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Isolation and identification of Foot and Mouth Disease Virus strains circulate in Egypt during 2021-2022 outbreaks

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ABSTRACT

Foot-and-mouth disease (FMD) is an acute viral animal disease; that causes huge economic consequences to the livestock sector. Totally, 118 samples (Epithelial Tissue (n = 38), Vesicles (n = 30), Heart (n = 31), Tongue (n = 9), Oral swabs (n = 10), and 232 samples (Epithelial Tissue (n = 108), Vesicular fluids (n = 36), Heart (n = 28), Tongue (n = 24), Oral swabs (n = 36) collected from suspected animals during FMD outbreak investigations in 2021, and 2022, respectively, constituted the material of the study. This study aimed to detect the Foot-and-mouth disease viruses (FMDVs) and their serotype using qRT-PCR, and antigen detection using ELISA, followed by virus isolation on BHK-21 cells then identification. The field samples were analyzed by FMDV screening primers/probes then the virus serotype-specific primers/probes revealing 57/118 (48.30%) during 2021, and 158/232 (68.10%) during 2022 positive samples with qRT-PCR and categorized into serotype O 46/57 (80.70%), 90/158 (56.96%) with the highest prevalence, followed by serotype A 7/57 (12.28%), 58/158 (36.70%), serotype SAT-2 4/57 (7%), 10/158 (6.32%) during 2021, and 2022, respectively. FMD antigen detection ELISA was performed giving results with 45/118 (38.14%) in 2021, and 120/232(51.72%) in 2022. The virus is propagated successfully in 4/10 (40%) in 2021, and 6/17(35.29%) in 2022 samples. Results illustrated in the present study help in general detection of FMDV serotypes currently circulating in Egypt, and aid in understanding of current status of epidemiological aspect of the disease to construct efficient control plan.

1. INTRODUCTION

Foot-and-mouth disease (FMD) is a trans-boundary viral disease that affects different cloven-hooved animals (Souley Kouato et al., 2018). The disease has high morbidity rate (up to 100%) in a susceptible population, and rare fatality. However, in young animals, it has a high mortality rate (up to 50%) even before the development of any classical signs of the disease, due to acute myocarditis (Aktas et al., 2015).

At present, FMD is regarded as the world's worst farm animal disease (Soltan et al., 2017), since it is one of the most contagious viral diseases with a major economic impact worldwide, as three-quarters of the world's agricultural population is concentrated in endemic areas (Asia, Africa) (Knight-Jones and Rushton, 2013), resulting in huge inefficiency in food security.

FMD is caused by foot-and-mouth disease virus (FMDV), that belongs to Genus *Aphthovirus*, *Picornaviridae* family. It is small, non-enveloped with an icosahedral capsid, and four different structural proteins VP1-4, the virus genome is positive-sense, single-stranded RNA about 8.3 kb length (Grubman and Baxt, 2004). The VP1 coding sequence can be used to identify the strain's serotype; as it is the most informative part of the FMDV genome for determining the relationship between different strains for epidemiological research (Reid et al., 2014).

FMDV is has a rapid mutation rate, it exists in seven serotypes: O, A, C, Asia 1 and SAT 1-3 (Balinda et al., 2010). Within each serotype, multiple variants can be recognized, some of which are unable to provide effective cross-protection against other same serotype viruses (Mahapatra et al., 2015).

FMD control differs based on the disease status, FMD-free countries focus on decreasing the risk of virus incursions from adjacent and trade-partner countries by limiting animal and their product mobility. While, endemic countries control is achieved by accurate and rapid diagnosis, regular, and compulsory mass vaccination with continuous surveillance to evaluate FMDVs circulating with the vaccine strain (Knight-Jones et al., 2016).

In North Africa, FMD epidemiology is complicated due to the co-circulation of endemic FMDVs, with sporadic incursions of exotic FMDVs from the Middle East and Sub-Saharan Africa (Ahmed et al., 2012), in addition to movements of livestock and wild animals that is influenced by several factors which increase the challenges of imposing restrictions on them as culture traditions, social and political disturbance (Ayebazibwe et al., 2010). In

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consequence, this virus spread into North Africa may represent a threat to the European countries.

In Egypt, FMD is enzootic, historical background started in 1950 with FMDV SAT2 outbreak. At present, the three FMDV serotypes A, O, and SAT2 are the greatest danger to susceptible animals. (El Nahas et al., 2021). Furthermore, repeated incursions of new lineages from other countries create an unstable condition that increases the chance of vaccine failure owing to strain mismatch (Abd El Rahman et al., 2020). Egypt has been infected by eleven various FMDV lineages since 2006; within serotype O (O/ ME-SA / Sharquia-72, and PanAsia-2), (O/ EA-3/ Qal-13, and Ism-16, and Alx-17), within serotype A (A/Africa/G-VII $^{KEN-05}$), (A/Africa/G-IV $^{ISM-12}$), (A/Asia/Iran-05^{BAR-08}), and the historical (A/Africa/G-II), within serotype SAT2 (SAT2/VII/Ghb-12, and Alx-12), and the recent detected (SAT2/VII/Lib-12) in Egypt 2018 (Abu-Elnaga et al., 2020).

The aim of this study was to detect and identify FMD viruses recovered from clinical samples collected from suspected animals during FMD outbreak investigations in 2021, and 2022 by molecular and virological techniques to gain more in-depth knowledge on FMDVs circulation and status in Egypt for better disease control.

2. MATERIAL AND METHODS

2.1. Sampling of animals in study areas

This study was conducted in six Egyptian governorates between January to December of 2021, and in five Egyptian governorates between January to July of 2022, where outbreaks were reported consistent with FMDV. All sampling sites' locations are presented in (Table 1) In total, 118 samples (Epithelial Tissue (n = 38), Vesicular fluids (n = 30), Heart (n = 31), Tongue (n = 9), Oral swabs (n = 10)), and 232 samples (Epithelial Tissue (n = 108), Vesicles (n = 36), Heart (n = 28), Tongue (n = 24), Oral swabs (n = 36)) were collected from clinically suspected cattle and buffaloes during FMD outbreak investigations in year 2021, and 2022, respectively.

Table 1 Samples collected from different Egyptian governorates in the period 2021 and 2022.

Year	Governorates	Sample Type					
		Epithelial Tissue	Vesicular fluids	Heart	Tongue	Oral swabs	Total
2021	Qalyubia	13	14	12	0	9	48
	Menofia	0	3	11	7	0	21
	Fayoum	12	5	6	0	0	23
	Giza	13	0	0	0	1	14
	Sohag	0	3	2	2	0	7
	Kafr El-Sheikh	0	5	0	0	0	5
	Total	38	30	31	9	10	118
			Total 118				
2022	Menofia	18	8	15	17	6	64
	Beni-seuif	27	0	10	7	10	54
	New Valley	26	21	0	0	0	47
	Behaira	14	7	3	0	16	40
	Assiut	23	0	0	0	4	27
	Total	108	36	28	24	36	232
			Total 232				

2.2. Samples Processing

Different samples were collected, put in a viral transport medium supplied with (antibiotics/ antimycotic) with final pH in the range of 7.2–7.6, because FMDV is labile in low pH; samples were labeled, and submitted to the laboratory in sterile ice container for the analysis. Tissue samples were grinded in sterile mortar and pestle by addition of suitable amount of sterile sand and tissue culture medium supplemented with (antibiotics/ antimycotic), further medium should be added to give a 10% suspension, then centrifuged (2000 rpm, 10 min, 4 °C.) to collect the supernatant then filtered, and stored at -80°C till use (OIE, 2017).

2.3. Extraction of viral genomes

RNA extraction from the FMD field outbreak clinical samples was performed using the Thermo scientific Kit (USA) while following manufacturers' instructions. RNA was eluted in 35 µl and kept at -80°C till further evaluation.

2.4. Pan- FMDV Screening by Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on extracted RNA for Pan-FMDV detection using the Primer Design™ Reverse Transcription Kit (USA) as guided. A universal set of primers and probe targeting the FMD virus non-structural protein 3D region was used (Callahan et al., 2002) as in (Table 2). Reactions were executed in (Applied Bio systems, USA), with the following (qRT-PCR) thermal profile, reverse transcription step at 55°C for 10 min, enzyme activation for 2 min at 95°C, then 40 cycles of denaturation and data collection at 95°C for 10 sec, 60°C for 1 minute.

2.5. Identification and serotyping performance evaluation

Following detection of the FMDV genome, the positive samples were analyzed through a second run in one-Step qRT-PCR for serotyping the O, A, and SAT2 virus using a panel of specific primers and probes set that amplify the VP1/1D gene of each serotype (Table 2). Briefly, a total of 5 µL of RNA, 10 µL of 2x mix, 1 µL of each primer (10 µM), 1 µL of probe (5 µM), and RNAse-free water up to a final volume of 20 µl. The assay was conducted with the same thermal protocol as described previously in the pan-FMDV. At the end of each annealing phase, the real-time fluorescence values of marked probes were measured in ongoing processes. The results were interpreted by calculating the threshold cycle (Ct) value for reporter dye identification, with a sample declared negative if no fluorescence signal was found after 40 cycles.

2.6. FMDV antigen detection by enzyme linked immune-sorbent assay (ELISA)

For further identification, the collected field samples were identified for FMDV and serotyped by the routine diagnostic method, commercial antigen detection ELISA for FMDV serotypes O, A, and SAT 2 (IZSLER, Brescia, Italy), the results were estimated as described by the manufacturer's guidelines.

2.7. Virus isolation

The prepared tissue homogenates were used to infect the Baby Hamster Kidney cell line (BHK-21) that is specific for FMDV propagation (OIE, 2017). Briefly, 1 ml tissue suspension was inoculated in confluent monolayer BHK-21 that was prepared one day before inoculation, by tilting for 1 hour for better adsorption then 10 ml of MEM Hanks' media was added, incubation is done in CO_2 incubator (37°C and 5% CO_2). The cells were examined twice daily for 24-72hour post-infection for appearance of characteristic cytopathic effect (CPE) under an inverted microscope (Germany). When a 90% CPE was detected,

the infectious fluid was then harvested by freezing and thawing cells for three times then titrated and kept at -80◦C until use. However, samples were regarded negative if no CPE was detected after 72 h on the third passage

Table 2 Primers/Probes targeting the 3D region and the variable region in the 1D gene of viral RNA.

Target	Primer sequence (5'-3')	Reference
Common 3D	(F) 5'-ACTGGGTTTTACAAACCTGTGA-3' (R) 5'-GCGAGTCCTGCCACGGA-3' (Probe) 5'-FAM-TCCTTTGCACGCCGTGGGAC-TAMRA-3'	Callahan et al., 2002
O (1D)	(F) 5-'CAACACACGGACGTCGCG-3' (R) 5'-GTTGGGTTGGTKGTGTTGTC-3' (Probe) 5'-FAM-GAGTTGGACCTGATGCAGACCC-BHQ1-3'	Reid et al., 2014
	(F) 5'-GCCACYGTKGAGAACTACGG-3' (R) 5'-GACCCASGTGAGGTTYCCCT-3' (Probe) 5'-HEX -ATGCARAYCCCCTCRCACAC -TAMRA-3'	El Bagoury et al., 2022
A (1D)	(F) 5'-ACGACCATCCACGAGCTYC-3' (R) 5'-RCAGAGGCCTGGGACAGTAG-3' (Probe) 5'-FAM-CGTGCGCATGAAACGTGCCG TAMRA-3'	Reid et al., 2014
SAT-2 (1D)	(F) 5'TGA AGA GGG CTG AGC TGTACT G-3' (R) 5'-CTC AAC GTC TCC TGCCAG TTT-3' (Probe) 5'-FAM-ACAGATTCGACG CGC CCATCG TAMRA-3'	Ahmed et al., 2012
	(F) 5'- GTGGGCGAHCACACAARGGTCT-3' (R) 5'- GGATGGCAA AGC GGGTYACG-3' (Probe) 5'- FAM-TGGCARCCCAACGGGGCA C-TAMRA-3'	El Bagoury et al., 2022

3. RESULTS

In this study, a total of 118, and 232 samples collected from FMDV suspect cases as in **(Table 1)**, taken from 10 different Egyptian governorates during FMD outbreak investigations in year 2021, and 2022, respectively, were analyzed for the detection and serotyping of FMDV by using different assays

3.1. Pan-FMDV screening using one-step qRT-PCR

The collected samples were initially diagnosed for FMDV genomic RNA using qRT-PCR 3D primers, revealing that 57 (48.30%) positive samples out of 118 suspected samples in year 2021, and 158 (68.10%) positive samples out of 232 suspected samples in year 2022, details of these positive samples are mentioned in (Table 3)

Table 3 Results of positive samples by Pan-FMDV qRT-PCR in the study period 2021 and 2022.

Year	Governorates	Sample Type					
		Epithelial Tissue	Vesicles	Heart	Tongue	Oral swabs	Total
2021	Qalyubia	4	4	4	0	2	14
	Menofia	0	1	4	3	0	8
	Fayoum	8	2	4	0	0	14
	Giza	13	0	0	0	0	13
	Sohag	0	1	1	1	0	3
	Kafr El-Sheikh	0	5	0	0	0	5
	Total	25	13	13	4	2	57
		Total 57					
2022	Menofia	11	6	10	11	5	43
	Beni-seuif	20	0	7	7	5	39
	New Valley	14	16	0	0	0	30
	Behaira	12	4	2	0	12	30
	Assiut	14	0	0	0	2	16
	Total	71	26	19	18	24	158
		Total 158					

3.2. Serotyping evaluation

Serotyping of positive samples by Common-FMDV assay (n= 57) in 2021, and (n= 158) in 2022, was conducted by qRT-PCR using a panel of specific primers/probes that targets the 1D gene which is responsible for FMDV serotypes differentiation. In year 2021, serotypes were determined for all samples yielding positive serotype O 46/57(80.70%), with the highest rate, followed by positive serotype A 7/57 (12.28%), positive serotype SAT-2 4/57 (7%). Whereas for 2022, serotype O 90/158 (56.96%) with the highest ratio, followed by positive serotype A 58/158 (36.70%), positive serotype SAT-2 10/158 (6.32%) (Table 4, Figure 1), with different threshold cycles values detected.

Table 4 Serotypes of FMDV detected from outbreaks that occurred in Egypt in the study period 2021 and 2022.

Year	Governorates	Suspected Cases		Serotypes detected		
		Examined	Positive	O	A	SAT-2
2021	Qalyubia	48	14	14	0	0
	Menofia	21	8	7	1	0
	Fayoum	23	14	14	0	0
	Giza	14	13	9	0	4
	Sohag	7	3	2	1	0
	Kafr El-Sheikh	5	5	0	5	0
	Total	118	57	46	7	4
2022	Menofia	64	43	30	6	7
	Beni-seuif	54	39	20	16	3
	New Valley	47	30	16	14	0
	Behaira	40	30	22	8	0
	Assiut	27	16	2	14	0
	Total	232	158	90	58	10

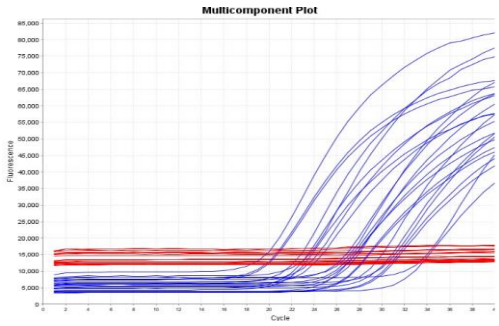

Figure 1 Representative multicomponent plot RT-qPCR assay targeting the VP1 (1D) for collected samples with CT range 20.02-34.23.

3.3 FMDV antigen detection by enzyme linked immune-sorbent assay (ELISA)

The collected field samples (n= 118) in 2021, and (n= 232) in 2022, were analyzed to be positive for FMDV and its different serotypes using antigen detection ELISA, giving 45 (38.14%) out of 118 samples undetermined suspected samples in 2021, and 120 (51.72%) out of 232 samples in 2022, The serotypes obtained by the antigen detection ELISA for the examined samples matched those obtained by the qRT-PCR, with high ratio to serotype O, followed by serotype A then SAT-2 (Table 5).

Table 5. Results of FMDV Antigen detection using ELISA.

Year	Positive Samples	Serotype O	Serotype A	Serotype SAT-2
2021	45/118(38.14%)	37/45(82.2%)	5/45(11.11%)	3/45(6.7%)
2022	120/232(51.72%)	73/120(60.83%)	43/120(35.83%)	4/120(3.3%)

3.4. FMDV propagation on BHK-21 cell line

Positive FMDV samples detected by ELISA, and qRT-PCR with (Ct values ≤ 27) where abundant viral RNA is present (Ularamu et al., 2020) were selected for further analysis by isolation on BHK-21. Virus propagation was performed for 10, and 17 positive samples in 2021, 2022, respectively. The virus is propagated successfully in 4/10 (40%) in 2021 samples (3 Epithelial Tissue, 1 Heart), positive samples were identified using qRT-PCR and antigen detection ELISA giving (3 serotype O, 1 serotype SAT-2). However, isolation positive samples were 6/17 (35.29%) in 2022

samples (3 Epithelial Tissue, 2 Vesicular fluids, 1 Heart), and were identified using qRT-PCR and antigen detection ELISA giving (3 serotype O, 2 serotype A, 1 serotype SAT-2) showing the characteristics FMDV CPE on BHK-21 in the form of rounding, clustering, and finally cells detachment, the virus titer range was 10^4-10^5 (Table 6, Figure 2).

Table 6 List of positive results after third passage on BHK cell line in the study period 2021 and 2022.

Year	Sample Type	No. of samples subjected for propagation	
		Examined	Positive
2021	Epithelial Tissue	5	3
	Heart	3	1
	Tongue	2	0
	Total	10	4
2022	Epithelial Tissue	8	3
	Vesicles	4	2
	Heart	5	1
	Total	17	6

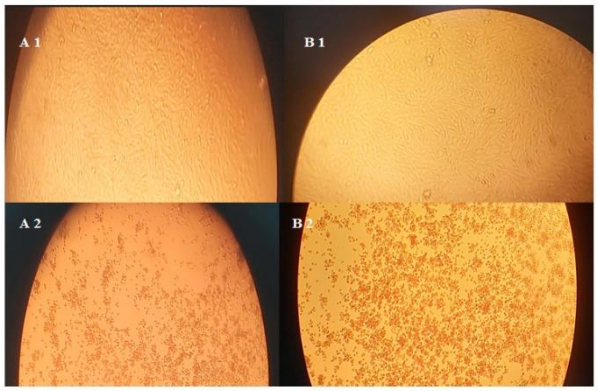

Figure 2 Microscopic images (40 X) of representative samples for FMDV Isolation on BHK 21 cell line. (A1, B1) demonstrate Normal control BHK 21 cell line, (A2, B2) demonstrate virus CPE, cell rounding, and clustering 48 hours post infection at the third passage.

4- DISCUSSION

FMDV is an RNA virus exhibiting a high genetic variability due to error-prone RNA replication. Many subtypes within each serotype are evolving rapidly with no cross-protection, and spread between different epidemiological zones by importing and exporting live animals and animal products (Jamal et al., 2020), this explains the cause of outbreaks all over Egypt although vaccination is executed (El-Bayoumy et al., 2014). In Egypt, FMD molecular epidemiology surveys can detect genetic variation and relationships among the circulating serotypes, with continuous monitoring of FMDV epidemic in surrounding countries for predicting strains that may enter Egypt.

Serotypes O, A, and SAT2 have been detected in Egypt since 2013, multiple FMDV serotypes and subtypes have been identified despite the control efforts, may be associated with the trade in animals or their products from FMD-endemic African countries to meet the meat demand, uncontrolled movement of live animals across borders, shortage in quarantines facilities, as well as the use of incompletely matching vaccines (Nampanya et al., 2013).

FMD diagnosis is typically based on laboratory detection by identification of the viral nucleic acid by RT-qPCR, which has higher analytical sensitivity, or the determination of the viral antigen, by ELISA, coupled with live virus propagation on tissue culture. These detailed diagnoses allow disease confirmation and virus serotyping for effective management (Wong et al., 2020). The use of early and precise diagnostic methods to quickly identify initial clinical indications of infection is a must in any epidemic FMD control.

During 2021–2022 periods, several outbreaks of FMDV were reported in different Egyptian governorates. Our results of molecular and virological investigation in year 2021 are in the same line with (Soltan et al., 2019), who stated that serotype O is the most predominant epidemic serotype circulating in the country. In addition, the results of serotyping were consistent with the qRT-PCR, with high prevalence to serotype O, followed by serotype A then SAT-2. The data obtained from this study demonstrates a superior analytical and clinical performance for qRT-PCR assay in detecting FMDV serotypes circulating in Egypt over other diagnostic tools, these data were in accordance with previous studies suggesting that qRT-PCR is a valuable diagnostic tool for the routine laboratory detection and quantification of FMDV-RNA because of its excellent sensitivity and specificity (Salem et al., 2021), qRT-PCR has many advantages as it has a simple workflow, automated in a closed system so reduces errors and contamination, it can detect and amplify a minute amount of the virus genome in short time; so, accelerating epidemiological surveillance, especially during outbreaks (El-Shehawy et al., 2012). Moreover, some samples that are unsuitable for direct ELISA analysis can be easily identified by qRT-PCR (Reid et al. 2001). In our study, the virus isolation gives negative results despite the presence of high to moderate viral RNA as detected by qRT-PCR. These results are in alignment with (Tesfaye et al., 2020), who implied that few samples were proliferated on BHK-21, may be due to changes in pH levels that lowers the infectivity of the isolated virus, varying susceptibility of virus to the cells used. Moreover, virus isolation depends on live virus; meanwhile qRT-PCR depends on nucleic acid, either dead or alive virus.

Despite control attempts, the study shows that throughout the study time period; the relative frequencies of the different FMDVs detected increases in cases of 2022, than 2021, this implies that greater restrictions must be imposed on animal transportation and vaccine; these results were in in line with (El Damaty et al., 2021) who mentioned a considerable high morbidity, and aggressiveness of the FMDV in Egypt outbreaks in early 2019 may be due to variation between the outbreak and vaccine strains.

Also, the current study doesn't depend on the phylogenetic analysis of amino acids residues to characterize the genetic relativity between the Egyptian FMDV isolates from the recent outbreaks and the bordering countries, other studies in last recent years demonstrate different strains circulating in Egypt during that period.

In Egypt, serotype A isolates from recent outbreaks fall under the Africa topotype, (A/AFRICA/G-IV lineage), this new lineage was identified for the first time in 2020, with genetic difference about 6.5% from that detected in Egypt in 2016-2017. Whereas, serotype O falls into EA-3 topotype, that entered in 2012 from Sudan, and continues spreading till now. However, SAT2 viruses are related to (SAT2/ VII/Lib-12 lineage) that entered in 2018 (Soltan et al., 2019) proposing that animal movement can contribute to the virus spread across the sub-Saharan Africa countries (Ahmed et al., 2012).

5. CONCLUSION

During FMD outbreak investigations in 2021, and 2022; a total of 118, and 232 samples were collected from

suspected cattle and buffaloes from 10 different Egyptian governorates and analyzed for the detection and serotyping of FMDV. The results revealed positive FMDV samples for serotype O 46/57(80.70%),90/158(56.96%)with the highest prevalence, followed by serotype A 7/57(12.28%), 58/158(36.70%), serotype SAT-2 4/57(7%), 10/158(6.32%) during 2021, and 2022, respectively, confirming the circulation of serotype O, A, and SAT-2 in the country. Whereas, the results of FMD antigen detection ELISA were 45/118(38.14%) in 2021, and 120/232(51.72%) in 2022. Current FMDV epidemiology and the circulation of serotype O, A, and SAT-2 in Egypt highlighted the necessity of borders and quarantines with strict hygiene and sanitation, accurate laboratory facilities to recognize the disease rapidly, effective control strategy with further stringency on animal movement and vaccine distribution to hinder the virus spread across countries.

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