International Journal of Veterinary Science and Medicine xxx (xxxx) xxx–xxx

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Contents lists available at ScienceDirect

International Journal of Veterinary Science and Medicine

journal homepage: www.elsevier.com/locate/ijvsm


Full Length Article

# Outbreaks of foot and mouth disease in Egypt: Molecular epidemiology, evolution and cardiac biomarkers prognostic significance

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ARTICLE INFO

*Keywords:*
Biomarkers
Cattle
Egypt
FMD
Myocarditis
Sequencing

ABSTRACT

Foot and mouth disease virus (FMDV) was isolated from sloughed tongue epithelium of Egyptian cattle presenting with mouth lesions and ropy salivation in two Egyptian governorates (El-Fayoum and Dakahlia). The virus was isolated in Madin-Darby bovine kidney (MDBK) cells and identified by reverse transcription-polymerase chain reaction (RT-PCR). The complete genome was obtained by next generation sequencing. The strains isolated from El-Fayoum and Dakahlia were serotype A and O, respectively and both isolates had identity with the previously reported Egyptian strains. This study reports successive outbreaks of FMDV that occurred in Egypt during 2015–2016 and describes the dynamics of two outbreaks in addition to the use of cardiac biomarkers in the diagnosis of FMD-related myocarditis in calves and its clinical relevance. Serum cardiac troponin1 (cTn I) and creatinine kinase myocardial band (CK-MB) were measured. Mean serum cardiac troponin1 (cTn I) showed significant increase ($P < 0.001$) in FMDV-infected calves. The increase in fatal and recovered cases was (2.794 ± 0.502 ng/mL) and (1.196 ± 0.443 ng/mL), respectively, compared to the healthy control cases (0.014 ± 0.002 ng/mL). Thus, the serum cTn-I successfully diagnosed FMD-associated myocarditis in calves but not prognostic for the fatal cases. The FMDV sequences described in this study should further help in studying FMDV endemicity in Egypt, tracking the source of infection, selection of control strategies and vaccine updates. The study also determines the clinical relevance of cardiac biomarkers in diagnosis of FMDV-related myocarditis in infected calves.

## 1. Introduction

Foot and mouth disease (FMD) causes huge economical loss in Egypt in terms of reduction in the production of red meat, milk and milk byproducts. The disease is caused by foot-and-mouth disease virus (FMDV), which is a member of the genus *Aphthovirus* under *Picornaviridae* [1]. Molecular epidemiology studies on FMDV in Egypt are lacking. Such studies are necessary to better understand the natural history of the disease, relationships among various serotypes, and to develop more effective prevention and control methods.

The FMDV is a small, non-enveloped, virus with an icosahedral capsid composed of 60 copies each of four structural proteins (VP1, VP2, VP3 and VP4), which surround a single stranded positive sense RNA genome of approximately 8500 nucleotides [2]. The virus is classified into seven immunologically distinct serotypes i.e., A, C, O, Asia 1, SAT 1, SAT 2, and SAT 3. The viral genome is further subdivided into P1, P2 and P3 regions. The P1 region encodes leader proteinase (Lpro) and structural proteins 1A (VP4), 1B (VP2), 1C (VP3), and 1D (VP1). The P2 region encodes non–structural proteins 2A, 2B, and 2C, while P3 region encodes 3A, 3B (VPg), 3C protease and 3D polymerase [3,4]. The VP1 gene at the surface of the virion is the most variable and contains serotype-specific amino acid sequence that can help differentiate various serotypes [3].

The FMD is a highly contagious transboundary disease that affects cloven-footed domesticated and wild animals in Africa, Asia and parts of South America [5]. Over the last 65 years, the disease has become endemic in Egypt with three strains (A, O and SAT 2). The first FMD outbreak in Egypt was reported in 1950 and it was due to serotype SAT2 [6]. The SAT2 reinvaded the country in 2012 and caused high mortality in ruminants [7].

Egypt suffered from FMD outbreaks in 1958 with serotypes A and O; in 1961, 1964, 1965, 1970, 1974, 1983, 1987, 1989, 1993 and 2000 with serotype O; and in 1967 and 1972 with serotype A [8]. On February 2006, serotype A caused six outbreaks in Ismailia and 12 additional outbreaks in seven other Egyptian governorates e.g., Alexandria (2 outbreaks), Behera (1 outbreak), Cairo (1 outbreak), Dakahlia (1

Peer review under responsibility of Faculty of Veterinary Medicine, Cairo University.
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https://doi.org/10.1016/j.ijvsm.2018.02.001
Received 17 October 2017; Received in revised form 3 February 2018; Accepted 4 February 2018

outbreak), Dumyat (5 outbreaks), Fayum (1 outbreak), and Menofia (1 outbreak). By April 6, 2006, 34 outbreaks of disease were reported. The results of the molecular typing suggested a relationship between Egyptian and East African strains [9]. In 2012–2013, A serotype of Asian origin related to Iranian strain was detected [10]. Serotype O has a long history of causing regular outbreaks in Egyptian livestock [11,12]. The outbreaks of FMD still occur all over Egypt although vaccination is obligatory in the country [10,13].

The FMDV is associated with sudden death in young calves. In such cases, no observable clinical signs are seen except for virus-induced damage in the myocardium [14]. Generalized myositis aphthosa affects only calves during the acute stage. Other muscles such as hind quarter, diaphragm and tongue are also affected [15]. Histopathological changes in the skeletal muscle are similar to those in heart during the acute stage of the disease [16]. Recently, serum cardiac troponin1 (cTn I) and creatinine kinase myocardial band (CK-MB) have been used as diagnostic biomarkers as they are released into the blood soon after any micro injury in the myocardium [14].

The reports of new FMD variants in the country have raised question related to genetic diversity of these variants as well as the need for better understanding of these variants. To answer some of these questions, we characterized FMDV isolates based on complete genome sequencing in order to understand the dynamics of serotypes A and O in calves. This follow up study also focused on mortality in calves and the relevance of cardiac biomarkers in diagnosis and prediction of mortality in infected calves.

## 2. Materials and methods

In the 2014–2015 outbreak, molecular epidemiology and evolution of FMDV in two provinces (El-Fayoum and Dakahlia) were investigated. During sampling, signalment and history from owners and veterinarians revealed the main complaint to be of high fatality rate in calves in spite of vaccination. In the 2015–2016 outbreak, the chief complaint was the same as above. Hence, we concentrated our efforts to predict the fatal cases before death to reduce the losses.

### 2.1. Molecular epidemiology and evolution study

#### 2.1.1. Clinical samples

In December 2014 and January 2015, 20 samples of sloughed tongue epithelium were collected from cattle showing fever, ropy salivation with slobbering, smacking of lips and lameness. Ten samples were collected from each of the two governorates i.e., El-Fayoum and Dakahlia. The samples were homogenized in Eagle's minimum essential medium (MEM) followed by centrifugation at 2000 × *g* for 20 min. The supernatants were inoculated in baby hamster kidney-21 (BHK21) cells followed by incubation for 2 h at 37 °C. This was followed by the addition of MEM with Earle's salts supplemented with 2% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin. Inoculated cells were harvested when cytopathic effects (CPE) appeared in about 80% of the monolayer (about three days post inoculation). The infected cells were frozen and thawed three times followed by centrifugation at 1200*g* for 20 min.

#### 2.1.2. Reverse transcription-polymerase chain reaction (RT-PCR)

The QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA) was used for viral RNA extraction from supernatants of infected cells. Conserved RdRp primer pair of forward **5′**-TTCGAGAACGGCACDGTCGGA-'3 and reverse **5′**-CACGGAGATCAACTTCTCCTG-'3 sequences was used to amplify a 881 bp product that confirmed the presence of FMDV infection [10]. The serotype differentiation was done by using VP1 primers (Table 1). Extracted RNAs were subjected to RT-PCR using one-step RT-PCR kit (Qiagen, Valencia, CA). Amplification reactions (25 μL reaction mixture) were performed in a thermocycler (Mastercycler Eppendorf) under the following conditions: 50 °C for 30 min for reverse transcription and initial denaturation at 95 °C for 15 min; then 35 cycles consisting of 94 °C for 1 min for denaturation followed by annealing for 1 min at 52 °C for serotype O and SAT2 while for RdRp and serotype A, the reaction run at 54 °C, 55 °C, respectively. Extension was done at 72 °C for 1 min followed by one final extension step of 10 min at 72 °C. The amplicons were analyzed by 1.2% agarose gel electrophoresis in Tris-acetate EDTA buffer followed by staining with ethidium bromide.

#### 2.1.3. Next generation sequencing (NGS)

One representative RNA sample from each of the governorates was subjected to whole genome sequencing. Total RNA was quantified by a fluorometric RiboGreen assay. The library was created using Illumina's Truseq RNA sample preparation kit (Cat. # RS-122-2001) and sequenced using Illumina MiSeq platform with 250 paired end cycle run. The CLC genomic workbench 6.0 (www.clcbico.com) was used to analyze the resulting NGS reads, including trimming, sequence quality, and *de novo* assembly. Assembled contigs were analyzed by BLASTx and open reading frame was predicted by the ORF finder tool (https://www.ncbi.nlm.nih.gov/gorf/orfig.cgi).

#### 2.1.4. Genome characterization and phylogenetic analysis

The study sequences were compared with previously reported FMDV sequences available in GenBank. Sequences were aligned by using the Clustal W in MEGA 6.0 (Molecular Evolutionary Genetic Analysis). A phylogenetic tree was constructed using the best fit Maximum Likelihood model in MEGA 6.0 based on lowest BIC score (Bayesian Information Criterion). The Maximum-Likelihood method used for constructing phylogenetic tree was GTR mode + G (Gamma distribution with 5 rate categories) for serotype O and GTR + G + I for serotype A [17]. The evolutionary distances were computed using the p-distance in MEGA 6.0. The FMDV A and O serotype sequences were submitted to GenBank with accession numbers KP940474 and KP940473, respectively.

### 2.2. Prognostic study (cardiac biomarker study)

#### 2.2.1. Animals used in follow up and cardiac marker detection

During the outbreak of 2015–2016, 119 saliva and nasal swabs were collected from suspected infected calves. Calves were aged 2–15 months belonging to El-Fayoum and Dakahlia governorates. Infection with serotypes A and O was confirmed by RT-PCR in 108 calves (40 males and 68 females). Blood samples were collected once upon admission. Serum samples were collected from blood and stored at -20 °C until used. Cardiac troponin I and CK-MB screening was performed in all confirmed infected calves. Ten clinically healthy calves with normal appetite, alertness, and good vital signs of the same age were used as a control. The control calves were belonged to a private farm in Dakahlia province, with no history of FMD infection. Follow up of all infected calves was done for up to 14 days.

#### 2.2.2. Clinical assessment and laboratory tests

All confirmed infected calves were subjected to thorough clinical examination with vital signs monitoring. Special attention was paid to heart and lung examination. Transthoracic ultrasonographic examination of the heart and lung was performed. Serum samples were examined for serum cTn I by an enzyme-linked immunosorbent assay (ELISA) kit (Monobind Inc, Lake Forest, CA, USA) and CK-MB activity was measured using commercially available kits (Spectrum diagnostics, Germany). These tests were applied according to the manufacturers' instructions.

#### 2.2.3. Postmortem and histopathological examination

Postmortem examination of lung and heart was performed for all dead calves. Lesions were observed and samples were collected. Lung and heart specimens were taken and fixed in 10% buffered formalin followed by histopathological examination [18].