Genetic identification of the foot-and-mouth disease virus caused 2006 outbreak in Egypt

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ABSTRACT

In early February 2006, a foot-and-mouth disease (FMD) outbreak has struck cattle and buffaloes in different localities of Egypt exerting severe economic losses to livestock industry. Representative specimens (tongue epithelium and foot vesicular fluid) were collected from severely infected foreign (imported from Ethiopia) and local cattle in different governorates (Ismailia, Sharqia and Behairah). Several assays of reverse transcription (RT) using random decamer primers, followed by FMDV VP1- based polymerase chain reaction (PCR), were used for rapid identification of the causative agent in clinical specimens, basically to circumscribe the countrywide spread of infection. The first PCR assay, utilizing a FMDV universal primer set, could identify the outbreak causative agent as a FMDV in all clinical specimens. FMDV specific primers were then utilized to determine the outbreak FMDV serotype. The specific PCR amplification products (amplicons) were purified and subjected to direct nucleotide sequencing. Blast searches, multiple alignments and phylogenetic analyses of the nucleotide sequence data revealed that outbreak FMDV is a serotype "A" which is a new serotype incursion to Egypt. Direct sequencing of the PCR amplicons was proved a relevant discriminative tool for genetic characterization of FMDV strains / isolates. Results of this endeavor initiated the potential to produce a bivalent FMDV vaccine, containing both of serotypes A and O_1, for the first time in Egypt.

Key words: *Foot -and-mouth disease– FMDV–RT-PCR – Nucleotide sequence–VP1 – Phylogeny.*

INTRODUCTION

For centuries, foot-and-mouth disesase virus (FMDV) has been known as one of the most fearful viral pathogens of animals since it is highly contagious among all species of cloven-hoofed animals (*Bachrach*, 1968; *Carrillo et al.*, 2005). FMDV is distributed worldwide and can be transmitted by direct and indirect contacts via multiple routes including wind-borne transmission, exerting a severe global economic impact on the livestock industry (*Alexandersen et al.*, 2000; *Sakamoto et al.*, 2002). FMDV infection is typically characterized by a rapid febrile illness with development of massive vesicular lesions in the mouth and on the feet. Morbidity of disease is nearly 100% among non-vaccinated animals. Although the disease is rarely fatal in adult animals (< 5%); mortality in young animals may reach 50%, due to myocardial collapse (*Sard,* 1978). FMDV is a small, non-enveloped RNA virus that is classified as a member of the *aphthovirus* genus within the family *picornaviridae* (*Hedger*, 1981; *Carrillo et al.*, 2005). FMDV

particles are naked icosahedrons comprising 60 copies each of four structural proteins VP1-4, encapsidating the viral genome which consists of an infectious molecule of single-stranded, positive-sense ribonucleic acid (RNA). The protein coding region is a continuous open reading frame of 6915 or 6999 nucleotides in length depending on which of two functional in-frame start codons is utilized. A polyprotein is synthesized from genomic RNA and processed by viral proteinases into four primary cleavage products, non-structural proteins (NSP) leader, Lab and Lb; Structural proteins (SP) P1: P1A, 1B, 1C and 1D equivalent to VP4, VP2, VP3 and VP1, respectively; NSP P2: (P2A, 2B and 2C); and NSP P3: P3A, P3B or VPg, P3C, and P3D (*Lewis et al.*, 1991; *Knipe et al.*, 1997).

Antigenically, FMDV is recognized as seven distinct serotypes namely: A, O, C, and Asia 1 (Euroasiatic serotypes), SAT1, SAT2, and SAT3 (South African Territories serotypes). Besides, there are considerable antigenic and genetic diversities within each serotype resulting in numerous subtypes (*Carrillo et al.*, 2005). Thus, an animal recovered from infection with one serotype becomes resistant to challenge by the same serotype but remains susceptible to infection by any other serotype (*Belsham*, 1993). Detection of FMDV with identification of the viral serotype must be rapid, sensitive and specific for effective disease control and proper selection of vaccines (*Reid et al.*, 1999). FMD can not be easily distinguished from other vesicular diseases by clinical findings only due to similarity of the clinical signs (*Alexandersen et al.*, 2000). Conventional laboratory diagnosis of FMD is achieved by concurrent cell culture virus isolation together with the enzyme-linked immunosorbent assay (ELISA) which indicates the type of viral antigen involved (*Roeder et al*, 1987; *Ferris et al.*, 1988; *Mackay et al.*, 2001) or by the virus neutralization test (*Golding et al.*, 1976). An appropriate and reliable test has to be able to detect all types of FMDV for rapid and precise diagnosis (*Clavijo et al.*, 2003). The reverse transcription - polymerase chain reaction (RT-PCR) assay has been proved rapid, specific and sensitive for detection and typing of the FMDV as well as for differentiating it from other vesicular diseases (*Marquardt et al.*, 1995; *Callens* and *De Clercq*, 1997; *Callens et al.*, 1998). Several reports described use of a wide range of primers in RT-PCR assays, utilizing specific viral genomic sequences as targets, for detection of FMDV with high sensitivity within 24 h in a wide range of specimens (*Reid et al.*, 1999).

In Egypt, since 1950s attention was drawn to economic importance of FMD after several outbreaks attacked cattle, buffaloes, sheep, goats and camels with predominant isolation of FMDV subtype O_1 (*Tantawi et al.*, 1984; *Daoud et al.*, 1988). Therefore, inactivated vaccines containing only FMDV serotype O_1 have been in use to immunize nearly all animals. In our region, the possible introduction of new strains of FMDV by the uninterrupted movement of animals across borders is a major constant threat. In February 2006, a severe FMD outbreak struck the vaccinated cattle and buffaloes in different Egyptian governorates causing enormous economic losses. Many confusing arguments have arisen about this invasive FMDV that needed to be solved.

The key objective of this endeavor is to genetically identify the causative outbreak FMDV using different RT-PCR assays followed by comparative nucleotide sequence analyses, as a basic step before being involved in our local vaccine production. That would secure proper vaccine formulation to confer appreciated protection of our livestock and to

control spread of this FMDV, recently intruded to Egypt.

MATERIALS AND METHODS

Clinical specimens

Representative specimens were collected form severely infected foreign (imported from Ethiopia) and local cattle in different governoates (Ismailia, Sharqia and Behaira) of Egypt, during the period of the 2006 outbreak (February and March). Main clinical symptoms observed were: profuse salivation, severe tongue and foot erosions, and lameness. The specimens were transported in glycerol buffer (50% glycerol analar and 50% PBS; pH 7.2). Part of these specimens was used for direct RNA extraction and the other was used for virus isolation on primary cell culture (IBRS-2), following the procedure described by *Woodbury et al.* (1994).

Virus and cells

The local vaccinal $FMDVO_1$ was used in this study as a positive virus control. FMDV was propagated on BHK_{21} clone 13 cell cultures. The IBRS-2 primary cell culture was used for FMDV isolation from clinical specimens. Both BHK_{21} and IBRS-2 cells (Institute for Animal Health, Pirbright, UK) were grown and maintained in minimum essential medium with Eagle's salts (MEME) supplemented with heat-inactivated 5% normal bovine serum (BCS), 100 U/ml penicillin and 100 µg/ml streptomycin, serum-free medium was used during virus growth (*Woodbury et al.*, 1994).

RNA extraction

RNA was extracted directly from the epithelial tissues by using RNeasy Mini kit (QIAGEN GmbH, Hilden, Germany) as per manufacture's instructions. RNA was eluted in RNase-free water and stored at – 80°C after addition of RNase inhibitor (Q-Biogene, USA). Additionally, the viral genomic RNA was extracted directly from the vesicular fluid and from clarified virus culture supernatants (from local vaccinal $FMDVO_1$ and IRBS-2 cells inoculated with processed clinical specimens) by using QIAamp viral RNA kit (QIAGEN GmbH, Hilden, Germany) according to the manufacture's instructions. RNA was eluted in 60µl RNase –free water and kept at – 80°C after addition of RNase inhibitor.

Oligonucleotide primers

Different primer sets were used for specific detection and serotyping of FMDV in the outbreak specimens. First, a specific FMDV universal primer set was used to verify whether the outbreak etiological agent was a FMDV or any other vesicular virus. The universal primers namely P1/P2 were selected to amplify a 216 bp cDNA fragment within sequences encoding for VP1 gene from any FMDV, regardless of its serotype (*Amaral-Doel et al.*, 1993). Second, *OIE/FAO*-cited FMDV serotype-specific primer sets were chosen according to *Knowles* and *Samuel* (1998) to amplify cDNA fragments within the coding sequences of 1D and 2AB region of FMDV genome. Primer set, SAT-1D209F and FMD-2B208R, is specific to detect any SAT serotype of FMDV, amplifying a cDNA fragment of approximately 730, 715 and 718 bp for serotypes SAT1, SAT2 and SAT3, respectively. Forward primers A- $1C_{612}$, C-$1C_{536}$,O- $1C_{124}$ and As1-$1C_{505}$ and the universal reverse primer FMD-$2B_{58}$ (NK61) produce RT-PCR amplification products of about 813-816, 877-883, 1301 and 908-914 bp for serotypes A, C, O and Asia 1, respectively. Primers sequences are presented in Table (1).

Reverse transcription (RT) and first strand cDNA synthesis

First strand complementary DNA (cDNA) was synthesized from all extracted

RNA samples using Reverse-iT™ 1st strand synthesis kit (AB-gene, Epsom, Surrey, UK). RNA (1 µg) was mixed with 400 ng random decamer primers and incubated at 70°C for 5 min, to remove any secondary structure, and then placed on ice. First strand synthesis buffer (50mM Tris-HCl pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, 0.5 mM TTP), dNTPs mix (10 mM each), 100 mM DTT and 50 U Reverse iT ™ RTase Blend enzyme were added to the RNA-primer mix and incubated at 47°C for 50 min as the manufacturer's instructions. The temperature was then elevated to 75°C for 10 min to inactivate the Reverse iT ™ RTase Blend enzyme.

Polymerase chain reaction (PCR) amplification

Following first strand cDNA synthesis, the PCR amplification was carried out with RED Taq™ Ready Mix™ PCR reaction mix (Sigma, USA) by using 2 µl of cDNA and 100 pmol of each forward and reverse primers; 25 µl of 2X RED Taq ready mix buffer (20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM Mg Cl_2, 0.002% gelatin, 0.4 mM dNTPs mix and 0.06 unit/µl of Taq DNA polymerase) and nuclease-free water to 50 µl total reaction volume. The thermal profile for PCR was 94°C for 3 min for initial denaturing, followed by 35 cycles of 94°C for 1 min, 60°C (for serotype O and C) or 55°C (for serotypes A, Asia and SAT) for 1 min, and 72°C for 2 min. The program was ended with a final extension cycle at 72°C for 10 min.

Analysis of RT-PCR amplification products (cDNA amplicons)

The RT-PCR cDNA amplicons were analyzed by 1.5% agarose gel electrophoresis in Tris acetate-EDTA buffer (TAE, 0.04 M Tris acetate, 1 mM EDTA), stained with ethidium bromide (0.5µg/ml) and visualized on UV transilluminator. A 100 bp DNA ladder (Invitrogen, Karlsruhe, Germany) was used to comparatively determine the molecular size of the PCR amplicons.

Direct sequencing of RT-PCR cDNA amplicons

The cDNA amplicons were purified from agarose gels using Ultra agarose spin kit (ABgene, Epsom, Surrey, UK) and directly sequenced in both directions with the same primers used to generate PCR amplicons. Sequencing was done in an ABI PRISM system using the dideoxy chain-termination method (*Sanger et al.*, 1977), based on the incorporation of fluorescent-labeled dideoxynucleotide terminators. The identity of each nucleotide was verified at least twice.

Computer-assisted sequence and phylogenetic analyses

The resulted nucleotide sequence data of the selected cDNA PCR amplicons were compiled and compared to each other. Further, nucleotide sequence representing the sequence majority of the outbreak causative FMDV was compared to those of other related FMDVs accessed by blast search for sequence homology via the GenBank database. The nucleotide sequences were aligned by the Clustal W (1.82) program (*Thompson et al.*, 1994) of European Bioinformatics Institute (EBI, EMBL). Clustal W is a fully automatic program for global multiple alignment of DNA sequences. Phylogenetic correlation and tree construction were done using the PHYLIP and Treeview 32 (1.6.6) programs (*Page*, 1996; *Felsentein*, 2001). All programs used in this study were accessed through their interactive web services.

Table (1): Oligonucleotide primers used for RT-PCR of foot -and -mouth disease virus.

Name	Primers sequence (5` to 3`)	Serotype specificity	Location on genome
P1	CCTACCTCCTTCAACTACGG	Universal-F	VP1
P2	GAAGGGCCCAGGGTTGGACTC	Universal-R	VP1
SAT-1D209F	CCACATACTACTTTTGTGACCTGGA	SAT-F	VP1
FMD-2B208R	ACAGCGGCCATGCACGACAG	SAT-R	2B
O- $1C_{124}$	ACCAACCTCCTTGATGTGGCT	O-F	1C
A- $1C_{612}$	TAGCGCCGGCAAAGACTTTGA	A-F	1C
C- $1C_{536}$	TACAGGGATGGGTCTGTGTGTACC	C-F	1C
As1-$1C_{505}$	TACACTGCTTCTGACGTGGC	Asia 1-F	1C
FMD-$2B_{58}$(NK61)	GACATGTCCTCCTGCATCTG	O,A,C,As1-R	2B

Table (2): Identity score table of multiple nucleotide sequence alignment of cDNA amplicons produced by RT-PCR using specific primers for FMDV serotypes A, C and SAT from outbreak (Egy2006) isolates using CLUSTAL W (1.82) program.

Seq. A Name	Len. (nt)	Seq. B Name	Len. (nt)	Identity (%)
Egy2006_SAT	691	Egy2006_C	858	75
Egy2006_SAT	691	Egy2006_A	574	98
Egy2006_C	858	Egy2006_A	574	82

Seq. = sequence **Len. (nt)** = length in nucleotides **Score (%)** = sequence similarity percent

Table (3): Identity score table of multiple nucleotide sequence alignment of the majority nucleotide sequence of outbreak (Egy2006) isolates versus those of other related FMDV serotypes using CLUSTAL W (1.82) program.

Seq. A Name	Len. (nt)	Seq. B Name	Len. (nt)	Identity (%)
Egy2006_ Majority	481	SAT2Ken_Kenya	960	58
Egy2006_ Majority	481	A21kenya_iso77	904	85
Egy2006_ Majority	481	A24Cruzeiro_Brazil55	734	73
Egy2006_ Majority	481	A3mecklenburg_iso81Germany	920	82
Egy2006_ Majority	481	Asia13kimron_iso61Israel	780	67
Egy2006_ Majority	481	C3indiso19_Brazil	840	71
Egy2006_ Majority	481	OGolaniso81_Israel	639	49
Egy2006_ Majority	481	O_1Sharquia72_EGYPT	639	50

Seq. = sequence **Len. (nt)** = length in nucleotides **Score (%)** = sequence similarity percent

RESULTS

The viral RNA was successfully extracted from specimens of tongue epithelia, vesicular fluids and virus culture supernatants and was utilized for reverse transcription (RT) using random decamer primers, followed by the polymerase chain reaction (PCR). The universal primer set amplified a cDNA fragment of 216 bp equivalent to the expected amplifi-cation product (amplicon) size from any FMDV genome. All specimens were positive for the FMDV universal primer set and yielded similar cDNA amplicons at the size of 216 bp, while no amplicons could be observed with any negative control (Fig. 1). Subsequently, it was certain that these specimens contained a FMDV. To identify this FMDV, several PCRs were conducted utilizing different FMDV type-specific primer sets. From each tested sample, specific cDNA amplicons equivalent to the expected sizes were amplified using serotype-specific primers for serotypes A, C and SAT, manifested as discrete bands at the molecular sizes of about 860 bp, 880 bp and 710 bp, respectively. The serotype O_1 was utilized as a control with all serotype-specific primer sets and yielded

amplicons of the right expected size, while no amplicons could be obtained with primer sets specific for neither Asia 1 nor O serotypes (Fig. 2).

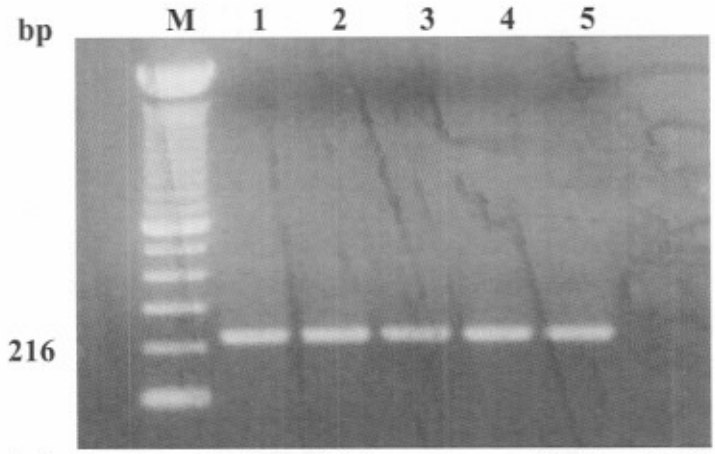

Fig.(1) : Agarose gel electrophoresis of the PCR amplicons of outbreak 2006 FMDV utilizing FMDV VP1 universal primers (P1 & P2), separated on 1.5 % agarose gel and stained with ethidium bromide. Lanes: (M) 100 bp DNA Ladder (Gibco-Invitrogen); (1) Control FMDVO$_1$ cDNA with specific FMDV "universal" primers; (2-5) Outbreak cDNA with the specific FMDV universal primers (~ 216 bp).

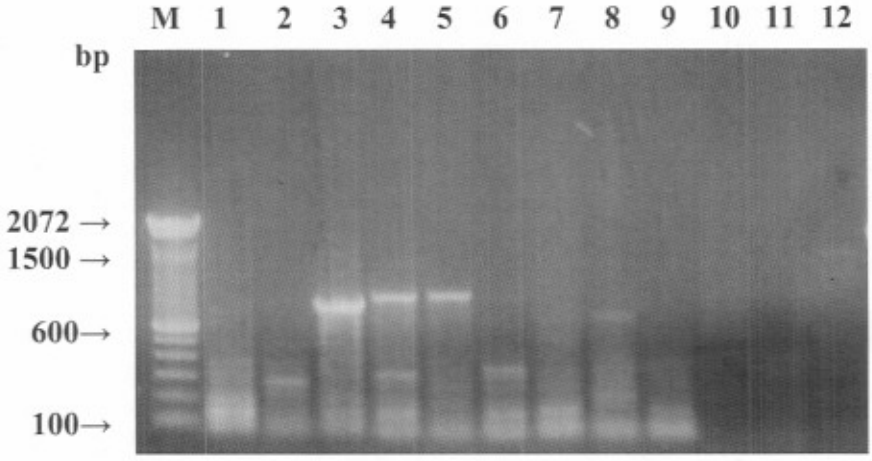

Fig.(2) : Agarose gel electrophoresis of the PCR amplicons of outbreak 2006 FMDV utilizing different FMDV serotype-specific primers, separated on 1 % agarose gel and stained with ethidium bromide. Lanes: (M) 100 bp DNA Ladder (Invitrogen); (1, 2, 6 and 9) control FMDVO$_1$ cDNA with the specific primers for Asia1, A, C and SAT, respectively (no amplicon); (3) outbreak cDNA with specific primers for A (~ 860 bp); (4&5) outbreak cDNA with specific primers for C (~ 880 bp); (7&8) outbreak cDNA with specific primers for SAT group (weak of > 700 bp); (10) outbreak cDNA with specific primers for Asia 1 (no amplicon); 11: outbreak cDNA with specific primers for O (no amplicon); (12) control FMDVO$_1$ RNA with the specific primers for O (~ 1301bp).

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Egy2006_Aprimer      ----------------------------A-CGGTA-CCCCTGAAAGCGCT 20
Egy2006_STAprimer    GC-TGAGG---ACCTGAC-TGGGTGCCCA-CGGTA-CCCCTGAAAGCG-T 75
Egy2006_Cprimer      GCATGAAGGTAACCTGACCTGGGTGCCCAACGGTAACCCCTGAAAGCGCT 400
                                                * ***** ************ *

Egy2006_Aprimer      CTCCAGAACGCGAGCAACCCCACGGCCTACCACAAGGCCCCGTTCACCAG 70
Egy2006_STAprimer    CTCCAGAACGCGAGCAACCCCACGGCCTACCACAAGGCCCCGTTCACCAG 125
Egy2006_Cprimer      CTCCAGAACGCGAGCAACCCCACGGCCTACCACAAGGCCCCGTTCACCAG 450
                     **************************************************

Egy2006_Aprimer      ACTTGCACTGCCTTACACCGCACCACACCGCGTGCTGGCTACGGTGTACA 120
Egy2006_STAprimer    ACTTGCACTGCCTTACACCGCACCACACCGCGTGCTGGCTACGGTGTACA 175
Egy2006_Cprimer      ACTTGCACTGCCTTACACCGCACCACACCGCGTGCTGGCTACGGTGTACA 500
                     **************************************************

Egy2006_Aprimer      ACGGGACAAGCAAGTACTCTGTGACTACCTCACCCAGACGGGGTGACTTG 170
Egy2006_STAprimer    ACGGGACAAGCAAGTACTCTGTGACTACCTCACCCAGACGGGGTGACTTG 225
Egy2006_Cprimer      ACGGGACAAGCAAGTACTCTGTGACTACCTCACCCAGACGGGGTGACTTG 550
                     **************************************************

Egy2006_Aprimer      GGG-TCCCTCGCGGCGAGAGTTGCCGCACAACTCCCTGCATCCTTCAACT 219
Egy2006_STAprimer    GGGGTCCCTCGCGGCGAGAGTTGCCGCACAACTCCCTGCATCCTTCAACT 275
Egy2006_Cprimer      GGG-TCCCTCGCGGCGAGAGTTGCCGCACAACTCCCTGCATCCTTCAACT 599
                     *** ********************************************

Egy2006_Aprimer      ACGGTGCAATTAGAGCCACGACCATCCACGAGCTTCTCGTGCGCATGAAG 269
Egy2006_STAprimer    ACGGTGCAATTAGAGCCACGACCATCCACGAGCTTCTCGTGCGCATGAAG 325
Egy2006_Cprimer      ACGGTGCAATTAGAGCCACGACCATCCACGAGCTTCTCGTGCGCATGAAG 649
                     **************************************************

Egy2006_Aprimer      CGGGCCGAGCTCTACTGCCCTAGACCGCTACTGGCAGTAGCAGTGTCATC 319
Egy2006_STAprimer    CGGGCCGAGCTCTACTGTCCTAGACCGCTACTGGCAGTAGCAGTGTCATC 375
Egy2006_Cprimer      CGGGCCGAGCTCTACTGTCCTAGACCGCTACTGGCAGTAGCAGTGTCATC 699
                     ***************** ********************************

Egy2006_Aprimer      AACAGACAGACACAAGCAAAAGATCATTGCACCCGCAAAACAACTCTTGA 369
Egy2006_STAprimer    AACAGACAGACACAAGCAAAAGATCATTGCACCCGCAAAACAACTCTTGA 425
Egy2006I_Cprimer     AACAGACAGACACAAGCAAAAGATCATTGCACCCGCAAAACAACTCTTGA 749
                     **************************************************

Egy2006_Aprimer      ATTTTGACCTACTCAAGTTGGCTGGAGACGTTGAGTCCAACCCAGGGCCC 419
Egy2006_STAprimer    ATTTTGACCTACTCAAGTTGGCTGGAGACGTTGAGTCCAACCCAGGGCCC 475
Egy2006_Cprimer      ATTTTGACCTACTCAAGTTGGCTGGAGACGTTGAGTCCAACCCAGGGCCC 799
                     **************************************************

Egy2006_Aprimer      TTCTTCTTCTCTGACGTTAGGTCAAATTTCACCAAGCTGGTAGAAACCAT 469
Egy2006_STAprimer    TTCTTCTTCTCTGACGTTAGGTCGAATTTCACCAAGCTGGTAGAAACTAT 525
Egy2006_Cprimer      TTCT-CTTCTCTGACGT-AGGTCGAATT-CACCAAGCTGAGA-------- 838
                     **** ************ ***** **** **********  *

Egy2006_Aprimer      CAACCAGATGCAGGAAGACATGTCAACAAAACACGGGCCCGACTTTAACA 519
Egy2006_STAprimer    CAACCAGATGCAGGAAGACATGTCAACAAAACACGGGCCCGACTTTAACA 575
Egy2006_Cprimer      ----CAGATGCAGGAGGACATGTG------------------------- 858
                         *********** *******
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Fig (3): Nucleotide sequences alignment of cDNA amplicons produced by RT-PCR using specific primers for FMDV serotypes A, C and SAT from outbreak (Egy2006) isolates using CLUSTAL W (1.82) program Numbers of the sequence indicate nucleotide positions in the resulted amplicon sequence data. Stars indicate that nucleotides in that column are identical in all sequences in the alignment.

```
OGolaniso81_Israel              GGCACCACACCGCGTGTTGGCTACTGTTTACAACGGGAACTGCAGGTATG 409
O1Sharquia72_EGYPT              GGCACCACACCGCGTGTTGGCTACTGTTTACAACGGGAACTGCAGGTATG 409
A24Cruzeiro_Brazil55            TGCGCCGCACCGTGTGCTGGCAACAGTGTACAACGGGACGAGTAAGTATG 436
A3mecklenburg_iso81Germany      TGCGCCGCACCGCGTGTTGGCAACTGTGTACAACGGGACAAGCAAGTACT 443
A21kenya_iso77                  CGCACCGCACCGTGTGCTGGCAACGGTGTACAACGGGACGAGCAAGTACT 436
Egy2006Majority_Abass           CGCACCACACCGCGTGCTGGCTACGGTGTACAACGGGACAAGCAAGTACT 141
SAT2Ken_Kenya                   AGCGCCTCACAGGTTGTTGTCCACGGTTTACAACGGGGAGTGCGAGTACA 378
Asia13kimron_iso61Israel        CGCTCCCCACCGTGTGCTGGCAACAGTGTACAACGGGAAGACAACGTATG 251
C3indiso19_Brazil               CGCACCACACCGCGTGCTGGCCACGGCGTACACCGGTACAACGGCCTACA 251
                                 ** ** *** *  ** ** * ** *  **** ***          **

OGolaniso81_Israel              GTAACGTTGCGGTGACCAACGTGAGAGGTGACCTGCAAGTGTTGGCCCAG 459
O1Sharquia72_EGYPT              GTAACGTTGCGGTGACCAACGTGAGAGGTGACCTGCAAGTGTTGGCCCAG 459
A24Cruzeiro_Brazil55            CTGTGG--GTGGTT-CAGGCAGAAGAGGCGACATGGGGTCTCTCGCGGCG 483
A3mecklenburg_iso81Germany      CCGTGA--GCGGTTCGAGAC----GAGGCGACTTGGGGACCCTCGCGGCG 487
A21kenya_iso77                  CCACAA--GTG-TCTCAAGCAGGCGCGGTGAGCTGGGGCCCCTCGCGGCG 483
Egy2006Majority_Abass           CTGTGACTACCTCACCCAG---ACGGGGTGACTTGGGGTCCCTCGCGGCG 188
SAT2Ken_Kenya                   CAAAAA---CAGTTACCGCAATTCGCGGTGATCGAGAGGTGTTGGCGCAG 425
Asia13kimron_iso61Israel        -GGGAA---CAGTCCACGCGA--CATGGTGATCTTGCCACCCTTGCACAA 295
C3indiso19_Brazil               --CTAC---CGGTGTACGCAG----GGGAGACCTAGCCCACTTGGCGGCG 292
                                              ** **          * **

OGolaniso81_Israel              AAG---------GCGGCGAGAACGCTGCCTACCTCCTTCAACTACGGTGC 500
O1Sharquia72_EGYPT              AAC---------GCGGCGAGAACGCTGCCTACCTCCTTCAACTACGGTGC 500
A24Cruzeiro_Brazil55            CGA---------GTCGTGAAACAGCTTCCTGCTTCATTTAACTACGGTGC 524
A3mecklenburg_iso81Germany      CGA---------GTCGCGACACAGCTTCCTACTTCTTTCAACTACGGTGC 528
A21kenya_iso77                  AGG---------ATCGCCGCACAGCTCCCTGCATCCTTCAACTACGGTGC 524
Egy2006Majority_Abass           AGA---------GTTGCCGCACAACTCCCTGCATCCTTCAACTACGGTGC 229
SAT2Ken_Kenya                   AAATACTCTTCCGCCAAGCACTCTCTACCATCCACCTTCAACTTCGGGTT 475
Asia13kimron_iso61Israel        GGG---------GTGAGCAACCGGCTGCCCACCTCCTTCAACTACGGTGC 336
C3indiso19_Brazil               GCGCAC------GCTCGGCAC---CTGCCGACGTCGTTCAACTTTGGTGC 333
                                           ** **  *  * ** **** **

OGolaniso81_Israel              CATCAAGNNGACTCGGGTGACTGAACTGCTTTACCGCATGAAAAGGGCTG 550
O1Sharquia72_EGYPT              CATCAAGGCCACTCGGGTGACTGAACTGCTTTACCGCATGAAAAGGGCTG 550
A24Cruzeiro_Brazil55            AATCAAGGCCGACGCCATCCACGAACTTCTCGTGCGCATGAAACGGGCCG 574
A3mecklenburg_iso81Germany      AATCAAGGCCCAGACCATCCACGAGCTTCTCGTGCGCATGAAACGGGCCG 578
A21kenya_iso77                  ACTTAAGGCCACGAACATCCACGAGCTTCTCGTGCGCATGAAACGGGCTG 574
Egy2006Majority_Abass           AATTAGAGCCACGACCATCCACGAGCTTCTCGTGCGCATGAAGCGGGCCG 279
SAT2Ken_Kenya                   TGTGACCGCCGACAAGCCAGTCGACGTTTACTACCGGATGAAGCGTGCAG 525
Asia13kimron_iso61Israel        TGTGAAGGCTGACACCATCACGGAGCTGTTGATCCGCATGAAGCGCGCAG 386
C3indiso19_Brazil               AGTTAAAGCAGAGACAATCACAGAGCTGCTTGTGCGCATGAAGCGTGCTG 383
                                  * *                 **  *       ** *****  * ** *

OGolaniso81_Israel              AGACATACTGCCCCCGGCCTCTGTTGGCCATTCAC---CCAAGTGAAGCC 597
O1Sharquia72_EGYPT              AGACATACTGCCCCCGGCCTCTTTTGGCCATTCAC---CCGGAACAGGCT 597
A24Cruzeiro_Brazil55            AGCTCTACTGCCCCAGACCGCTGCTGGCAATAGAGGTGTCTTCGCAAGAC 624
A3mecklenburg_iso81Germany      AGCTCTACTGTCCCAGGCCGCTGCTGGCAATAGAGGTGTCTTCACAAGAC 628
A21kenya_iso77                  AACTTTACTGCCCTAGGCCACTACTGGCAGTAGAGGTCACTTCACCAGAC 624
Egy2006Majority_Abass           AGCTCTACTGTCCTAGACCGCTACTGGCAGTAGCAGTGTCATCAACAGAC 329
SAT2Ken_Kenya                   AGTTGTACTGCCCCCGTGCCCTGCTTCCCGCGTAC---ACACACGCGGGC 572
Asia13kimron_iso61Israel        AGACATACTGCCCCAGGCCTTTGCTAGCTCTTGAC---ACCACCCAAGAC 433
C3indiso19_Brazil               AACTCTACTGCCCCAGACCGGTCCTTCCGGTCCAA---CCAGCGGGCGAT 430
                                *    ***** **  *  *  *  *  *           *       *

OGolaniso81_Israel              AGACACAAAC---AAAAGATTGTGGCACCTGTGAAACAGCTTCTG----- 639
O1Sharquia72_EGYPT              AGACACAAGC---AGAAGATTGTGGCACCTGTGAAACAGCTTCTT----- 639
A24Cruzeiro_Brazil55            AGGCACAAGC---AAAAGATCATTGCACCAGCAAAGCAGCTTCTGAATTT 671
A3mecklenburg_iso81Germany      AGGCACAAGC---AAAAGATCATTGCACCCGCAAAACAGTTGCTGAACTT 675
A21kenya_iso77                  AGACACAAGC---AGAAGATCATTGCACCCGCAAAACAACTTCTGAACTT 671
Egy2006Majority_Abass           AGACACAAGC---AAAAGATCATTGCACCCGCAAAACAACTCTTGAATTT 376
SAT2Ken_Kenya                   GGGGACAGATTCGACGCGCCTATCGGCGTCGCGAAACAGCTTTTGAACTT 622
Asia13kimron_iso61Israel        CGCCGTAAGC---AGGAGATCATTGCACCTGAGAAACAGGCTTTGAACTT 480
C3indiso19_Brazil               AGGCACAAAC---AACCGCTCATTGCGCCAGCGAAACAGCTGCTGAACTT 477
                                 *    *      *   *    * *     *  ** **     *
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Fig (4): Alignment of the majority nucleotide sequence of the outbreak (Egy2006) isolates versus those of other related FMDV serotypes, generated from the most identical nucleotide sequences encoding for the viral capsid protein VP1 (1D) of the analyzed viral genomes, using CLUSTAL W (1.82) program. Numbers of the sequence indicate nucleotide positions in their sequence data. Stars indicate that nucleotides in that column are identical in all sequences in the alignment.

Representative outbreak cDNA amplicons, resulted from the A, C and SAT specific primers-directed RT-PCR amplification, were analyzed for their nucleotide sequences (Fig. 3). These nucleotide sequence data were comparatively aligned to each other and revealed variable percentages of sequence identity (Table 2). All sequences in the alignment, of the outbreak FMDV, were subjected to blast search versus the GenBank database, each separately. The closest sequences to the outbreak FMDV were those of isolates/strains of FMDV serotype "A". Also, some isolates/strains of other FMDV serotypes showed lesser degrees of nucleotide sequence similarities. The nucleotide sequence that was most identical among all sequences in the alignment was extracted and utilized as the sequence majority of the outbreak FMDV.

The nucleotide sequence alignments between the outbreak FMDV sequence majority and its counterparts of other FMDV serotypes revealed variable similarity percenttages as presented in (Fig. 4 and Table 3). The phylogenetic tree produced confirmed the results obtained from both nucleotide sequence alignments and blast search as illustrated in (Fig. 5). Phylogenetic analysis strongly indicated that FMDV A21 Kenya "isolate 77" was the closest FMDV serotype to the outbreak FMDV (Fig. 5).

DISCUSSION

The highly contagious nature, accompanied with the dramatic economic losses, make FMDV of a primary animal health concern worldwide. Effective vaccines and stringent control measures have enabled FMD eradication in most developed countries, which maintain unvaccinated, seronegative herds in compliance with strict international trade policies. However, the disease remains enzootic in many regions of the world, posing a serious problem for commercial trade with FMD-free countries.

A severe FMD outbreak has struck cattle and buffaloes in Egypt on early February 2006. It has been established that rapid control of FMD is foremost to reduce dissemination of the causative virus to other non-infected regions (Howard and Donnelly, 2000). Also, characterization of the FMDV serotype is essential for tracing source of the virus with proper selection of effective vaccine (*Clavijo et al.*, 2003). Accordingly, this work was conducted in the process of rapid diagnosis, virus isolation and serotyping of the causative agent for proper formulation and production of a local emergency vaccine to control severe losses of this outbreak among livestock. Diagnosis of FMD depends basically on clinical findings, isolation of the causative agent and specific antigen detection (*Clavijo et al.*, 2003). Infected animals from which the specimens were collected, exhibited typical and severe signs of FMD. The collected specimens (tongue epithelia and foot vesicular fluid) were used for virus isolation and RT-PCR. Virus isolation takes several days and must be followed by an immunoassay for identification of the viral antigen. Although many ELISAs have been developed for detection of FMD viral antigen, field specimens might not contain enough viral antigens to be directly detected by an ELISA (*Alonso et al.*, 1991). Therefore, RT-PCR was the test of choice for rapid detection and serotype identification of the outbreak causative agent. RT-PCR results confirmed that there was a FMDV infection by amplifying the FMDV VP1-derived 216 bp fragment and excluded possibilities for presence of other vesicular diseases. Selection of the PCR target nucleotide sequences is critical as it should be highly conserved among

all FMDV strains (*Clavijo et al.*, 2003). Consequently, primer set P1/P2, selected from VP1 conserved region of FMDV genome, was used for primary diagnosis of the FMD since it detects most of O, A, Asia 1 as well as some of C, SAT1, SAT2 and SAT3 of FMDV serotypes.

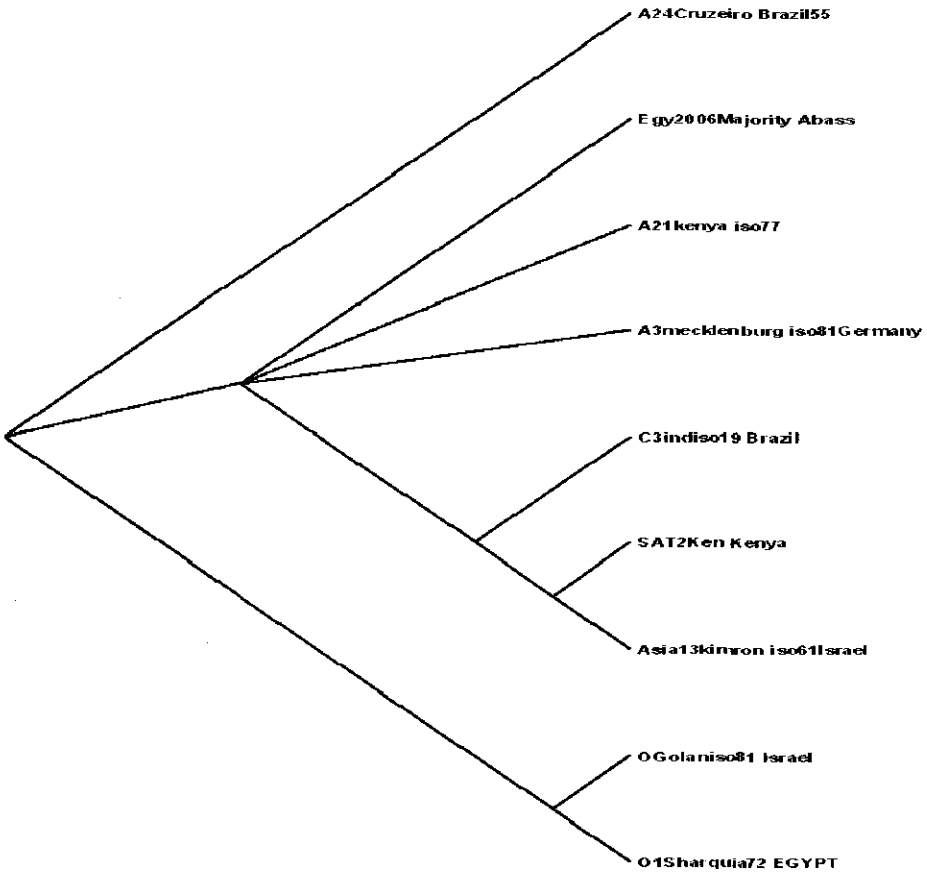

Fig (5): Phylogenetic cladogram of the majority nucleotide sequence of the outbreak (Egypt 2006) isolates versus nucleotide sequences of other related FMDV serotypes, generated from nucleotide sequences enco-ding for the viral capsid protein VP1 of the analyzed viral genomes. The seque-nces were first aligned using Clustal W (1.82) program and the phylogenetic analyses were performed using the PHYLIP package and Treeview 1.6.6 prog-rams.

Nevertheless, it distinguishes them from other vesicular viruses (*Amaral-Doel et al.*, 1993; *Reid et al.*, 2000). There was a primary suggestion that FMDV which caused the Egypt 2006 outbreak is not a serotype "O" because nearly all cattle and buffaloes in Egypt are obligatory vaccinated with the local inactivated FMDV O_1 vaccine. This suggestion was confirmed by the result of RT-PCRs for the tested outbreak specimens, giving no amplicon with the primer set specific for FMDV serotype "O". Using primer sets specific for FMDV serotypes A, C, and SAT, three different RT-PCR cDNA amplicons were

obtained from each tested sample providing a suggestion for the presence of a mixed infection with these three serotypes. Representative serotype-specific cDNA amplicons were sequenced and subjected to multiple nucleotide sequence alignment versus each other. The resulted alignments revealed high percentage of nucleotide sequence similarity (75%-98%) among the three cDNA amplicons (Fig. 3 and Table 2). However, the blast searches in the GenBank database demonstrated that nucleotide sequences of the three cDNA amplicons from the outbreak FMDV samples belong to serotype "A" of FMDV. Hence, cDNA amplicons obtained in RT-PCR with the primer sets specific for serotypes C and SAT group were false positive amplicons. These primers were chosen to specifically amplify cDNA fragments within the 1D and 2AB encoding sequences of FMDV genome (*Knowles* and *Samuel*, 1998). 1D is the most significant FMDV protein for serotype specificity that exhibits about 26% invariant residues among FMDVs (*Jackson et al.*, 2003; *Carrillo et al.*, 2005). While, 2A is a highly conserved FMDV protein, 2B contains about 117 invariant residues among FMDVs (*Carrillo et al.*, 2005). Thus, it could be explained as such primers are non-specific enough to each FMDV serotype that allowed their cross-annealing to homologous complementary sequences in genome of serotype "A". Similar false positive RT-PCR results were reported by other investigators (*Reid et al.*, 1999 & 2000).Virtually, these reference (*OIE/FAO*-cited) FMDV primer sets, although highly specific to FMDV, are non-sensitive enough to stringently distinguish FMDV serotypes and require revision.

Most of the data used for molecular epizootiological studies of FMD has been obtained by direct RNA sequencing (*Knowles*, 1990). The majority nucleotide sequence, which is the most identical sequence observed among all serotype-specific cDNA amplicons, was subjected to multiple nucleotide sequence alignment versus other related FMDVs in the GenBank. The results came in accordance with the previous ones as a high percent of nucleotide sequence similarity was observed among the outbreak FMDV sequence majority and FMDV serotypes A21 Kenya isolate 77 (85%), A3 Mecklenburg-Germany isolate 81 (82%) and A24 Cruzeiro-Brazil isolate 55 (73%). While, a lower percentage of nucleotide sequence similarity (50-67%) was observed with other FMDV serotypes SAT 2, C, Asia 1 and O as presented in (Table 3). It has been suggested that approximately 85% identity is a realistic cut-off to differentiate major genotypes of *picornavirus* infections (*Rico-Hesse et al.* 1987; *Vosloo et al.* 1992). These data were strongly supported by phylogenetic analysis manifested as a distinguished phylogenetic tree. Comparative genetic analyses were useful not only to trace serotype of the new outbreak FMDV versus other related ones but also to establish genetic tools for national-wide epidemiological studies and development of novel efficient FMDV vaccines.

From the previous results, it is concluded that the virus caused Egypt 2006 outbreak is doubtless a FMDV serotype "A" which is closely related to FMDV serotype A21 Kenya as revealed by the high nucleotide sequence identity (85%) and close branch distance in the phylogenetic tree. This conclusion is supported by history of importing live cattle from Ethiopia, shortly before the outbreak beginning, as both imported and local breeds expressed the disease. Furthermore, direct sequencing of the 1D/2AB-derived PCR amplicons was a relevant discriminative tool for genetic characterization of FMDV strains/ isolates. Future work, considering complete nucleotide sequencing of the isolated virus genome, is essential to determine the exact topotype of this new invasive FMDV.

Moreover, genetic analysis of field FMDVs should be routinely conducted to ensure that local vaccine strains will be appropriately matched

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الملخص العربي

" التعرف الوراثى على فيروس الحمى القلاعية المسبب لوباء ٢٠٠٦ فى مصر"

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فى مطلع شهر فبراير من عام ٢٠٠٦، هاجم وباء مرض الحمى القلاعية قطعان الأبقار والجاموس فى مناطق عديدة من مصر مسبباً خسائر إقتصادية فادحة فى صناعة الماشية. و قد تم تجميع عينات عينية (الغشاء الخارجى المسبطن للسان، سائل الفقاعة بين أظلاف القدم) من أبقار شديدة الأصابة بالمرض، منها المستورد (من أثيوبيا) و منها المحلى، من مختلف محافظات مصر (الأسماعيلية، الشرقية، البحيرة).وللقيام بهذا العمل تم إستخدام العديد من إختبارات النسخ العكسى (RT) بإستخدام بادئات عشارية عشوائية و بعدها تفاعل البلمرة المتسلسل (PCR) المبنى على الجين المشفر لبروتين فى. بى – ١ VP1 ، وذلك للتعرف السريع على المسبب المرضى فى العينات الاكلينيكية كخطوة أساسية للسيطرة على هذا الوباء ومنع إنتشاره فى باقى أنحاء الجمهورية. وبإستخدام بادئات عامة لفيروس الحمى القلاعية فى أول إختبار لتفاعل البلمرة المتسلسل تم التعرف على المسبب المرضى للوباء على أنه فيروس الحمى القلاعية، فى جميع العينات الاكلينيكية. وبعد ذلك تم إستخدام مجموعة من البادئات المتخصصة لأنواع فيروس الحمى القلاعية العديدة ، وذلك لتحديد نوع الفيروس المسبب للوباء . وقد تم تنقية نواتج إختبارات تفاعل البلمرة المتسلسل المتخصصة (amplicons) ثم إجراء تحليل التتبع النيوكليوتيدى المباشر لناتج كل نوع على حدة. وقد أظهرت نتائج التحاليل الوراثية بما فيها تطابقات التتبع النيوكليوتيدى العديدة والبحث على مشابهاتها فى بنك الجينات وكذلك التشعب الوراثى الفيلوجينى أن فيروس الحمى القلاعية المسبب للوباء هو من النوع " أ " (A) والذى يعتبر نوع جديد وافد على مصر. و قد أثبتت هذه النتائج أن تحليل التتبع النيوكليوتيدى المباشر لنواتج إختبار النسخ العكسى- تفاعل البلمرة المتسلسل المشتق من الجين المشفر لبروتين فى. بى – ١ VP1 هو أداة حاسمة يعتمد عليها للتوصيف الوراثى لمعزولات و عترات فيروس الحمى القلاعية. وقد أدت نتائج هذا العمل إلى دفع الجهود لبدء إنتاج لقاح ثنائى لمرض الحمى القلاعية يحتوى على نوعى الفيروس " أ " و " و " (A & O1) وذلك لأول مرة فى مصر .