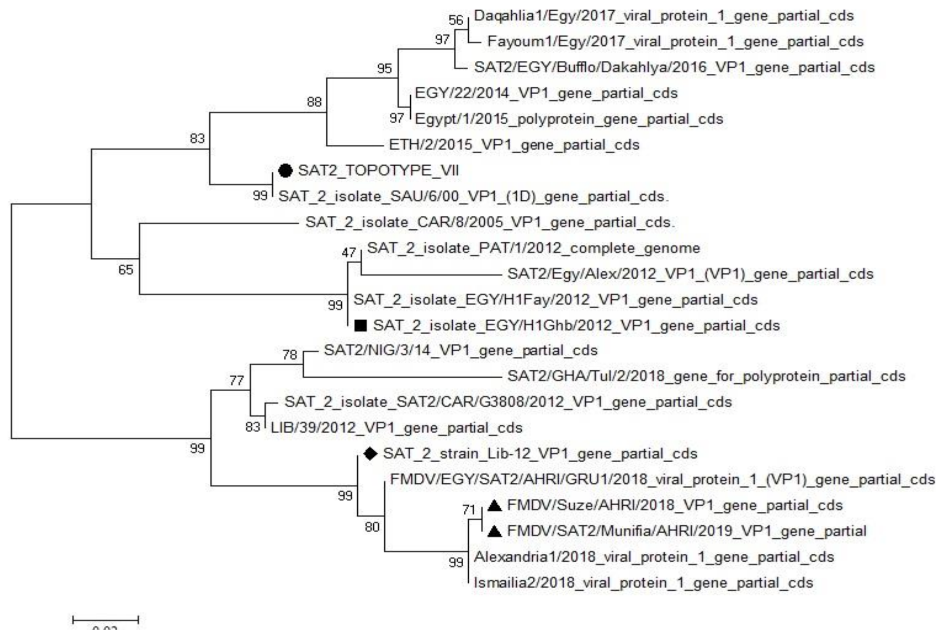

Fig. (3): Maxium Likelihood Phylogenetic analysis of our isolated Egyptian FMD virus serotype SAT2 compared with other closely related viruses. The two serotype SAT2 sequences of this study are indicated by a triangle (▲) while the vaccine strain is indicated by a square (■). While Prototype strain for the topotype related to our isolates is indicated by circle (●) and the related lineage is indicated by diamond (◆)

DISCUSSION

Foot and mouth disease (FMD) is a transboundary highly transmissible animal disease that can spread very quickly among cloven-foot animals through the movement of infected animals, animal products, and contaminated objects (such as animal transportation vans) and even by air currents. Vaccination is impaired due to the antigenic variations between serotypes and even subtypes(**Otte *et al.*, 2004**). Worldwide distribution of FMD can be categorized by seven "virus pools" scattered in Africa, Eurasia, and parts of South America; within these pools, Egypt is at risk for jumps of infection from Virus Pools 3, 4, and 5, from West Eurasia and sub-Saharan Africa (**Lockhart *et al.*, 2012**) Egypt adopts the immunization-dependent prevention and quarantine program for imported animals(**Aidaros, 2002**) Despite mandatory vaccination against FMD, regular outbreaks of either newly introduced serotypes or previously resistant ones still occur from time to time(**El-Khabaz and Al-Hosary, 2017**). Molecular epidemiology research studies on FMDV in Egypt are needed to help us understand the evolutionary history of the disease, the relationship between different serotypes, and to develop more effective strategies of prevention and control. In the current study, fourteen samples were selected to be positive for FMDV antigen detection ELISA three of them positive for serotype O and three for serotype A and eight for serotype SAT2 one sample for each serotype selected to be from a vaccinated animal. Our findings revealed the co-circulation of serotypes A, O, and SAT2 in Egypt among vaccinated and unvaccinated animals. The BHK-21 cell line is preferred for isolation trials for its ability to sustain the growth of FMDV and to produce a CPE within 72 h, which is demonstrated by cell rounding, granulation, and detachment. All A and O serotypes sample in the current study were successfully propagated on the BHK cell line and give good CPE while all SAT2 samples failed to give any CPE after 6 successive passages this in agree with that, the three SAT serotype viruses, endemic in Africa, being difficult to adapt to BHK-21 cells. (**Maree *et al.*, 2010**).

Viral protein 1 is the most diverse region of virus capsid, with nucleotide alternatives of up to 1% per year (**Abdul-Hamid *et al.*, 2011**). It has an important role in the host humoral immune response (**Sobrino *et al.*, 2001**). In the current study, the VP1 gene was selected for amplification due to its importance in the molecular characterization of FMDV strains and its significance in the virus serotype specificity. Evolution and evolution analysis based on the VP1 sequence can help in understanding the epidemiology of the disease and inferring the

source of the strains causing the outbreak. Moreover, the VP1 gene sequence serves to predict the antigenic synthesis of FMDV epitopes **(Bazid *et al.*, 2014)**.

All detected serotype O isolates in this study (O/Behera/AHRI/2016, O/Port Said/AHRI/2016, and O/Giza/AHRI/2018) belonged to the East Africa 3 (EA-3) topotype Fig. (1). The identity percent between detected isolates were 91% to 94% to each other, they had a percentage of identity ranging from 88% to 90%, 83% to 86%, 88% to 97%, and 87% to 93% with East Africa 3 (EA-3) prototype strain, used Egyptian vaccine strain (O Panasia 2 prototype of ME-SA topotype), Egypt/21/2016 and O/Qaliubia/EGY/2013, respectively**.**

When deduced amino acid sequence for each sequence obtained revealed that our recent O isolates (O/Behera/AHRI/2016, O/Port Said/AHRI/2016, and O/Giza/AHRI/2018) found to be related to the EA-3 prototypes (O/SUD/2/86) with identity percent of 96%, 94%, and 94% respectively, and related to each other with a percent of 96% to 98%, and with a percent of 92% to 94% identity with vaccine strain (O Panasia 2 prototype of ME-SA topotype)**.**

These results were agreed with the results obtained from previous studies on the FMD virus in Egypt **(Soltan *et al.*, 2017; Diab *et al.*, 2019)**

All serotype A isolates of the current study (Fayoum/AHRI/2016, Giza/AHRI/2016, and A/Giza/AHRI/2018) are related to genotype IV belonging to the African A topotype (A/SUD/3/77) Fig (2), with a percentage of 84%, 84%, and 83% respectively and with a percentage of 75%, 74% and 75% to A-Iran-05 lineage of the Asian A topotype(vaccine strain), and had a percentage of identity ranging from 97% to 100% and 96% to 98% with Giza 1/Egy/2016 and FMDV/EGY/type A/RL-39/AHRI/2018 respectively, The identity percent between A isolates of the current study was around 97% to 99% to each other.

The deduced amino acid sequence for each A serotypes revealed that recent study isolates (Fayoum/AHRI/2016, Giza/AHRI/2016, and A/Giza/AHRI/2018) are related to genotype IV of the African A topotype with a percentage of 87%, 85%, and 87% respectively and with a percentage of 81%, 80% and 81% to A-Iran-05 lineage of the Asian A topotype(used vaccine strain), The identity percent between A isolates of the current study was around 98% to 99%**.**

This is in agreement with previous FMDV results declared in previous studies **(Soltan *et al.*, 2017; Abdulrahman *et al.*, 2018)**.

The two sequences of SAT2 samples of the current study are related to topotype VII, Lib-12 lineage Fig (3), with a percentage of 96% that differ from the previously circulating SAT2

types (Used vaccine strain SAT 2 isolate EGY/H1Ghb/2012) with an identity percent of 86%. The identity percent between the two SAT2 strains of the current study was 99% and had a percentage of identity of 83% to 84% and 99% to 100% with Daqahlia1/Egy/2017 and Alexandria1/2018, respectively.

when the deduced amino acid sequence of SAT2 strains obtained it revealed that our recent study strains were identical to each other with a percentage of 99% and with a percent of 90% to 91% to SAT2 topotype VII prototype, the identity between the used Egyptian SAT2 vaccine strain (SAT 2 isolate EGY/H1Ghb/2012) and the recent strains was 91%, but recent strains were identical to Libyan strain (topotype VII, Lib-12 lineage) with 96% identity. This newly emerged SAT2 strain could have been transmitted from Libya to Egypt through borders and could explain the cause of the severe SAT2 outbreaks that occurred in Egypt in this period. These findings matched with other studies in Egypt **(Soltan *et al.*, 2019)**. The two current SAT2 sequences are related to each other by a percentage of 99%.

CONCLUSION

The results of the present study record the co-circulation of FMDV serotypes O, A, and SAT2 among the vaccinated and non-vaccinated animals population in Egypt, with no significant difference between FMDV viruses from vaccinated and non-vaccinated animals elevating the need to analyze the possible causes of the failure of vaccine strategy. Nevertheless, the Egyptian veterinary authorities adopted vaccination campaigns using trivalent vaccines that are used regularly (serotype O, topotype ME-SA, Panasia2 lineage; serotype A, Asian topotype, Iran05 lineage; and serotype SAT2, topotype VII, Gharbia12 lineage) and locally produced monovalent vaccine containing newly emerged SAT2 strain (Serotype SAT2, topotype VII, Lib-12 lineage), even though FMD epidemics still occurred. The sequences of SAT2 detected in the present work ensure the circulation of SAT2(Lib-12 lineage) so more control measures should be applied for the prevention of transboundary introduction of FMDV, and close monitoring of the circulated FMDV strains in Egypt is essential to select the appropriate vaccines to be used for the disease control programs.

Recommendations:

Continuous Surveillance of FMDV circulating strains should be done besides more studies of the suitability between the used vaccine strains and the field circulating strains through the estimation of the r_1 value.

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