



Original Research Article

Molecular characterization and VP1 sequence analysis of Foot and Mouth disease new virus strains isolated during 2015-2017 in Beni-Suef, Egypt

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ABSTRACT

Outbreaks of Foot and Mouth Disease (FMD) cause severe economic loss to the livestock industries in terms of loss of meat, milk production and the high mortality rates especially in calves. This study was intended for detection, isolation and molecular characterization of FMDV circulated strain among different regions in Beni-Suef governorate, Egypt during 2015 and 2017. One hundred field samples were collected from clinically diseased cattle and buffaloes including vesicular fluid and sloughed epithelial membrane. Molecular detection and differentiation of FMDV serotypes by RT-PCR showed that (71%) were positive for serotype O indicating its predominance in Beni-Suef, Egypt. Also (18%) were positive for serotype SAT2 and (7%) were un-typed serotype being only positive for universal primer. Twenty samples were isolated on BHK-21 cells clone 13. Three samples showed the characteristic CPE of FMDV after blind passage 4 times. Sequences of VP1 coding-region of the three isolated FMD virus showed that the three isolated viruses were serotype O. The phylogenetic analysis of the isolated viruses revealed that they were closely related to type O of those reported in Ismailia, Behira, Giza and Cairo during 2016 with identity ranged from 97.7% - 99.8%. The most relevant outboard isolate was SUD/8/2008 with 93% identity after Blast homology search. However, the phylogenetic tree analysis revealed that the three FMDV type O isolates FMDV/serotype O/Beni-Suef/2017 differs partially from all other Egyptian serotypes of 2016. In conclusion, serotype O was the most prevalent FMDV serotype in Beni-Suef, Egypt.

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Introduction

Foot-and-mouth disease (FMD) is a highly contagious and economically viral disease of cloven-hoofed animals that is characterized by fever, lameness and vesicular lesions of the feet, tongue, snout and teats[1]. The high debilitating effects, rather than high mortality rates of FMD, are responsible for severe economic losses associated with FMD [2]. The causative agent, Foot and mouth disease virus (FMDV) is classified as small icosahedral non-enveloped virus of the *Aphthovirus* genus within the *Picornaviridae* family. FMDV has seven clinically indistinguishable serotypes (A, O, C, Asia1, Southern African Territories SAT1, SAT2 and SAT3), and multiple subtypes reflecting significant genetic variability in each serotype [3]. These different serotypes have no cross-protection between each other following infection or vaccination [4]. FMDV has a linear single-stranded positive-sense RNA genome approximately 8.5 kb in length including the 5' untranslated region (5'UTR), a large single open reading frame (ORF), and the 3' untranslated region (3'UTR)[5]. The 5'UTR consists of a short (S) fragment, a poly (C) tract, and a long fragment (5'LF-UTR), which contains three or four tandemly repeated pseudoknots and an internal ribosome entry site (IRES) [6]. The ORF encodes a polyprotein that can be cleaved to form four structural proteins (VP4, VP2, VP3 and VP1) and 8 non-structural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C and 3D) [6]. The 1D gene encodes VP1 protein which is the most surface-exposed capsid protein and contains three important immunogenic sites at amino acid

positions 40-60, 140-160 and 200-213 [7] as well as it contains the G-H loop spanning residues 134-158 contains conserved Arg-Gly-Asp (RGD) tripeptide, which is considered to be a ligand for cell-surface attachment [8]. VP1 plays an important role in virus attachment, protective immunity and serotype specificity. Nucleotide sequencing of VP1 has been used for molecular epidemiological studies on FMDV[9]. The FMDV serotypes subdivided into clades (topotypes) based on the sequence analysis of the VP1 structural protein gene which reflects genetic, antigenic and geographical relationships among strains [10]. In addition, FMDV 3A region has been implicated in virus virulence and host range similar to the 3A proteins of other Picornaviruses [11] the UTR of about 90 residues with a poly (A) tail (35-100nt) at 3'-end is likely to be a site of interaction with viral and host proteins for RNA replication [3].

In Egypt, between 1964 and 2005, serotype O was the most predominant reported while serotype A was firstly introduced in 1972. In 2006, widespread outbreaks, due to serotype A, as a result of importation of live infected cattle were reported[10]. During 2012, severe FMD outbreaks by SAT2 serotype were occurred for the first time in Egypt[12].

The rapid and accurate detection of FMDV is of ultimate importance. Clinical samples are routinely tested and serotyped in the OIE/FAO World Reference Laboratory for FMD (WRL), Pirbright, by combined use of virus isolation in cell culture and ELISA [13]. However, there have been limitations of the classical diagnostic techniques of

antigen detection ELISA (Ag ELISA)[14]. RT-PCR assays are alternative or are complementary to the classical serological and viral isolation methods due to their higher sensitivity and speed [15]. The nucleotide sequence and the phylogenetic tree based on the VP1 (1D) region of FMDV are widely used for the genetic relationships between strains[16].

The present study aimed to demonstrate the last 3 years situation of FMD in Egypt based on isolation, molecular identification and genotypic characterization of the isolated viruses to highlight the current status of the FMDV circulated in Egypt for effective containment of the disease and selection of a target vaccine.

Material and methods

Clinical samples

One hundred field samples (55 tongue&gum epithelial tissues and 45 vesicular fluids&saliva) were collected from FMD clinically infected cattle and buffaloes of various ages during the outbreaks from different centers in Beni-Suef governorate in Egypt during the period from April 2015 to March 2017. Infected cattle and buffaloes were shown oral and feet lesions. The collected samples were transported to the laboratory in transport medium with antibiotics and kept at -80 °C until used[17].

Extraction of Viral RNA using RNeasy Spin-Columns

RNA was extracted using ZYMO RESEARCH "ZR" Viral Kit™ (Catalog No. R1034) provided for rapid isolation of high-quality viral RNA from biological sources. Viral RNA extraction

was done according to the manufacturer's protocol.

Molecular detection and differentiation of FMDV serotypes by RT-PCR

RT-PCR was done using FMDV universal and type specific primers synthesized by Integrated DNA Technologies "IDT" (table 1). The RT-PCR was carried out using MyTaq™ One-Step RT-PCR Kit that has been formulated for highly reproducible first-strand cDNA synthesis according to the manufacturer's protocol (table 2) to perform the reverse transcription and the subsequent PCR in a single reaction tube. The reaction was done in 50 µl reaction volume, containing 5 µl RNA templates[18]. The PCR conditions in case of universal FMDV primers included reverse transcription step at 45°C (30 min.), initial denaturation step at 94 °C (2 min.), followed by 45 cycles of denaturing at 94 °C (20 sec.), annealing at 55 °C (30 sec.) for all primers, extension at 72 °C (60 sec.), and followed by completion of DNA synthesis at 72 °C (5 min.). Conditions were the same for the other serotypes SAT2, A and O except that annealing temperature that was 56°C for serotype O primers.

Agarose Gel Electrophoresis

The RT-PCR product was detected and purified by agarose gel electrophoresis using 2% (w/v) agarose gels in presence of 3µl of ethidium bromide (10 mg/ml) with reference to 100 bp DNA ladder. Loading dye (2µl) was mixed with 10 µl of PCR product. The technique was run at 100 volts for 60 min. The PCR products were visualized and photographed using UV-trans illuminator[19-21]. The positive sample

was recorded based on the appearance of expected size of bands in the gel.

Virus isolation

In Middle East for Veterinary vaccines (ME-VAC) research laboratory, established cell line of BHK-21 cells in 25T flasks were inoculated with 0.5 ml of the twenty-two samples of vesicular fluids representing the two groups according to PCR typing results (twenty samples were representative for A&O group and two samples were representative for SAT-2 group). The cell cultures were examined for Cytopathic effects (CPE) for 48 hours. Samples showed CPE were used for subsequent identification while samples of no CPE were subjected for another set of virus isolation. The infected BHK-21 monolayer cells were subjected to three freeze-thaw cycles to release the viral particles. The viral suspension was clarified from the cell debris by centrifugation at $800 \times g$ for 10 min and stored at -70°C for further examinations.[22].

Sequencing and phylogenetic analysis of selected RT-PCR products of FMDV isolated sample

The RT-PCR products were purified using QIAquickPCR purification Kit (Qiagen, Valencia, CA) as per manufacturer's instructions to remove primer-dimer and free nucleotides. The concentration of amplified genes was determined using NanoDrop-ND1000-Spectrophotometer (Thermo Scientific, Wilmington, USA). The purified PCR products were sequenced at (MACROGEN® Company, Korea) using the same forward and reverse primers as used in RT-PCR using ABI Prism BigDye terminator sequencing kit version 3.1 and analyzed with ABI Prism

3100 generic analyzer according to manufacturer's instructions. The obtained sequence was assembled by BioEdit software v. 7.0.9.0 and CLC sequence analyzer program, then homology search was done using BLAST search at NCBI database (www.ncbi.nlm.nih.gov/blst). Multiple sequence alignment and molecular phylogeny were constructed using MEGA7 software[23] using the Neighbor-Joining method and the evolutionary distances were computed using the Kimura 2-parameter method[19].

Results

Samples collection and molecular determination of FMD serotypes

All examined animals, from which samples were collected, were found to show the characteristic clinical picture of FMD. The results of universal RT-PCR of FMD showed that ninety-one samples (91%) of collected samples were positive which yield positive band at 328 bp after gel electrophoresis (**Figure 1**). Only 9% of collected samples were negative for universal FMD primers.

All positive samples by universal primers were typed using serotype-specific primers. The results revealed that 71 samples were positive for serotype O which yielded positive bands at 830bp and 402 bp for their both primers (**Figure 2 – 3**). Only 4 samples gave positive bands at 830 bp while gave no bands at 402 bp indicating their possible relatedness to serotype A. In the same time there were eighteen samples positive for SAT 2 specific primers and gave the specific bands at 715:730-bp (**Figure 4**). On the other hand, seven samples were un-typed by the used primers (**Table 3**) (**Figure 5**).

Virus isolation and identification.

Twenty samples were isolated on BHK-21 cells clone 13. Three samples out of twenty samples of molecular typed A&O groups were shown the characteristic CPE of FMDV after blind passage 4 times as shown in **Figure (6)**. However the molecular typed SAT-2 group were not isolated. Therefore, three strains of FMDV were isolated, the isolated virus were identified again by RT-PCR and sequencing.

Sequences manipulations and phylogenetic analysis

Sequences of VP1 coding-region of the three isolated FMD virus strain revealed that the three isolated virus strains from Beni-Suef governorate were serotype O. The phylogenetic analysis of the isolated virus strains revealed that they were closely related to type O isolates of those reported in Ismailia, Behira, Giza and Cairo during 2016 with identity ranged from 97.7% - 99.8% as illustrated in **table (4)**. The most relevant outboard isolate was SUD/8/2008 with 93% identity after Blast homology search. However, the phylogenetic tree

analysis revealed that the three FMDV type O isolates FMDV/serotype O/Beni-Suef/2017 differs partially from all other Egyptian serotypes of 2016 as shown in **figure (7)**. Nucleotide's alignments showed some point mutations at different positions especially when comparing of the isolated viruses with those of 2016 which is clarified in **figures (8 and 9)**.

- 1- Amino acid alignment of the three FMD isolated virus strains showed that RGD motif at amino acid residues 144–146 as well as the cysteine residue at the base of the G-H loops (position 134) was completely conserved as shown in **figure (10)**. Deduced amino acid sequence analysis of the major VP1 protein antigen epitope of type O FMDV demonstrated a high similarity existed in VP1 of the three identified isolates when they compared with those isolated during 2016. However some minor differences were observed especially at the amino acids 43, 45 and 46 in the isolated virus strain. The VP1 sequences have been submitted to GenBank and accession numbers were assigned to MF962874, MF962875 and MF962876.

List of tables:

Table 1:FMDV universal and serotype specific oligonucleotide primers for RT-PCR. These primers sets were designed according to [12, 37].

| Primer Name | Sequence 5'-3' | Gene | Position | Product Size (bp) | Purpose | Annealing Temp |
|------------------|--|-------|-----------|-------------------|---------------|----------------|
| 1F | 5'- GCCTGGTCTTTCCAGGTCT -3' | 5'UTR | 652-670 | 328 bp | Universal | 55° |
| 1R | 5'- CCAGTCCCCTTCTCAGATC -3' | 5'UTR | 958-976 | | | |
| A-1C612F | 5'- TAGCGCCGGCAAAGACTTTGA -3' | VP3 | 3173-3988 | 815- | Serotype | 55° |
| EUR-2B52R | 5'- GACATGTCCTCCTGCATCTGG TTGAT -3' | 2B | 3963-3988 | 830 bp | A&O | |
| P33 R | 5'-AGCTTGTACCAGGGTTTGGC-3' | 2B | 3832-3851 | 402 bp | Serotype O | 56° |
| P38 F | 5'-GCTGCCTACCTCCTTCAA-3' | 1D | 3451-3467 | | | |
| 1D209F | 5'- CCACATACTACTTTTGTGACC TGGA-3 | 1D | 3451-3475 | 715- | Serotype | 55° |
| 2B208 | 5'-ACAGCGGCCATGCACGACAG-3' | 2B | 4176-4195 | 730 bp | SAT-2 | |

| Reagent | Volume | Final concentration |
|--------------------------|-------------|---------------------|
| 2x MyTaq One-Step Mix | 25.0µl | 1x |
| Forward Primer (10µM) | 2.0µl | 400nM |
| Reverse Primer (10µM) | 2.0µl | 400nM |
| Reverse transcriptase | 0.5µl | - |
| RiboSafe RNase Inhibitor | 1.0µl | - |
| DEPC-H2O | Up to 45µl | - |
| Template | 5µl | - |
| Final volume | 50µl | |

Table3: Distribution of different FMDV serotypes during the periods 2015-2017 in Beni-Suef, Egypt.

| | Total Samples | Negative samples | FMD universal primer | Serotype O | Serotype SAT2 | Serotype A | Positive not detected serotype | Mixed O & SAT2 |
|-----------|---------------|------------------|----------------------|------------|---------------|------------|--------------------------------|----------------|
| 2015-2016 | 50 (100%) | 4 (8%) | 46 (92%) | 37 (74%) | 7 (14%) | 4 (8%) | 2 (4%) | 3 (6%) |
| 2016-2017 | 50 (100%) | 5 (10%) | 45 (86%) | 34 (68%) | 11 (22%) | --- (0%) | 5 (10%) | 5 (10%) |
| Total | 100(100%) | 9 (9%) | 91 (91%) | 71 (71%) | 18 (18%) | 4 (4%) | 7 (7%) | 8 (8%) |

Table 4: Similarity percent between different Egyptian isolates from different Governorates during the period 2015 - 2017.

| Seq-> | MF962874 | MF962875 | MF962876 | KX447138 | KX447132 | KX447135 | KX447125 | KX447122 | KX447121 | KX447134 | KX447130 |
|---|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| MF962874 FMDV - type O isolate Beni-Suef 7/Egy/2016 viral protein 1 gene, partial cds | | 98 | 97.9 | 99.8 | 99.8 | 99.6 | 99.6 | 99.5 | 99.5 | 99.2 | 99.2 |
| MF962875 FMDV - type O isolate Beni-Suef 8/Egy/2017 viral protein 1 gene, partial cds | 98 | | 99.8 | 98.2 | 98.2 | 98 | 98.4 | 98.2 | 98.2 | 97.9 | 97.9 |
| MF962876 FMDV - type O isolate Beni-Suef 10/Egy/2017 viral protein 1 gene, partial cds | 97.9 | 99.8 | | 98 | 98 | 97.9 | 98.2 | 98 | 98 | 97.7 | 97.7 |
| KX447138.1 FMDV - type O isolate Ismailia 11/Egy/2016 viral protein 1 gene, partial cds | 99.8 | 98.2 | 98 | | 100 | 99.8 | 99.8 | 99.6 | 99.6 | 99.3 | 99.3 |
| KX447132.1 FMDV - type O isolate Giza 2/Egy/2016 viral protein 1 gene, partial cds | 99.8 | 98.2 | 98 | 100 | | 99.8 | 99.8 | 99.6 | 99.6 | 99.3 | 99.3 |
| KX447135.1 FMDV - type O isolate Ismailia 9/Egy/2016 viral protein 1 gene, partial cds | 99.6 | 98 | 97.9 | 99.8 | 99.8 | | 99.6 | 99.5 | 99.5 | 99.3 | 99.2 |
| KX447125.1 FMDV - type O isolate Ismailia 2/Egy/2016 viral protein 1 gene, partial cds | 99.6 | 98.4 | 98.2 | 99.8 | 99.8 | 99.6 | | 99.5 | 99.5 | 99.2 | 99.2 |
| KX447122.1 FMDV - type O isolate El-Behira 2/Egy/2016 viral protein 1 gene, partial cds | 99.5 | 98.2 | 98 | 99.6 | 99.6 | 99.5 | 99.5 | | 100 | 99 | 99.3 |
| KX447121.1 FMDV - type O isolate El-Behira 1/Egy/2016 viral protein 1 gene, partial cds | 99.5 | 98.2 | 98 | 99.6 | 99.6 | 99.5 | 99.5 | 100 | | 99 | 99.3 |
| KX447134.1 FMDV - type O isolate Ismailia 8/Egy/2016 viral protein 1 gene, partial cds | 99.2 | 97.9 | 97.7 | 99.3 | 99.3 | 99.3 | 99.2 | 99 | 99 | | 98.7 |
| KX447130.1 FMDV - type O isolate Cairo 1/Egy/2016 viral protein 1 gene, partial cds | 99.2 | 97.9 | 97.7 | 99.3 | 99.3 | 99.2 | 99.2 | 99.3 | 99.3 | 98.7 | |

List of figures:

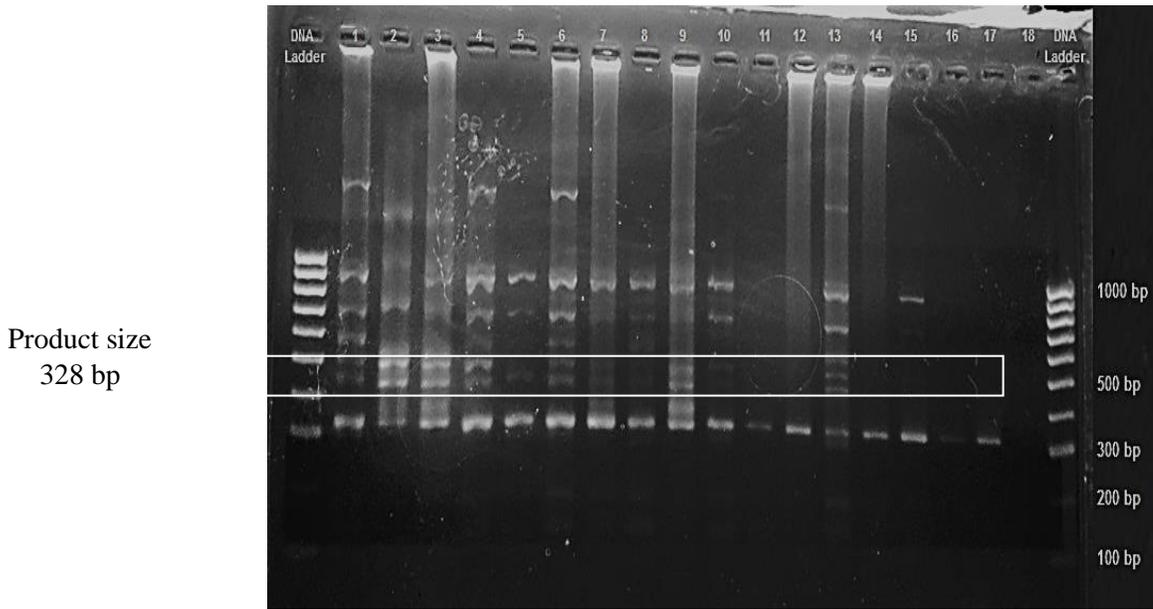


Figure 1: Agarose gel electrophoresis of RT-PCR products using universal primer (1F & 1R). Lanes 1-30 are tested PCR products flanked with 100 bp DNA ladder. For examples; lanes 1-15 gave positive bands of 328 bp while lanes 18 and 28 gave negative bands.

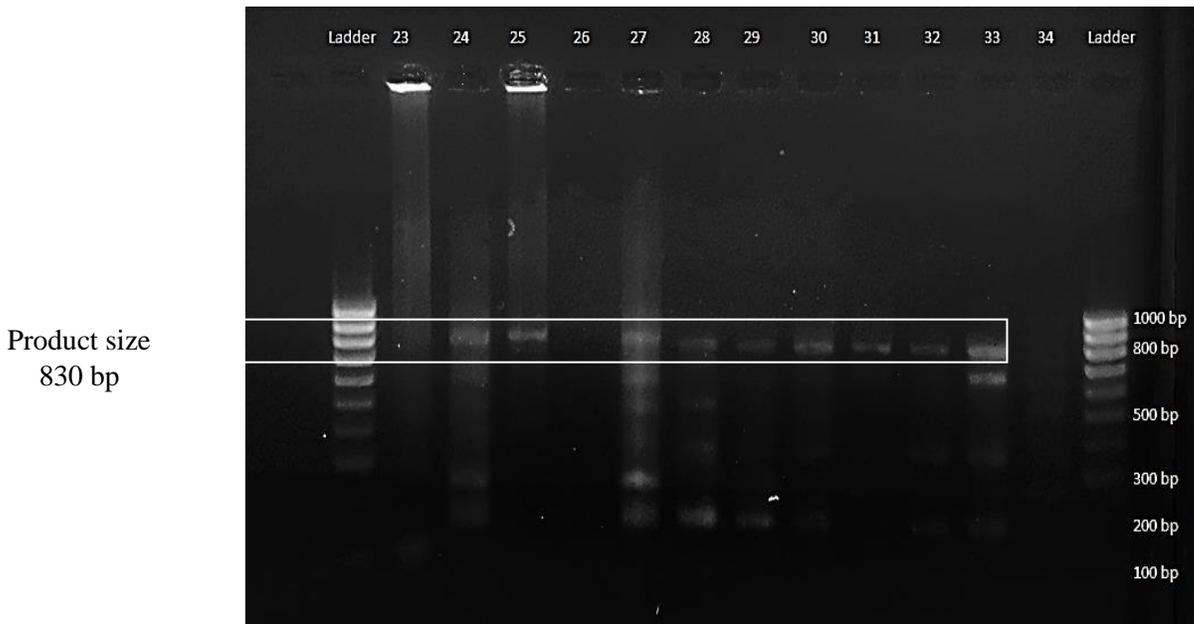


Figure 2: Agarose gel electrophoresis of RT-PCR products using specific primers for serotypes A & O compared with 100 bp DNA marker. For some samples, positive bands were appeared at 815-830 bp.

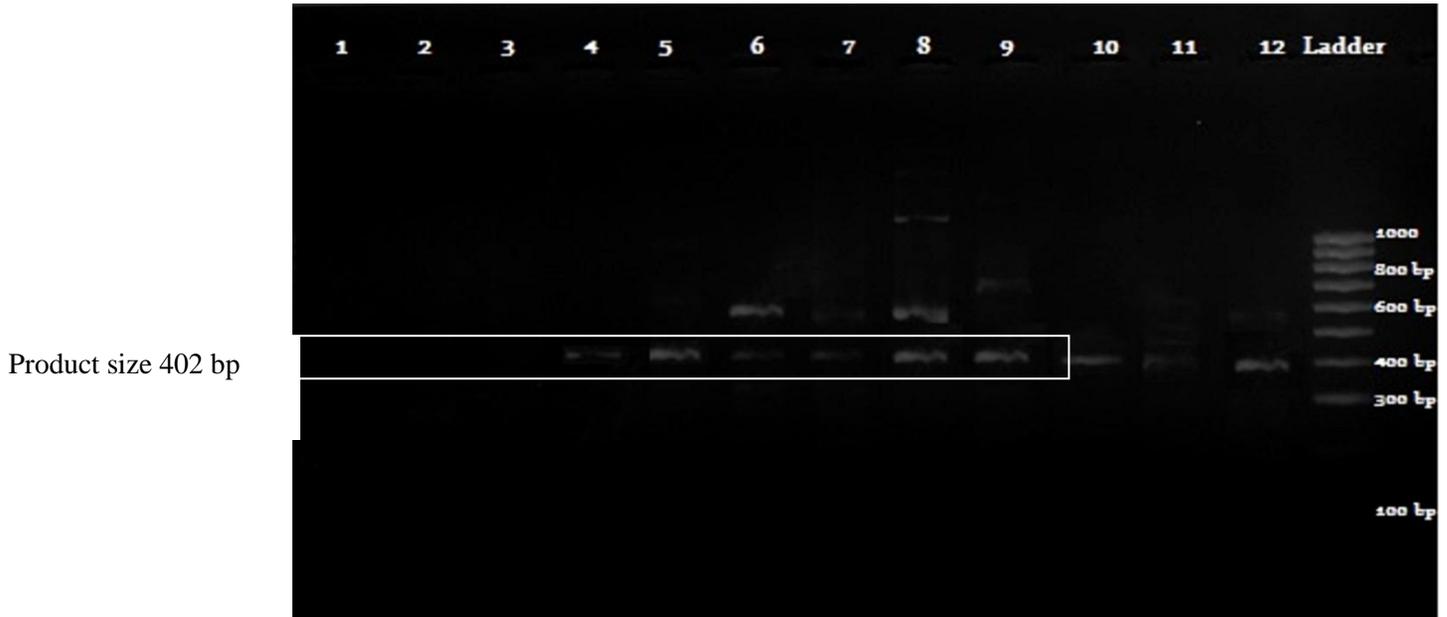


Figure 3: Agarose gel electrophoresis of RT-PCR products using specific primers for serotypes O compared with 100 bpDNA marker. For some samples, positive bands were appeared at 402 bp.

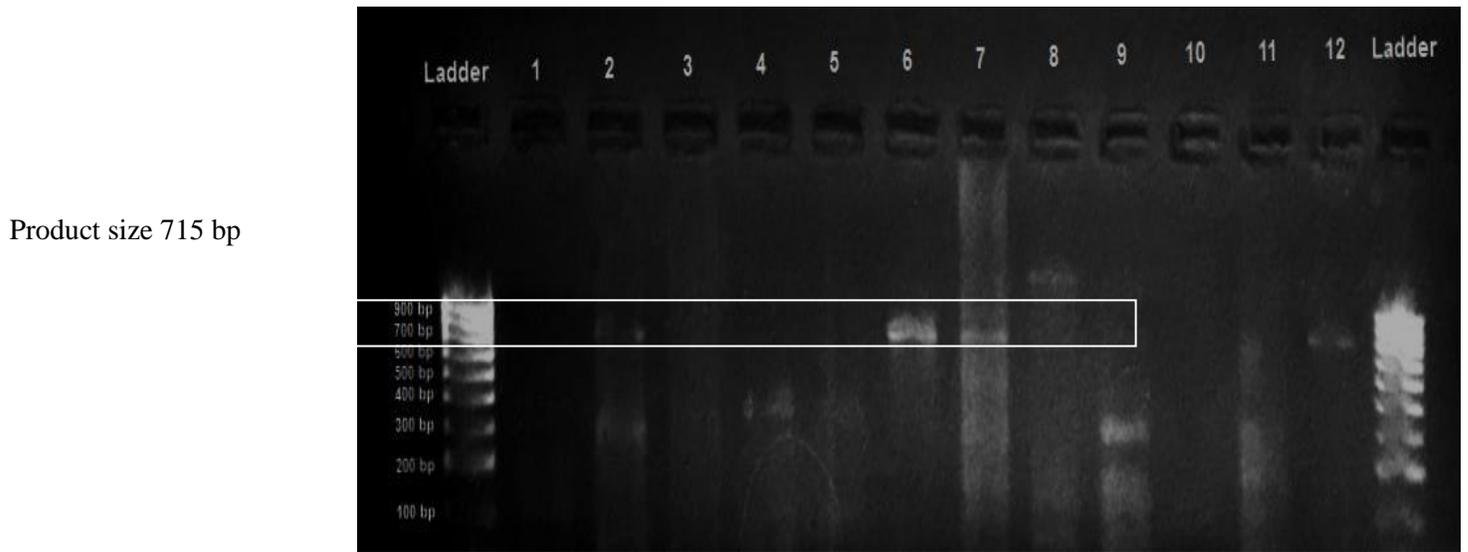


Figure 4: Agarose gel electrophoresis of RT-PCR products using Serotypes SAT-2 primers in presence of 100 bp DNA ladder. For examples, lanes 2, 6, 7 and 12 showed positive bands at 715 – 730 bp while lanes 1, 3, 4, 5 and 10 showed negative bands.

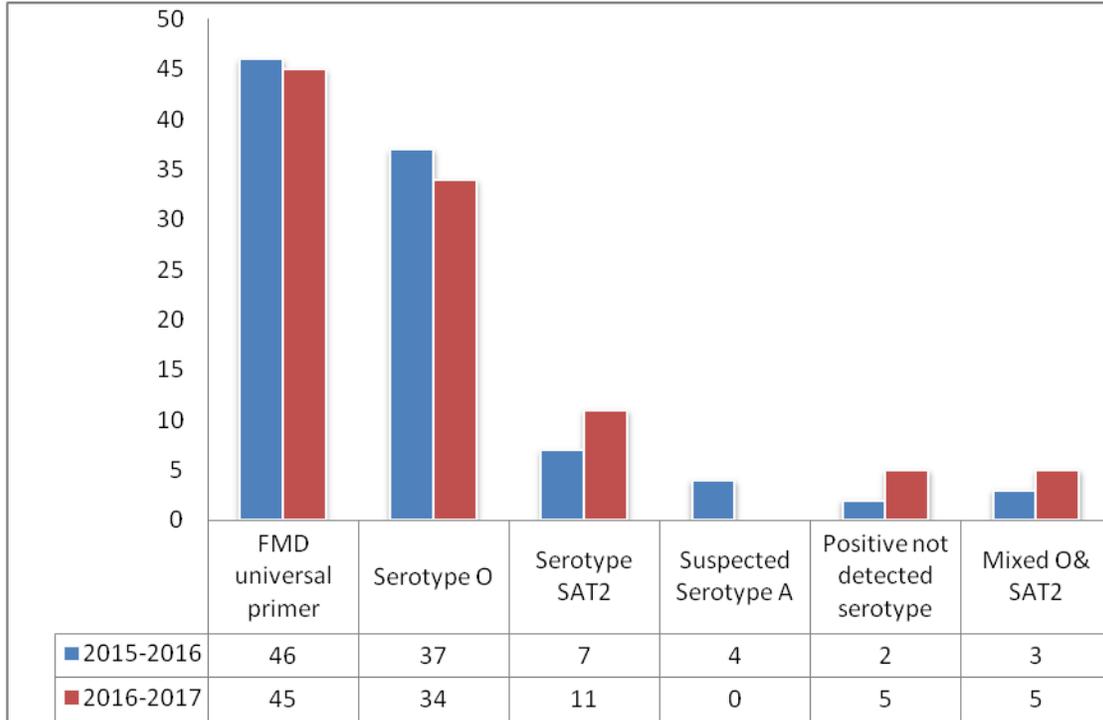


Figure 5: Comparative representation of FMD different serotypes distribution in between collected isolates in Egypt.

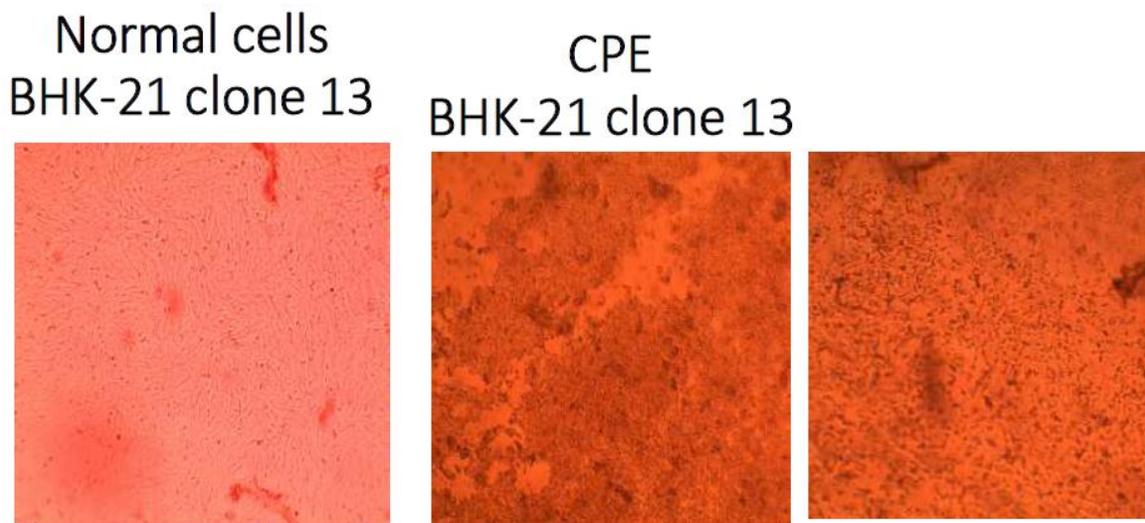


Figure 6: The effect of FMDV on BHK-21 cells inoculated with FMDV (Serotype O)

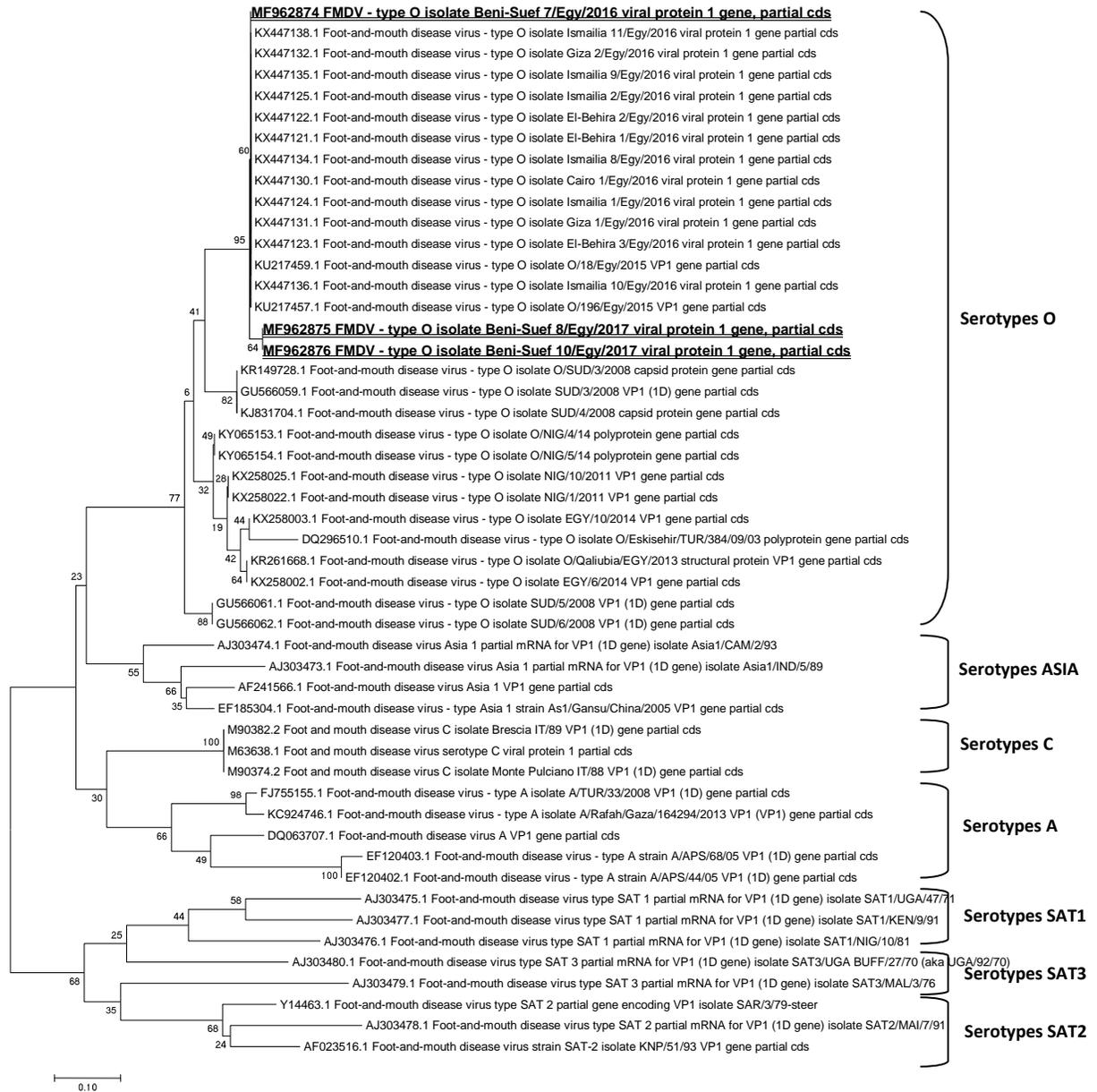


Figure 7:Phylogenetic tree of partial VP1 sequences of FMDV serotype O isolates from Egypt and other countries in compared with other FMDV serotypes. Phylogenetic relationship bootstrap trial of 1000 were conducted using MEGA version 7 using clustal W alignment algorithm and Neighbor-joining for tree construction [23].

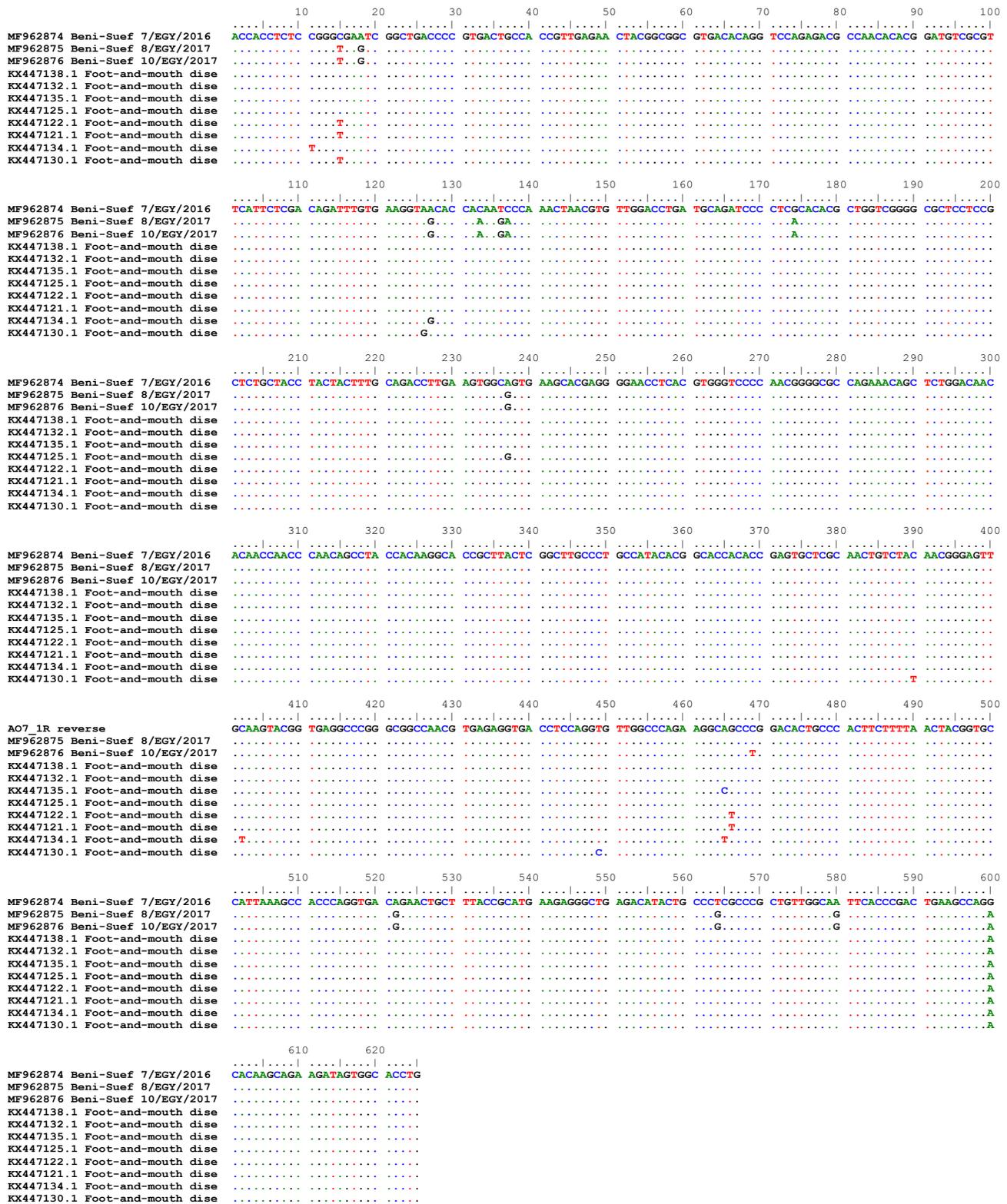


Figure8: Nucleotides multiple sequences alignment showing some point mutations in between Egyptian isolates.

| | | | | | | | | | | |
|--------------------------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |
| MF962874 Beni-Suef 7/EGY/2016 | ACCACCTCTC | CGGGCGAATC | GGCTGACCCC | GTGACTGCCA | CCGTTGAGAA | CTACGGCCGC | GTGACACAGG | TCCAGACAGC | CCAACACACG | GATTCGCGT |
| MF962875 Beni-Suef 8/EGY/2017 | | | | | | | | | | |
| MF962876 Beni-Suef 10/EGY/2017 | | | | | | | | | | |
| KX447138.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447132.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447135.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447125.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447122.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447121.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447134.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447130.1 Foot-and-mouth dise | | | | | | | | | | |
| | 110 | 120 | 130 | 140 | 150 | 160 | 170 | 180 | 190 | 200 |
| MF962874 Beni-Suef 7/EGY/2016 | TCATTCTCGA | CAGATTGTG | AAGTAAAC | CACAATCCCA | AACTAACGTG | TTGGACCTGA | TGCAGATCCC | CTCGCACAGC | CTGGTCGGGG | CGCTCCTCCG |
| MF962875 Beni-Suef 8/EGY/2017 | | | | | | | | | | |
| MF962876 Beni-Suef 10/EGY/2017 | | | | | | | | | | |
| KX447138.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447132.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447135.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447125.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447122.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447121.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447134.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447130.1 Foot-and-mouth dise | | | | | | | | | | |
| | 210 | 220 | 230 | 240 | 250 | 260 | 270 | 280 | 290 | 300 |
| MF962874 Beni-Suef 7/EGY/2016 | CTCTGCTACC | TACTACTTTG | CAGACCTTGA | AGTGGCAGTG | AAGCACCAGG | GGAACTCAC | GTGGTCCCC | AACGGGGCCG | CAGAAACAGC | TCTGGCAAC |
| MF962875 Beni-Suef 8/EGY/2017 | | | | | | | | | | |
| MF962876 Beni-Suef 10/EGY/2017 | | | | | | | | | | |
| KX447138.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447132.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447135.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447125.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447122.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447121.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447134.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447130.1 Foot-and-mouth dise | | | | | | | | | | |
| | 310 | 320 | 330 | 340 | 350 | 360 | 370 | 380 | 390 | 400 |
| MF962874 Beni-Suef 7/EGY/2016 | ACAACCAACC | CAACAGCCTA | CCACAAAGCA | CCGTACTACT | GGTTGGCCCT | GCCATACACG | GCACCACACC | GAGTGCCTCC | AACTGCTTAC | AACGGGAGTT |
| MF962875 Beni-Suef 8/EGY/2017 | | | | | | | | | | |
| MF962876 Beni-Suef 10/EGY/2017 | | | | | | | | | | |
| KX447138.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447132.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447135.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447125.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447122.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447121.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447134.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447130.1 Foot-and-mouth dise | | | | | | | | | | |
| | 410 | 420 | 430 | 440 | 450 | 460 | 470 | 480 | 490 | 500 |
| AO7_1R reverse | GCAAGTACGG | TGAGGCCCGG | GCGGCCAACG | TGAGAGTGA | CCTCCAGGTG | TTGGCCAGAG | AGGCAGCCCG | GACACTGCCC | ACTTCTTTTA | ACTACGGTGC |
| MF962875 Beni-Suef 8/EGY/2017 | | | | | | | | | | |
| MF962876 Beni-Suef 10/EGY/2017 | | | | | | | | | | |
| KX447138.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447132.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447135.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447125.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447122.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447121.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447134.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447130.1 Foot-and-mouth dise | | | | | | | | | | |
| | 510 | 520 | 530 | 540 | 550 | 560 | 570 | 580 | 590 | 600 |
| MF962874 Beni-Suef 7/EGY/2016 | CATTAAGGCC | ACCCAGGTGA | CAGAATGCT | TTACCCATG | AAGAGGGCTG | AGACATAGT | CCCTCGCCCG | CTGTTGGCAA | TTCACCCGAC | TGAGGCCAGG |
| MF962875 Beni-Suef 8/EGY/2017 | | | | | | | | | | |
| MF962876 Beni-Suef 10/EGY/2017 | | | | | | | | | | |
| KX447138.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447132.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447135.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447125.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447122.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447121.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447134.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447130.1 Foot-and-mouth dise | | | | | | | | | | |
| | 610 | 620 | | | | | | | | |
| MF962874 Beni-Suef 7/EGY/2016 | CACAAGCAGA | AGATAGTGGC | ACCTG | | | | | | | |
| MF962875 Beni-Suef 8/EGY/2017 | | | | | | | | | | |
| MF962876 Beni-Suef 10/EGY/2017 | | | | | | | | | | |
| KX447138.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447132.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447135.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447125.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447122.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447121.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447134.1 Foot-and-mouth dise | | | | | | | | | | |
| KX447130.1 Foot-and-mouth dise | | | | | | | | | | |

Figure10: Amino acids multiple sequence alignment showing antigenic alteration in between 2017 isolates at sites 43, 45 and 46 when compared with 2016 isolates

Discussion

FMD is an endemic disease in Egypt and usually occurs as recurrent outbreaks causing huge economic losses. A precise and rapid diagnosis was a mandatory requisite to avoid more economic losses. From April 2015 till, March 2017 susceptible animals in Egypt suffered from many FMD outbreaks, so the present study aimed to isolate and molecularly type of FMDV which is the causative agent of these outbreaks in Beni-Suef governorate in Egypt. One hundred samples were collected for isolation and molecular typing of the isolated FMDV which have caused wide-spread outbreaks in Egypt during the period of the study.

FMD clinical signs in diseased animals include fever, rosy salivation, vesicles and erosions in gums, dorsum of the tongue and in the interdental spaces [24].

The accurate and early diagnosis of infected animals is essential for effective control of FMDV. Rapid and sensitive diagnosis depend upon detection of viral nucleic acid or virus isolation [25]. Several molecular techniques were used for diagnosis of FMD outbreaks such as RT-PCR for detection of FMD virus genomic RNA in cell culture fluids or field samples [26, 27].

In this study, clinically positive FMD samples were subjected to the molecular identification using RT-PCR based on universal primer set at 5'UTR with specific band appeared at 328 bp to detect the FMDV regardless to the serotype; followed by specific primers for each serotype present in Egypt including serotype O, SAT2 and A. The obtained results revealed that 91% of the examined samples were positive for universal FMDV.

From the presented results it was reported that conventional PCR was a very effective confirmatory diagnostic tool and this agreed

with the results obtained by Knowles and Samuel [10]. Serotype O was the most common serotype isolated during last outbreaks in Beni-Suef Governorate, while a few were found to be serotype SAT2. Predominance of FMDV serotype O in this study supports that serotype O is still endemic in Egypt from the first detection in Egypt since 1964 and still circulated in different governorates of Egypt [28, 29]. These findings might be related to several causes; one of them is related to the insufficiency of vaccination program. Also genetic mutation of the virus may result in a new antigenic structure which can be escaped from the animal immune system, especially with no cross protection in between the different serotypes of FMDV [3].

Some samples were positive for the universal primer but negative for other serotypes primer sets, this might be attributed to different possibilities, one of which might be simple point mutations at a critical site leading to failure of the primer to bind to the viral nucleic acid [26] at which the success in PCR depends mainly on the efficiency of the primer and template to bind and amplify [30] especially when high nucleotide mutation rate is a common character of FMDV [31].

The amplification of whole VP1 coding region was followed by direct sequencing in order to stand upon the recent changes in nucleotide sequence of VP1 coding region. Representative serotype specific cDNA amplicon was sequenced and subjected to multiple nucleotide sequence alignment against other related FMDV in the GenBank database. The sequences were first aligned using the clustal W (1.82) program and the phylogenetic analysis were performed. Phylogenetic analysis of the VP1 region of FMDV has been used extensively to investigate the molecular epidemiology of the disease worldwide. These techniques have assisted in studies of the genetic relationships between different FMDV isolates, geographical

distribution of lineage & genotype, the establishment of genetically and geographically linked topotypes and tracing the source of the virus during outbreaks[32].

The nucleotide sequence of VP1 region of serotype O isolated from Beni-suef during 2016 showed a high similarity to those isolated and reported in 2016 from Ismailia, Behira, Giza and Cairo, while those isolated during 2017 from Beni-suef showed a slight difference than those isolated during 2016 and the minor variation of sequences of isolated FMDV in this study is agreed with Bronsvoort et al., [33] who reported that VP1 capsid protein of FMDV alone had provided valuable insight into the emergence of various strains and serotypes worldwide. The nucleotide sequence of VP1 of serotype O from 2016 and 2017 isolates showed great similarity with other type O Egyptian isolates in 2016 but relatively far from FMDV type O isolate SUD/8/2008 (identity 93%) obtained by blast of nucleotide sequence in Gene bank, and this was confirmed by the neighbor joining phylogenetic analysis including different mutations from Sudan isolates. These changes explain why animals acquired infection smoothly as they didn't have previous antibodies against this serotype in presence of no cross-reaction between them. Phylogenetic tree revealed that FMDV type O isolate/VP1/Beni-Suef/2017 has branched differently from all other Egyptian serotypes especially those of 2016.

These isolates also branched far away from type O isolate SUD/8/2008, revealing that the isolate of our study differs from all previously serotypes that were detected in Egypt before 2016. So it is advisable to include these isolates in the vaccine production to induce complete protection against circulating viruses. These results are in agreement with [34] who found that viruses which are endemic appear to evolve more slowly. These viruses evolve is crucial to interpreting the genetic relationships used to infer virus phylogeny and molecular epidemiology. It has been used to individually

characterize strains of FMDVs in **figures (7, 8)** and track their movement across international trade.

Isolation of FMDV was done on BHK21 cell line because they are susceptible for FMDV and most desirable for diagnostic system and resulted in CPE in the form of rounding of cells, granularity of the cytoplasm and complete cell lysis [35]. These results reported that virus isolation on BHK-21 cells is the most reliable diagnostic method [36]. Mixed infection (O and SAT 2) was observed in 3 samples during outbreak 2016 and in 5 samples in outbreak 2017 as shown in **figure (5)**.

Conclusion

FMDV serotype O is the main prevalent serotypes in this report in Beni-Suef governorate, Egypt with minor nucleotide variation was observed as shown in the amino acids multiple sequence alignment that showing antigenic alteration in between 2017 isolates at sites 43, 45 and 46 when compared with 2016 isolates. Also, subtypes A and SAT 2 were detected and mixed infection with subtype O and SAT 2 is considered a dangerous line of infection. The current FMD situation may give an indication about the degree of efficiency of vaccination campaigns. Further works are essential to explain the exact serotype of the samples that gave negative results with different primer sets used in this study.

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