A nucleotide sequencing of foot-and-mouth disease virus Egyptian strains

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Abstract: Foot and mouth disease virus (FMDV) has been annually isolated during the recent years in Egypt. There is no sufficient local data about the genetic changes occurred in the virus, which makes tracing its origin difficult. In this work, FMDV serotype O and A were isolated from bovine clinical samples, collected from Ismailia, Dakhlia, Monoufia and Sharkia during 2010. RNA extracted from either clinical or cell culture grown virus was subjected to RT-PCR. Nucleotide sequencing of FMDV 1D gene was determined using standard automated sequencing technique. Phylogenetic analysis revealed that the isolated serotype O viruses were closely related to O1/Sharquia/EGY/72 and had same lineage with the O Turkish Manisa strains. Deduced amino acids of 2009 and 2010 Egyptian A isolates in relation to the Egyptian A 2006 strains showed several replacements (I42V, N43S, S44N, L45Q, P141S and A156P). From the obtained result, it was clear that FMDV serotypes O and A isolated in 2010 were closely related to the Egyptian FMDV vaccinal strains and they emerged from the same ancestor.
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1. Introduction

Foot-and-mouth disease (FMD) is an animal viral disease worldwide affecting ungulates animals and is caused by *Foot-and-mouth disease virus* (FMDV). It can cause vesicles in the mouth, on the nose and on the coronary bands of the hoofs. It has severe economic impacts. Milk production drops dramatically and animals become lame. Mortality can be high in young animals in unvaccinated herds. Trade of livestock and animal products must be blocked and the export of agricultural products must be banned from areas where the disease occurs [1]. The virus is a picornavirus (genus *Aphthovirus*, family *Picornaviridae*) which exists as seven immunologically distinct serotypes (A, O, C SAT1, SAT2, SAT3 and Asia) and multiple subtypes reflecting significant genetic variability [2]. The high potential for genetic and antigenic variation is one of the most important FMDV features, which derives from the quasispecies structure of its viral populations [3]. This structure poses important implications on its biology and control; among them, the high antigenic diversity of viral population that is reflected in seven serotypes and a multitude of variants [4]. The virus has a linear single-stranded RNA genome of about 8.5 kb that consists of 12 protein-coding genes encoding a leader peptide, structural proteins (1A, 1B, 1C, 1D), and non- structural proteins. 1D encodes VP1, which is the most surface exposed capsid protein and contains three important immunogenic sites at amino acid positions 40-60, 140-160 and residues 200–213 [4-6]. Variation at specific amino acid sites within those immunogenic regions contributes to the antigenic variability of the virus. Nucleotide sequence data have been used to understand the transboundary nature of FMDV, recreating temporal movement patterns of virus spread within countries and across international borders. At the broadest scale, phylogenetic analyses of VP1 (1D) sequences have been shown to be a useful tool to categorize field strains into discrete topotypes which frequently show geographical clustering based on the historical distribution of the virus [7].

In Egypt, FMDV has been endemic since 1952 and evoked outbreaks every few years [8-11]. Recently, the disease were annually recorded either sporadic or epizootic without sufficient national database of the origin of the causative agent and the genetic changes occurred in the main immunogenic sites of the virus that have a negative reflection on the control strategy of the disease in Egypt. In this paper, molecular detection, nucleotide sequencing and phylogenetic analysis of FMDV suspected clinical samples in differentEgyptianprovincewere performed to study molecular epidemiology of virus.

2. Materials and methods

2.1. Samples

Clinical samples were received from different Egyptian governorates **(Table 1)**. These samples were 18 tongue epithelium and 20 oeso-pharyngeal fluid (OP) collected from cattle. The clinical samples were investigated by ELISA and RT-PCR coupled to nucleotide sequencing for detection and identification of FMDV.

Table 1 Suspected FMDV samples from different governorates.

Clinical samples	Governorates				Total
	Ismailia	Dakhlia	Monoufia	Sharkia	
Tongue Epithelium	6	4	2	6	18
OP	6	5	2	5	20

2.2. Preparation of samples

All virus samples were prepared for FMDV detection. In brief, tongue epithelium were minced, suspended in veronal buffer and centrifuged. Also, OP samples were undergone several centrifugation until clear transparent supernatant was obtained. Afterward, all the prepared supernatants were inspected by sandwich ELISA kit purchased from Institute for Animal Health, Pirbright, UK. Virus supernatants were filtered and isolated on low passage monolayer BHK cells.

2.3. Nucleic acid recognition of virus samples

RT-PCR was used to amplify genome fragment from the prepared samples followed by nucleotide sequencing using FMDV serotype specific primers. These oligos were synthesized by BioBasic, Canada. Primers PH1 (5´- AGC TTG TAC CAG GGT TTG GC -3´) and PH2 (5´- GCT GCC TAC CTC CTT CAA -3´) were designed to bracket a 402-bp region in the 1D/2B genome sequence for serotype O [12]. For serotype A, primers PH9 (5´- TAC CAA ATT ACA CAC GGG AA -3´) and PH10 (5´- GAC ATG TCC TCC TGC ATC TG -3´), designated to whole 1D gene, were used to amplify the expected sequence fragment of 863-866 bp in the 1C/2B regions [13]. RNA Extraction was done using QIAamp® Viral RNA kit (Qiagen, Germany) according to the manufacturer's protocol. RT-PCR was carried out using One-Step RT-PCR Kit (Qiagen, Germany). The cycling parameters were 50 °C for 30 min and 95 °C for 15 min; then 30 cycles consisting of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 60 s, and then a final incubation at 72 °C for 10 min. Amplified products were analyzed on agarose gel. Negative control specimen and DNA ladder were involved in agarose gel electrophoresis.

2.4. Nucleotide sequence and Phylogenetic analysis

Amplified viral products were sent to Macrogen (Korea) for DNA sequencing. The sequenced samples represented different FMDV isolates from different governorates. These samples were designated O/EGY/2010 iso1031 - O/EGY/2010 iso1038 for serotype O and A/EGY/2010 iso1031- A/EGY/2010 iso1032 for serotype A. All the received sequence results were aligned with nucleotide sequences database at the National Centre for Biotechnology Information site (NCBI) using Basic Local Alignment Search Tool programs to assert the new sequences FMD virus/serotype specificity. Analysis of the sequences identity, divergence and phylogenetic relationship was performed using the clustal W method with weighted residue table provided in the MegAlign program (DNASTAR, Inc. Madison, WI, USA). For multiple alignment and analysis of the deduced amino acid of the received sequence, the sequences of some related viruses and reference virus strains were involved in the analysis.

3. Results

Epithelia and OP samples were collected in this study during 2010. All epithelial samples were positive for FMDV by isolation on low passage monolayer BHK cells and ELISA. Three of these isolates were of serotype A from Ismailia and Dakhlia governorates, while 16 isolates were serotype O. All OP samples were negative except one sample that was serotype A from Ismailia (Table 2). All ELISA positive samples were amplified by RT-PCR with the production of the expected amplicons of either 402 bp for serotype O or 863-866 bp for type A (Fig 1).

Table 2 Positive FMDV epithelium and OP samples using isolation on tissues culture and identification by ELISA

Governorates	**No of Tongue Epithelium samples**	**No of OP samples**	**Tissue culture**	**ELISA**	
				FMDV serotype O	**FMDV serotype A**
Ismailia	6	6	7 (6+1*)	5	2
Dakhlia	4	5	4	3	1
Monoufia	2	4	2	2	-
Sharkia	6	5	6	6	-
Total	18	20	19	16	3

*One OP sample was positive for serotype A from Ismailia governorate

Partial nucleotides sequences of type O 1D gene was obtained and gave the easiness to select from FMD viruses partial and complete sequences found on genebank in order to align deduced amino acid residues **(Fig. 2)** and to construct the phylogentic tree. For serotype A, complete deduced amino acid of VP1 region **(Fig. 3)** was investigated to study genetic relationship between the different strains. Phylogenetic analysis of serotype O revealed 97% identity among the compared 2010 Egyptian strains **(Fig. 4)**. While there was 0.8 % divergence between the studied serotype A Egyptian viruses isolated in 2009 and that were isolated in 2010 **(Fig. 5)**.

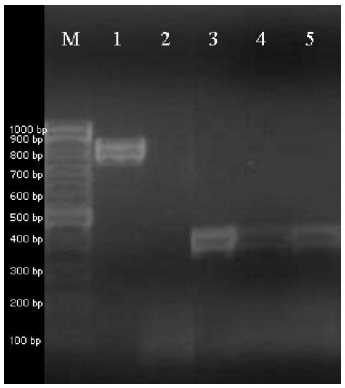


Fig. 1 RT-PCR for detection of FMDV serotype A and O. M: 100 bp ladder, lane 1: serotype A (863 bp), lane 2: negative control, lanes 3-5: serotype O (402 bp)

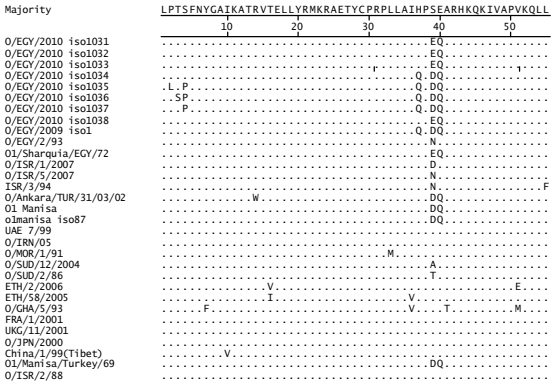


Fig. 2 Alignment of partial deduced amino acid sequences of VP1 gene of FMDV serotype O isolated from Egyptian governorates

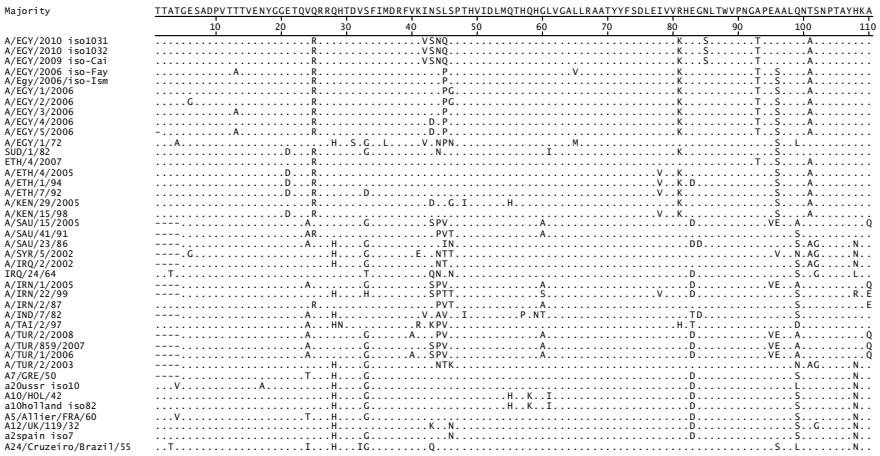


Fig. 3 Alignment of complete deduced amino acid sequences of 1D gene of FMDV serotype A isolated from Ismailia and Dakhlia

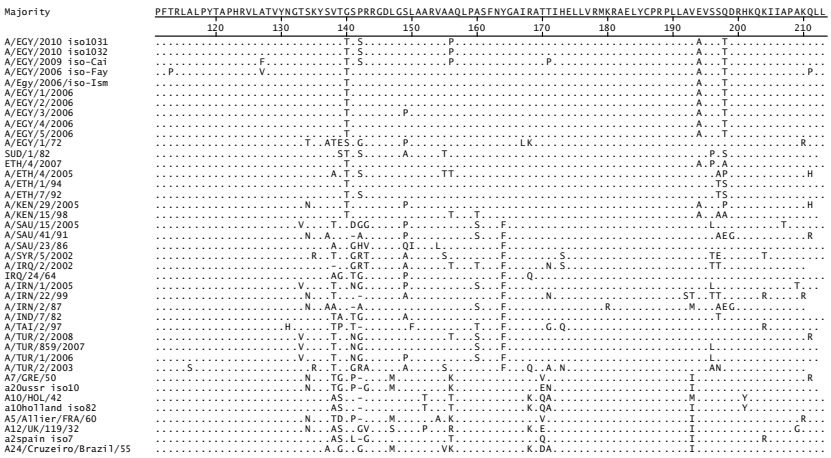


Fig. 4 Phylogenetic tree showing relationship among FMDV O depending on the virus partial 1D gene sequence

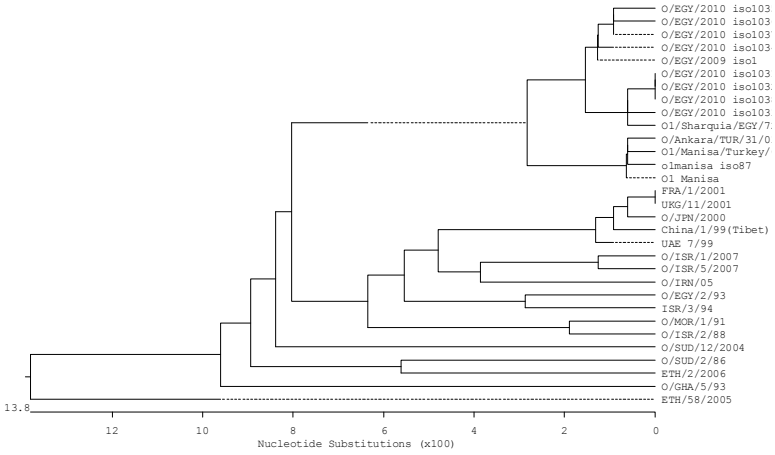
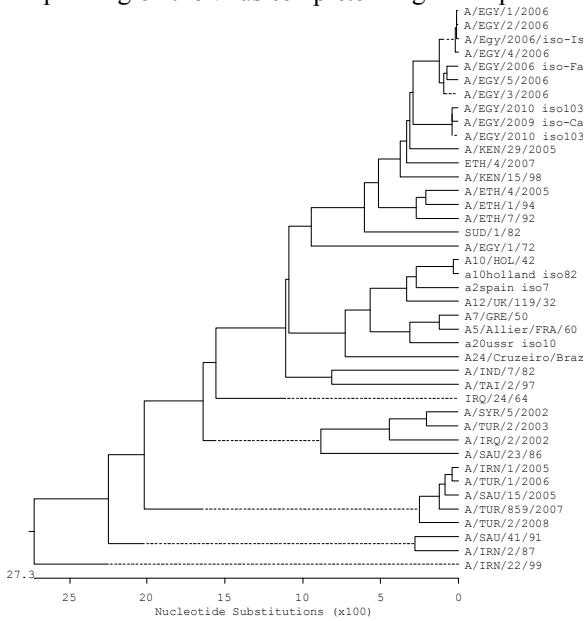


Fig. 5 Phylogenetic tree showing relationship among FMDV A depending on the virus complete 1D gene sequence



4. Discussion

FMDV represents an annual challenge in Egypt to the authorities, veterinarians and farmers. The virus causes the disease in sporadic cases in different areas. Monitoring the strains of FMDV is important to enable appropriate vaccine to be selected and control measures to be implemented as rapidly as possible. In this paper, virus samples from clinically infected cattle were tested by ELISA and RT-PCR. They were found to be either FMDV serotype O or A.

FMDV serotype A was detected in two epithelial samples from Ismailia and Dakhlia and one OP samples from Ismailia while serotype O was detected in 16 samples (Table 2). Recent articles [11, 14] recorded that serotype O and A still persist and circulating in Egypt. RT-PCR is an important diagnostic tool for laboratory detection. RT-PCR was performed by using specific primers for FMDV serotype O and A to amplify the VP1 coding region fragment of FMDV. The amplified fragments confirmed all ELISA positive samples for FMDV. FMDV was detected in 50 % of the collected samples, where 7.89% were serotype A from Ismailia and Dakhalia governorates while 42.1% were serotype O from Ismailia, Dahklia, Monofia and Sharkia. Phylogenetic analysis of isolates were performed to provide further characterization data and to support epidemiological investigation of the source of outbreak in the country. FMDV type A outbreak in Egypt in 2006 was due to indigenous African virus [15]. The molecular epidemiology of circulating viruses was monitored by sequencing the VP1 region of FMDV genome and comparing the recovered sequences to sequence data held in gene bank [16].

In this study, FMDV strains O/EGY/2010 iso1031- O/EGY/2010 iso1038 were homologous with the vaccinal strain O/EGY/2009 iso1 and had conserved amino acid residue at positions 198, where Glutamine replace the consensus residue Glutamic acid (Fig. 4). In addition, the Egyptian strains, constructed the phylogenetic tree, suffered variable residue between them at positions 161, 195, 197, nevertheless, these changes had no impact on the immunogenicity of these strains. Phylogenetic analysis revealed that the former strains were closely related to O1/Sharquia/EGY/72 and had the same lineage with the O Turkish Manisa strains with nucleotide sequence identity less than 5%. This analysis oppose the suggested concept of a constant evolutionary rate, where studies of FMDV types O, A and C in the Philippines and type A in Turkey have shown that the VP1 gene changes by approximately 1% per year [17]. The partial nucleotide sequence of type O VP1 gene gave some informative content; however, it is worthiness in future to execute complete nucleotide sequence of 1D gene to have more data.

The sequenced samples of FMDV serotype A revealed that they were closely related to the vaccinal strain namely, A/EGY/2009 iso-Cai with homology 99.2% and they had no significant changes in amino acid residues between them (Fig. 5). However, at antigenic sites 40-60 and 140-160, deduced amino acids of serotype A 2009 and 2010 Egyptian strains in relation with the 2006 isolates showed several replacements (I 42→V, N 43→S, S 44→N, L 45→Q, P 141→S and A 156→P). Analysis of phylogenetic tree demonstrated that A/EGY/2010 strains have the same lineage with serotype A 2006 and 2009 strains and compose with the other compared African strains, the Africa topotype with more than 85% resemblance. It has been reported that serotype A could be grouped according to genetic and geographic distinct evolutionary lineage into three topotypes, (i) Euro-/SA; (ii) Asia and (iii) Africa, although occasional spread between these continents may take place [18].

From this study, it was found that FMDV serotypes O and A isolates collected in 2010 were closely related to the Egyptian FMD bivalent vaccinal strains, which were able to challenge the sporadic circulating variants. However, more clinical samples are required from all over the country to have a wide view on the molecular epidemiology of FMDV in Egypt.

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