Benha Veterinary Medical Journal 43 (2022) 86-90

Official Journal Issued by
Faculty of
Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

Since 1990

Original Paper

Isolation and Genetic characterization of foot and mouth disease virus causing an outbreak at Qalyabia government in 2021

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ARTICLE INFO

Keywords

FMDV

BHK-21

O/EA-3/Alx-17

Phylogenetic analysis

Received 30/06/2022
Accepted 06/08/2022
Available On-Line 09/10/2022

ABSTRACT

Foot and mouth disease (FMD) is an extremely contagious transboundary disease that affects cloven-hoofed animals. An uncontrolled animal movement has a significant role in the disease circulation in endemic areas. An accurate and early diagnosis is critical for FMD control. Seventeen tongue epithelium and three vesicular fluid samples were collected from five cattle farms, and nine tongue epithelium and one vesicular fluid samples were collected from three buffalo farms suspected to be infected with FMDV at Qalyubia government in 2021. Trail of virus isolation was carried out on BHK-21 cell line followed by conventional RT-PCR for typing of the obtained virus isolate. Partial sequencing and phylogenetic analysis of VP1 for the field isolate revealed that it was serotype O topotype EA-3 lineage Alx-17 (FMDV/O/EGY/Qalyubia/2021/OM681353) and closely related to the previously local Egyptian isolate in 2017 (FMDV/O/EGY/2017/OM221230.1) with nucleotide identity 99.36% and sharing nucleotide similarity 99.72% with the previously isolated virus from Sudan (FMDV/O/SUD/2017/MK422569.1) and Ethiopia (FMDV/O/ETH/2017/ MN987453.1) during 2017 that suggested the transboundary incursion. The isolated FMDV/O/EA-3/Alx-17 shared 85.65% nucleotide identity with the sequenced FMDV serotype O topotype ME-SA lineage Sharqaui-72 (FMDV/O/EGY/OM681355), while 15.28% nucleotide difference between the isolated FMDV/O/EA-3/Alx-17 and the vaccine strain Pan-Asia-2 (FMDV/O/ME-SA/Pan-Asia-2/OK642671.1). The sequenced Sharqaui-72 virus revealed 86.01% sequence identity with the Pan-Asia-2 vaccine strain. The authors recommend for periodical molecular and genetic characterization between field isolates and vaccine strains, more cross-matching (R-value) and challenge studies between EA-3 viruses and PanAsia-2 vaccine strain, in addition to strict quarantine measures for the imported animals

1. INTRODUCTION

Foot and mouth disease is one of the most fearful viral diseases. It is a highly contagious disease affects cloven-hoofed animals. FMD is characterized by fever, vesicular eruption in the oral cavity, feet, and teat on females' udder. FMD causes severe economic hardship in endemic countries as a result of decreasing milk production, weight loss, cost of treatment, fatalities especially in young animals (tiger heart), and prevents importing of animals or animal products from FMD endemic countries (Bronsvoort and Radford, 2004; OIE, 2018).

FMDV belongs to *Aphthovirus* genus, family *Picornaviridae*, and it is immunologically classified into seven serotypes: O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1. Each serotype contains several variants frequently constrained to specific geographical locations (topotypes) and lineage (Knowles, 2003).

FMDV is a single-stranded positive-sense RNA virus, without an envelope, The RNA genome is approximately 8.5 kb, surrounded by an icosahedral capsid formed of 60 copies (VP1 (1D), VP2 (1B), VP3 (1C), and VP4 (1A)) structural proteins and eight non-structural proteins (NSP) genes L, 2A, 2B, 2C, 3A, 3B, 3C and 3D responsible for proteolytic cleavage and viral replication (Zell et al., 2017) FMDV genome is prone to replication errors during the replication process resulting in genetic diversity between FMDV serotypes (Castro et al., 2005; Belsham, 2020).

The source of MDV can be determined by using the sequence of the G-H loop region present on VP1 which acts as a fingerprint (Brown, 2003). The viral protein (1D) coding region of FMD virus strains is immunogenically significant and accountable for antigenic heterogeneity, protective immunity, serotype specificity and cell virus attachment. The phylogenetic analysis based on VP1 nucleotide sequences has already been widely applied to deduce evolutionary dynamics, molecular characterization and epidemiological relationships among the genetic lineages of outbreak strains (Cottam et al., 2008). Serotype-specific amino acids are also found in VP1, helping in the differentiation between different serotypes, topotypes, and lineages (Carrillo et al., 2005; Freimanis et al., 2016)

Egypt is endemic with FMDV since 1958 (Vosloo et al., 2002). FMDV circulates in the form of three serotypes, with different topotypes, and lineages. Recorded FMDV serotypes included Serotype O contains two topotypes

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Middle East-South Asia topotype (ME-SA) with two lineages (PanAsia-2 and Sharquia-72) and East Africa 3 topotype (EA-3) with three lineages (Qal-13, Ism-16, and Alex-17). Serotype A circulates under two topotypes, Africa topotype with two lineages (G-VII, and G-IV) and topotype Asia, lineage Iran-05. Serotype SAT-2 in the form of topotype VII with three lineages (Ghb-12 lineage, lineage Alx-12, and lineage Lib-12) (Hagag et al., 2019; Tekleghiorghis et al., 2016; AbuElnaga. et al., 2020; Abd El Rahman et al., 2020; Ismael et al., 2021; Wasfy, 2021).

Vaccination is one of the most practical and effective methods of preventing FMD outbreaks (Paton et al., 2009). The inactivated FMD vaccines were quadrivalent (O panAsia-2, An Iran O5, SAT2/ Ghb/2012, and SAT2/Lib/2018) till 2020 (Wasfy, 2021). In 2020 severe FMD outbreak was reported in vaccinated and unvaccinated animals in Egypt caused by serotype A, topotype Africa, Genotype IV (Hassan, et al., 2022). Infection or vaccination with one serotype does not confer immunity to other serotypes and may also fail to protect fully or at all against other strains of the same serotype (Paton et al., 2005). So, periodical isolation, characterization of the FMDV causing outbreaks and studying the genetic relationship between the FMDV isolates and vaccine strains is essential for FMD control.

This study was designed for isolation, molecular characterization, and phylogenetic analysis of FMD virus causing an outbreak at Qalyubia government in 2021 comparED with the field isolated virus and other FMD viruses related to the same serotype in Egypt including the vaccine strain.

2. MATERIAL AND METHODS

2.1. Samples

A total of 30 tongue epithelium and vesicular fluid samples (20 from cattle and 10 from buffalo) were collected from 5 cattle farms and 3 buffalo farms from Qalyubia government, in winter, 2021 (Table 1). Samples were collected from vaccinated and unvaccinated farms. About 80% of animals from unvaccinated farms showed fever and vesicular eruption on the oral cavity and feet. These samples were collected, transported, and stored according to recommendations by OIE (2021).

Table 1 Data of collected samples

Farm	Cattle		Buffalo	
	Tongue Epithelium	Vesicular Fluid	Tongue Epithelium	Vesicular Fluid
1	5	2	4	1
2	5	1	2	-
3	2	-	3	-
4	3	-	-	-
5	2	-	-	-
Total	17	3	9	1

2.2. Processing of the collected samples

Tongue epithelium samples were weighted and grinded in a sterile mortar with sterile sand and MEM media with antibiotics and antimycotic were added till reached 10% tissue suspension then cooling centrifugation at 4000 RPM for 15 minutes. The supernatant was collected, filtrated at a 0.22 µ Millipore filter and stored at -80 °C till used. The vesicular fluid samples were clarified after being treated with an antibiotic-antimycotic solution and stored at -80 °C till subjected to experimental work.

2.3. Baby hamster kidney cell line (BHK-21)

BHK-21 obtained from VSVRI, Abasia, Cairo was used for virus isolation and titration using Eagle's minimum essential medium (MEM) supplemented with 8-10% bovine serum as described by Macpherson and Stoker (1962).

2.4. Virus isolation and titration

BHK-21 cell culture was used for FMDV isolation as previously described by OIE (2021) where the prepared epithelium and vesicular fluids samples were inoculated in confluent BHK-21 flasks and incubated in a CO_2 incubator at 37 °C for 24-72 hours and examined microscopically for the development of a cytopathic effect (CPE). CPE was observed for three consecutive blind passages on the BHK-21 cell with three cycles of freezing and thawing between each passage. BHK-21 was used for virus titration and expressed as $\log_{10}TCID_{50}$/ml as previously described by Reed and Muench (1938).

2.5. Archived FMD viruses

Two archived FMD viruses were serotyped as O by the IZLER ELISA kit (The Kits were produced and packaged at IZSLER Biotech laboratory, pirbright institute, UK and The plate read at 450 nm wave length using a micro plate reader. The positive controls are expected to give OD values of 1.0 unit or higher in the type-specific reactions and in the pan-FMDV reaction, the negative control usually gives OD values lower than 0.1 in wells) , and the R-value was estimated against the vaccine strain Pan-Asia-2 by El-Bagoury et al. (2018). In the present study, further investigation of the ELISA results was confirmed by RT-PCR and followed by sequence analysis.

2.6. Viral RNA extraction

The total RNA was extracted from the FMDV isolate, the previously typed FMDV serotype O used as a positive control, and BHK-21 negative control using a QIAamp® Viral RNA Kit (QIAGEN, Germany) according to the manufacturer's instructions. The extracted RNA was eluted in 60 µl of AVE kit elution buffer and kept at –80°C until used.

2.7. Identification and serotyping of FMDV nucleic acid using conventional RT- PCR

Conventional one-step RT-PCR was followed up according to the manufacturer's directions for serotyping of the FMD virus field isolates and the two archived FMD viruses. The viral VP1-RNA was amplified by RT-PCR using specific primers for serotype O, A, and SAT2 as shown in table (2). The reaction was done in 50 µl reaction volume containing 10 µl RNA template, 4 µl gene specific primers 1 µM, 2 µl dNTP (mix), 2 µl enzyme mix, 10 µl 5x RT buffer, and RNase-free water Up to 50 µl was performed using the following cycling program 60 °C for 30 min and 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 sec. and 60 °C for 55 sec.

Table 2 Oligonucleotide FMDV-specific primers used for typing by RT-PCR Technique

Primer	Orientation	Sequence 5`-3`	Band size	Serotype specificity	Genomic location	References
Pan FMDV	Reverse	GCCTGGTCTTTCCAGGTCT	328 bp	Pan FMDV	1D	Reid et. al. (2000)
	Forward	CCAGTCCCCTTCTCAGATC				
P1	Reverse	AGC TTG TAC CAG GGT TTG GC	402	FMDV O	1D	Knowles et al. (2007)
P2	Forward	GCT GCC TAC CTC CTT CAA				
P3	Reverse	TAC CAA ATT ACA CAC GGG AA	866	FMDV A	1D	Knowles et al. (2005)
P4	Forward	GAC ATG TCC TCC TGC ATC TG				
P5	Reverse	CCA CAT ACT ACT TTT GTG ACC TGG	715	FMDV SAT2	1D	Shawkey et al., (2013)
P6	Forward	ACA GCG GCC ATG CAC GAC AG				

2.8. Sequencing:

It was performed using BigDye® Terminator v3.1 Cycle sequencing kit (Thermo Fisher, USA); steps were done according to the manufacturer's instructions.

2.9. Phylogenetic Analysis

The resulting sequences were compared to FMDV sequences already reported in GenBank. ClustalW/Bio-edit software - version 7.1 was used to align the sequences. MEGA version X software for Neighbor-joining phylogenetic trees constructions was used to create the phylogenetic tree using the Maximum Likelihood method (Tamura et al., 2021).

3. RESULTS

3.1. Virus isolation and titration

Six samples (four tongue epithelium and two vesicular fluid) out of 30 samples showed specific CPE of FMD virus on BHK-21 cell culture characterized by cell rounding, granulation, and cell detachment (Fig. 1, panel B) as compared to the normal spindle uninfected BHK-21 (Fig. 1, panel A). The infectivity titer of the obtained virus isolates ranged from 5-6 $\log_{10}TCID_{50}$/ml.

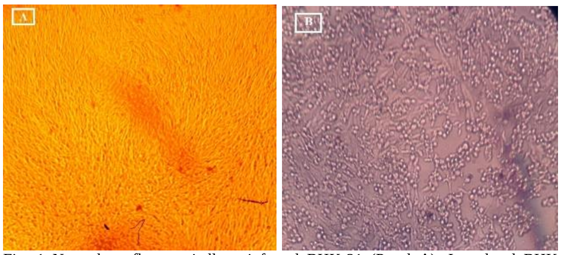

Fig. 1 Normal confluent spindle uninfected BHK-21 (Panel A). Inoculated BHK-21 showed the characteristic CPE for FMDV exhibited rounding, granulation, and cell detachment (Panel B).

3.2 FMD virus serotyping using conventional RT-PCR

The six isolated FMD viruses and the two archived FMD viruses gave positive bands with the specific primer for serotype O, while no results with the specific primers for serotype A and SAT-2 as shown figure (2).

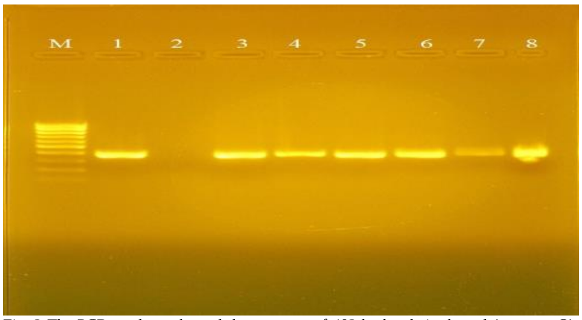

Fig. 2 The PCR products showed the presence of 402 bp bands in the gel (serotype O). (M: Marker), Lane 1 represents positive control; Lane 2 represents negative control, and lanes (3, 4, 5, 6, 7 and 8) showed a positive reaction.

3.3. Sequencing

The positive RT-PCR bands for the isolated virus and the archived viruses were selected for 1D (VP1) sequencing and submitted in GenBank under Accession numbers (OM681353) for the field isolated FMD virus and (OM681354), (OM681355) for the two archived FMD viruses.

3.4. Phylogenetic analysis

The phylogenetic tree revealed that the isolated FMD virus was related to serotype O, East Africa 3 (EA-3) topotype, lineage Alx-17, and the archived viruses belonged to serotype O, Middle East-South Asia topotype (ME-SA), lineage Sharquia-72 as illustrated in figure (3).

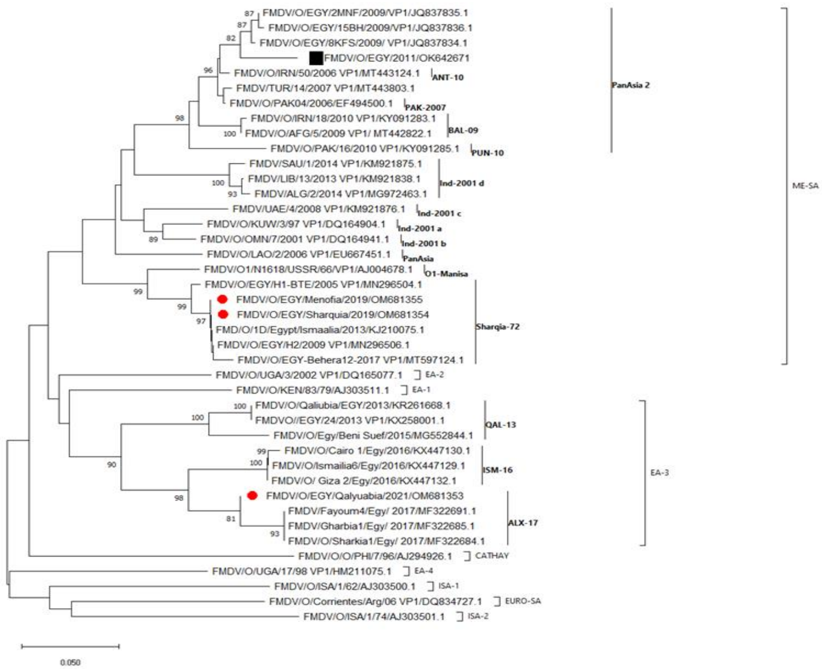

Fig. 3 The phylogenetic tree based on 1 D sequence using the neighbor-joining method illustrated that the field isolated virus belongs to serotype O, East Africa 3 (EA-3) topotype, lineage ALX-17, and the archived viruses belong to serotype O, Middle East-South Asia topotype (ME-SA), lineage Sharquia-72 (tagged by circular) and the vaccine strain related to Pan-Asia -2 lineage, Middle East-South Asia topotype (ME-SA) (tagged by square)

4. DISCUSSION

In winter season 2021, a severe FMD outbreak was recorded in cattle and buffalo in Qalyubia government, where these animals suffered from fever, salivation, lameness, and vesicular eruption on the oral cavity, between the coronary bands of the feet, and teat of the female udder. These signs strongly suggested FMDV infection as described by OIE (2021).

Trials of virus isolation on BHK-21 cell culture through three successive passages revealed that six samples (four TE and two VF) showed specific characteristic CPE of FMDV represented by cell rounding, cell aggregation followed by a detachment of the cell sheet within 24-72 hours post cell infection. These findings came confirmed with the normal spindle uninfected BHK-21, and what was described by Ismael et al. (2021). The infectivity titer of the obtained virus isolates ranged from 5-6 $log_{10}TCID_{50}$/ml.

RT-PCR was used for serotyping the six isolated viruses using specific primers for serotype O, A, and SAT2 (Table 2) that gave positive bands with the specific primer for serotype O (Figure 2). Strong positive bands were selected for virus sequencing and the phylogenetic analysis revealed that the isolated virus belongs to serotype O, East Africa 3 (EA-3) topotype Lineage Alx-17 (FMDV/O/EGY/ Qalyubia/2021/OM681353) as shown in Figure (3) and closely related to the local Egyptian isolate during 2017 (FMDV/O/EGY/2017/OM221230.1) with nucleotide similarity of 99.36% and sharing 98.72% nucleotide sequence with the isolated virus from Sudan (FMDV/O/SUD/2017/ MK422569.1) and Ethiopia (FMDV/O/ETH/2017/ MN987453.1) during 2017. This result could be attributed to the transboundary incursions.

The Two archived FMD viruses were confirmed by RT-PCR and followed by sequence analysis and phylogenetic tree revealing that the two viruses were serotype O topotype Middle East-South Asia (ME-SA) lineage Sharquai-72 accession numbers (OM681354), (OM681355) as illustrated in Fig. (3) and shared 99.76% nucleotide sequence within each other. The sequenced Sharquai-72 virus (OM681354) is closely related to the previously local Egyptian isolate in 2014 (FMDV/O/EGY/Dakhlia/ 2014/KP940473.1) with nucleotide identity 99.77%. The sequenced Sharquai-72 virus (OM681355) was closely related to the local Egyptian isolate in 2017 (FMDV/O/ EGY/2017/MN296510.1) with nucleotide similarity 99.46%.

The isolated FMDV EA-3 revealed nucleotide identity 84.72% with FMDV serotype O topotype ME-SA lineage PanAsia-2 vaccine strain (FMDV/O/EGY/2011/ OK642671) while showing 85.65%nuclotide sequence with the sequenced sharqaia-72 virus (OM681355). The sequenced sharquia-72 virus (OM681355) showed 86.01% nucleotide sequence with the PanAsia-2 vaccine strain (OK642671). These results are in close agreement with the findings of Abu Elnaga et al. (2020) that illustrated viruses belong to EA-3 topotype sharing 80.9-83.6% nucleotide sequence with Pan-Asia-2 viruses, EA-3 viruses revealed 83.5-86.5.3% nucleotide identity with sharquia-72 viruses, and PanAsia-2 viruses showing 87.3-88.6% sequence similarity with sharquia-72 viruses. We agreed with Abu Elnaga et al. (2020) suggestion that any vaccine incorporating the PanAsia-2 virus support complete or partial protection against sharquia-72 viruses and vice versa while vaccines containing PanAsia-2 viruses support partial or no protection against EA-3 viruses. Pan-Asia-2 virus and Sharquai-72 virus belong to the same ME-SA topotype with a nucleotide difference of less than 15% while EA-3 viruses belong to a different topotype with a nucleotide difference of more than 15% from the PanAsia-2 vaccine strain (ME-SA topotype). These findings were in accordance with Samuel et al. (1999), who illustrated that FMD virus strains that differ in the sequenced genomic region by less than 15% are thought to belong to the same genotype, while those that differ by less than 5% are thought to be closely related.

Egypt has reported with multiple EA-3 outbreaks since its incursion in 2012 (Soltan et al., 2019; WRLFMD.2019). and some outbreaks were reported in vaccinated animals less than six months post-vaccination with vaccines incorporating Pan-Asia-2 (Abdulrahman et al., 2019; AL-Hosary et al., 2019).Repeated occurrence of FMD outbreaks although obligatory governmental vaccination programs may be due to the viral genetic variation (EL-Bayoumy et al., 2014; Sobhy et al., 2014; Soltan et al., 2017).

5. CONCLUSION

We concluded that the FMDV causing an outbreak at Qalyubia government in 2021 was related to serotype O topotype East Africa 3 (EA-3) Lineage Alx-17 and our recommendations are directed toward periodical isolation, molecular characterization of the field circulating FMDV to keep up dated vaccine strains in addition to monitoring FMD virus in surrounding countries for predicting strains that may escape to Egypt.

ACKNOWLEDGMENT

We are a pleasure to thank FMD Research Department, Veterinary Serum, and Vaccine Research Institute, Abasia, Cairo, Egypt where this work was carried out, and Animal Health Research Institute; Giza where sample sequencing was done. The authors did not obtain any funds for this study.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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