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## Rapid Molecular Detection of Genetically Diverted Foot and Mouth Disease Virus Serotype O During the Outbreak of 2012 in Egypt

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### ABSTRACT

Increasing the international trade of animals and their products and the continuous changing of Foot and Mouth Disease Virus (FMDV) lead to the rise of disease introductions and create an urgent need for laboratory methods facilitating a swift and sensitive confirmation of suspect outbreaks and fast characterization of new isolates. As FMDV is extremely contagious and affects all cloven-hoofed animals, it provides a continuous burden and risk to the livestock industries of the developing world, in particular due to mortality in young animals, weight and milk loss, lameness and the trade restrictions necessary to control the disease. The control of FMD in Egypt requires an accurate analysis of the newly introduced viral strains and an analysis of their relationship with the current circulating strains and the routinely used vaccines. This study evaluates a recently developed rapid Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) for the identification of FMDV in samples from suspect cases. Samples were collected from gum, tongue epithelium, vesicular fluid, buccal and gum swabs, as well as saliva of infected cattle, buffalo and sheep and examined using two RT-qPCR of routine and high-speed formats using “IRES1” and “3D-OIE” primers. In addition, partial VP1 sequencing of some selected isolates was carried out for phylogenetic analysis. The high-speed RT-qPCR allowed a reliable diagnosis of FMDV in less than half an hour and can be used as a fast and valuable method for the monitoring and controlling of the foot and mouth disease. A new variant genotype (FMD-O/Eg/Mans/14) was circulating in Egypt during the outbreak of 2012 showing 98.5% identity to the isolated strain in Sudan (SUD\_6\_2008).

**Key words:** Foot and mouth disease virus, RT-qPCR, VP1 sequencing, FMD/O/Eg/Mans/14

### INTRODUCTION

Foot and Mouth Disease (FMD) is a highly contagious disease, affects cloven-footed animals causing severe economic losses whereby its potential zoonotic effect cannot be ignored (Brown, 2001). The causative agent of FMD is a virus belongs to genus *Aphthovirus*, family

Picornaviridae, order Picornavirales and is immunologically classified to seven distinct serotypes (O, A, C, SAT1, SAT2, SAT3 and Asia1). The viral genome is a linear single stranded RNA of about 8.5 kb that contains a 12 protein-coding genes in the plus orientation with a poly A tract at its 3' end and a viral genome protein (VPg) at its 5' end (Carroll *et al.*, 1984; Forss *et al.*, 1984; Li *et al.*, 2007). Foot and mouth disease serotypes SAT2 and A were recorded in Egypt since 1950 and reported as the main causes of outbreaks during, 1953, 1958 and 1960, followed by serotype O which was circulating in livestock till 2006 (OIE., 2000; Aidaros, 2002). In February, 2006, FMD outbreaks of serotype A were reported in cattle and buffalo (OIE., 2006; Knowles *et al.*, 2007). Although, a bivalent vaccine containing both O1 and A/Egypt/2006 local isolates was locally produced and used for routine vaccination of Egyptian animals in May, 2006, other outbreaks of serotype O were reported in Alexandria and El-Buhayrah governorates in September, 2007 and January, 2008 (FAO., 2008).

The molecular tests used for detection of FMD provide rapid, accurate and more sensitive tools for diagnosis and assist in studying the epidemiology and pathogenesis of FMD (Kitching and Mackay, 1994). Polymerase Chain Reaction (PCR) followed by nucleotide sequences and its antigenic nature using expressed proteins and monoclonal antibodies, helps in identifying new circulating FMD strains in cattle and buffalo and also strains causing outbreaks in cattle over extended periods (Dawe *et al.*, 1994; Hargreaves, 1994).

The control of FMD requires rapid, more accurate and continuous evaluation of the circulating serotypes using diagnostic laboratory tools for preventing its spread into huge geographic areas (Belak, 2007). The application of reverse transcription quantitative PCR (RT-qPCR) provides a high sensitivity, reduced risk of cross-contamination and considers as a valuable tool for the detection of viruses (Mackay *et al.*, 2002; Mackay, 2004). The time required for the common PCR protocols is about 90-120 min, this time could be reduced using recently developed high-speed (PCR) assays for detection of e.g., Influenza, adenoviruses or classical swine fever virus (Hoffmann *et al.*, 2005, 2010; Wernike *et al.*, 2013; Fujimoto *et al.*, 2010; Sakurai *et al.*, 2011).

Egypt is one of the endemic countries for FMD expressing many outbreaks of the disease almost every year causing great economic losses. The routine used vaccinations are not efficiently controlling the spreading of the disease because of the absence of cross-protection between the different viral serotypes and subtypes (Mason *et al.*, 2003) and the high rate of viral mutations, especially in VP1 gene (Dopazo *et al.*, 1988; Domingo *et al.*, 1990; Samuel *et al.*, 1999; Carrillo *et al.*, 2005).

The role of sheep and goats, as a possible FMD carriers, in the epidemiology and transmission of the disease had been mentioned by Ganter *et al.* (2001) while, Ptail *et al.* (2002) reported them to constitute the majority of the world's FMD-susceptible livestock. Frequently mild or no clear clinical signs of FMD appeared in sheep (Kitching and Hughes, 2002), even so, they can secrete and excrete considerable amounts of FMDV (Burrows, 1968; Donaldson and Sellers, 2000; De Rueda *et al.*, 2014) and therefore may play a significant role in FMD virus transmission. The FMD type O transmission from sheep to cattle was reported in Greece during 1994 (Donaldson, 2000) and Morocco during 1999 (Blanco *et al.*, 2002) and UK during 2001 (Gibbens *et al.*, 2001).

During the Egyptian outbreaks of 2012, a huge threat and spreading of FMD were reported in many villages and farms with vesicular lesions, salivation, loss of body weight, decrease in milk production in adult cattle, buffalo and sheep and high mortality in young calves. Molecular investigations for the outbreaks of 2012 could identified FMD serotype SAT2 (Ahmed *et al.*, 2012; Kandeil *et al.*, 2013; Elhaig and Elsheery, 2014; El-Shehawy *et al.*, 2014).

In this study, we have evaluated routine and high-speed RT-qPCR techniques using IRES1 and 3D-OIE primers for detection of FMD serotype in the collected samples and characterizing the circulating serotype during the outbreak of 2012 occurred in Egypt.

## MATERIALS AND METHODS

**Viruses and samples:** During the outbreaks of 2012 in Egypt, seventeen different samples collected from gum, tongue epithelium, vesicular fluid, buccal and gum swabs and saliva of infected cattle, buffalo and sheep in El-Dakahlyia and Damietta governorates. All infected animals suffered from lameness, high fever and presence of some vesicles in the oral cavity and hoof during the collection of samples. Collected tissues were homogenized in transport buffer, centrifuged and filtered before genome extraction; saliva, fluids and soaked swabs in PBS were centrifuged. The supernatants of all fluids were kept in -20°C till use.

**Extraction of viral RNA:** The viral RNA was extracted from all collected supernatants by QIAamp®Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the mini spin protocol according to the manufacturer’s instructions and eluted in 50 µL of elution buffer.

**Primers and probes used for RT-qPCR:** Two sets of primers were used for the identifying of the two different genomic regions of FMDV. The FMD-IRES1 and FMD-3D-OIE primers were selected for detecting the IRES and the3D (polymerase RNA gene) fragments respectively (Callahan *et al.*, 2002; Wernike *et al.*, 2013). The used probes were labeled with 6-carboxyfluorescein (FAM) at the 5' end and the quencher tetramethylrhodamine (TAMRA) at the 3' end. Table 1 shows the names, sequences and concentrations of the used primers and probes required for a single reaction.

**Routine and high-speed RT-qPCR analysis:** The real time reaction combined with reverse transcription in the routine RT-qPCR and without reverse transcriptase step in the high-speed RT-qPCR, as the short time between PCR mix preparation and *Taq* polymerase activation by the initial denaturation step was sufficient for an effective cDNA generation, were carried out using Superscript™III with Platinum® *Taq* RT-PCR kit (Invitrogen, Darmstadt, Germany) in a total volume of 12.5 µL reaction mixtures, using an Eco™ Real-Time PCR system (Eco™ Software v3.0.16.0; Illumina, Inc., San Diego, CA, USA). The reagents mixture for the high-speed RT-qPCR consists of 6.25 µL 2×reaction mix, 1 µL 5 mM magnesium sulfate, 0.25 µL RT/Platinum® Tag Mix, 1.5 µL RNase-free water, 1 µL Primer-probe-mix and 2.5 µL Template RNA with the following thermal profile: Initial activation step at 95°C for 1 min, 45 cycles of a two-step cycling consisting

Table 1: Primers and probes used in RT-PCR for detection of FMD

References	Primer	Name	Sequences	Concentration (µmol/reaction)
Wernike <i>et al.</i> (2013)	FMD-IRES	FMD-IRES1-3F	5'-ACCTGGWGRCCAGGCTAAG GA-3'	10.00
		FMD-IRES1-3.2R	5'-CCYRGTCCTTCTCAGAT-3'	10.00
		FMD-IRES1-3FAM	5'-FAM-CCCTTCAGGTACCCCGA GGTAACA-BHQ1-3'	2.5
Callahan <i>et al.</i> (2002)	FMD-3D-OIE	FMD-3D-OIE-F	5'-ACTGGGTTTTACAAACCTGTGA-3'	10.00
		FMD-3D-OIE-R	5'-GCGAGTCTGCCACGGA-3'	10.00
		FMD-3D-OIE-FAM	5'-FAM-TCCTTTGCACGCCGTGGGAC-TAMRA-5'	2.5

of denaturation at 98°C for 1 sec and annealing and extension at 54°C for 1 sec. In order to compare the results with routine RT-qPCR and conventional PCR methods, an identical reverse transcription set-up was carried out using the following thermal profile: Reverse transcription step at 50°C for 15 min (one cycle), PCR initial activation step at 95°C for 2 min (one cycle), 45 cycles of a three step cycling consisting of denaturation at 95°C for 15 sec, annealing at 56°C for 20 sec and extension at 72°C for 30 sec.

**Conventional PCR, nucleotide sequencing and Phylogenetic analysis:** Some samples were selected for conventional PCR, sequencing and phylogenetic analysis. By one step reaction in a total volume of 50 µL reaction mix including: 1.5 µL of each primer against VP1 gene, 1 µL enzyme, 2 µL MgCl<sub>2</sub>, 1 µL dNTPs, 5 µL 10x Buffer, 6 µL extracted RNA and 32 µL DEPC treated water in the same thermal profile described in the routine RT-qPCR. The PCR amplification was performed by thermocycler machine (GeneAmp PCR system 9700, Applied Biosystems).

Finally, the PCR products run in 1.5% agarose gels and staining with ethidium bromide (1 µg mL<sup>-1</sup>) followed by PCR fragment cleaning using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Formamide 10% was added to dry purified samples which analyzed by an ABI Prism 3100 Avant Genetic Analyzer sequencing machine for nucleotides sequencing according to the manufacturer's procedure. Identity of the VP1 nucleotides (amplicon size of 765 pb) and the amino acids sequencing were manually edited and calculated using the basic local alignment search tools BLASTn and BLAST, respectively, which are available at the webpage of the National Center for Biotechnology (NCBI).

Some selected reference FMD viruses were used for the nucleotide and amino acid alignments. BFS-89/68 (accession number JX869188.1), BFS-1860 B5.9D.V (JX570655.1), ISR/2/2011 (JX040501.1), BUL/30/2011 (JX040489.1), Israel 07-6380 (FJ175662.1), SAU/3/2013 (KJ206910.1), LIB/2/2013 (KJ206909.1), BHU/1/2013 (KJ206908.1), OUGA2009LIRA (JN974308.1), O/ISL/PAK/L1573/2009 (HQ113232.1), Manisa (FN594747.1), O/Nigde/TUR/441/05/00 (DQ472652.1), Tibet/CHA/99 (AJ539138.1), UAE 7/99 (EU140964.1), O/SKR/2000 (AY312587.1), o1skr iso85 (AY593824.1), IRN\_073/2001 (JF749851.1), HKN15/2010 (KF112889.1), BAN/NA/Ha-156/2013 (KF985189.1), Kumi District (FJ461344.1), O/K/117/1999 (HM625676.1), FMDV (EF611987.1), FMDV (NC\_004004.1), FMDV (AF308157.1), KEN/7/98 (K30/98) (KF135266.1), FMDV (AF292107.1), O/India/R2/75 (AF204276.1), SOM/1/80 (AY344595.1), ERI/2/96 (AY283391.1), O/ETH/30/94 (HM211078.1), O/SUD/1/99 (HM211078.1) and SUD/6/2008 (GU566062.1).

## RESULTS

The collected FMD samples utilized in this study were all isolated during the outbreak of 2012 in Egypt, from susceptible clinically infected cattle, buffalo and sheep. All samples were initially evaluated by real time RT-qPCR assays, which showed positive for FMD virus by both routine diagnostic RT-qPCR and new rapid RT-qPCR using 3D-OIE and IRES1 primers. Some selected samples were further analyzed by partial sequencing of VP1 coding region to determine the serotype and subtype of the isolates.

**Comparative analysis of the samples by real time RT-qPCR techniques:** Using of real time-RT-qPCR techniques for diagnosis of FMD viruses by two different primers against IRES1 and

Table 2: Sequence identity of the nucleotides and amino acids of the FMD-O/Eg/Mans/14 Egyptian isolate to selected reference isolates based on their partial VP1 sequences

Isolate	Nucleotide (400) identity	Amino acid (133) identity
BFS_89_68	81.0	91.0
BFS_1860_B5_95_V	81.0	91.0
ISR_2_2011	83.5	94.0
BUL_30_2011	84.3	94.0
Israel_07_6380	83.0	95.5
SAU_3_2013	85.0	84.7
LIB_2_2013	85.3	94.7
BHU_1_2013	86.0	94.7
OUGA2009LIRA	85.5	93.2
O_ISL_PAK_L1573_2009	84.3	94.0
Manisa	85.0	94.0
Tibet_CHA_99	85.0	97.0
UAE_7_99	84.0	93.2
O_SKR_2000	86.5	97.0
O1SKR_ISO85	86.5	97.0
IRN_073_2001	84.5	96.2
HKN15_2010	82.3	91.0
BAN_NA_Ha_156_2012	85.3	94.0
Kumi_District	84.8	93.2
O_K_117_1999	87.3	95.5
EF611987_1	86.0	94.0
NC_004004_1	79.3	88.0
AF308157_1	79.3	88.0
KEN_7_98	87.8	97.0
AF292107_1	87.0	95.5
O_India_R2_75	84.8	94.0
SOM_1_80	89.3	92.5
ERI_2_96	91.0	96.2
O_ETH_30_94	91.8	97.0
O_SUD_1_99	94.3	97.7
SUD_6_2008	95.0	98.5

Numbers indicate the total sequence identity (%)

3D gene regions shows no significant differences between the used techniques and primers. The new advanced RT-qPCR is a rapid, accurate and sensitive technique for diagnosis of FMD virus within 30 min. The two different thermal profiles using the Eco<sup>TM</sup> Real-Time PCR system resulted in a total reaction time of 90 min (routine RT-qPCR) and 28 min (high-speed RT-qPCR) respectively.

**Comparative analysis of the nucleotides and amino acids sequence identity:** The genetic relationship analysis based on VP1-gene of both FMDV isolate and some viruses, was shown in Fig. 1. From the resulting list of matching isolates, we used a total of thirty one reference sequences of isolates from different neighbor countries as Israel, Sudan, Kenya, Ethiopia and Iran. The sequences were processed to match the length and the total sequence identity of nucleotides. Additionally, the conservation of each (aa) residue of the VP1-protein of the new FMD isolate to the reference viruses was compared and aligned using the NCBI database (BLASTp search) of the isolate to each of the reference viruses (Fig. 2). The VP1 sequence identity of the new Egyptian isolate FMD-O/Mans/Eg/14 to the other reference strains ranged from 81% (BFU\_89\_68) to 95% (SUD\_6\_2008) on (nt) level and 91% (BFU\_89\_68) to 98.5% (SUD\_6\_2008) on (aa) level (Table 2). By this method we could show







(d)

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IRN_073_2001      GAAGAGGGGCTGAAGCATACTGCCCCCGGCTCTTTTGGCTATTACCCCGAGCGAAGCTAG 360
O_SKR_2000        GAAGAGGGGCCGAAACATACTGTCCCCGGCCTCTTTTGGCTATTACCCCGAGCGAAGCTAG 360
o1skr_iso85       GAAGAGGGGCCGAAACATACTGTCCCCGGCCTCTTTTGGCTATTACCCCGAGCGAAGCTAG 360
SAU_3_2013        GAAGAGGGGCCGAAACATACTGTCCCTCGACCTCTGCTCGCCATTACCCCGGACGAGGCCAG 360
LIB_2_2013        GAAGAGGGGCCGAAACATACTGTCCCTCGACCTCTGCTCGCCATTACCCCGAACGAGGCCAG 360
BHU_1_2013        GAAGAGGGGCCGAAACATACTGTCCCTCGGCCCTGCTCGCCATTACCCCGAGCCAGGCCAG 360
BAN_NA_Ha_156_2013 GAAGAGGGGCCGAAACATACTGTCCCTCGGCCCTGTTGGCCATCCACCCGAGCCAAAGCCAG 360
AF292107_1        GAAGAGGGGCTGAACATACTGCCCCCGGCTCTTTTGGCCATCCACCCGAAATGAGGCCAG 360
O_India_R2_75     GAAGAGGGGCTGAGACGTAAGTGTCCCCGGCCTCTTTTGGCCATTACCCCGAACGAGGCCAG 360
Manisa            GAAGAGGGGCTGAGACATACTGTCCCCCGGCTCTTTTGGCCATTACCCCGGACGAGGCTAG 360
HKN15_2010       GAAGAGGGGCCGAGACGTAAGTGTCCCTCGGCCCTCTTTGGCTGTTACCCCGAGTGCAGGCCAG 360
NC_004004_1      GAAGAGAGCCGAGACATACTGTCCAGGCCCTTCTCGCCATTCAACCCGAGTGACGCTAG 360
AF308157_1       GAAGAGAGCCGAGACATACTGTCCAGGCCCTTCTCGCCATTCAACCCGAGTGACGCTAG 360
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OUGA2009LIRA      ACACAAACAAAAGATTGTGGCACCTGTCAAACAACTTCTA 400
EF611987_1        ACACAAACAAAAGATTGTGGCACCTGTCAAACAACTCCTA 400
Kumi_District     ACACAAGCAAAGATTGTGGCACCTGTCAAACAACTTCTA 400
O_K_117_1999      ACACAAACAAAAGATTGTGGCACCTGCCAAACAACTCCTA 400
BFS_89_68         ACACAAACAGAAAATTGTGGCACCGGTGAAACAGACTTTG 400
BFS_1860_B5_9D_V ACACAAACAGAAAATTGTGGCACCGGTGAAACAGACTTTG 400
KEN_7_98          GCACAAACAAAAGATAGTGGCGCCTGTGAAACAACTCCTG 400
SOM_1_80          ACACAAACAGAAAATAGTGGCGCCTGTGAAACAACTCTTG 400
ERI_2_96          ACACAAACAAAATTGTGGCACCTGTGAAACAACTCCTG 400
O_ETH_30_94       ACACAAACAGAAATAGTGGCACCTGTGAAGCAACTCCTG 400
SUD_6_2008        ACACAAGCAGAAATAGTGGCACCTGTGAAACAACTCCTG 400
FMD-O/Eg/Mans/14 ACACAAACAGAAATAGTGGCACCTGTGAAACAACTCCTG 400
O_SUD_1_99        ACACAAGCAGAAATAGTGGCACCTGTGAAACAACTCCTG 400
ISR_2_2011        ACACAAGCAAAGATAGTGGCACCTGTGAAGCAACTCCTA 400
BUL_30_2011       ACACAAGCAAAGATAGTGGCACCTGTGAAGCAACTCCTG 400
Israel_07_6380    ACACAAACAAAAGATAGTGGCACCTGTGAAGCAGCTCTTG 400
O_ISL_FAK_L1573_2009 ACACAAGCAAAGATAGTGGCACCTGTGAAACAGATTTTG 400
Tibet_CHA_99      ACACAAACAAAAGATTGTGGCGCCTGTGAAACAGCTTTTG 400
UAE_7_99          ACACAAACAAAAGATTGTGGCGCCTGTGAAACAGCTTTTG 400
IRN_073_2001     ACACAAACAAAAGATTGTGGCACCTGTCAAACAGCTTTTG 400
O_SKR_2000        ACACAAACAAAAGATTGTGGCGCCTGTGAAACAGCTTCTG 400
o1skr_iso85       ACACAAACAAAAGATTGTGGCGCCTGTGAAACAGCTTCTG 400
SAU_3_2013        ACACAAACAAAAGATTGTGGCACCTGTGAAACAGCTCTTG 400
LIB_2_2013        ACACAAACAAAAGATTGTGGCACCTGTGAAACAGCTCTTG 400
BHU_1_2013        ACACAAACAAAAGATTGTGGCACCTGTGAAACAACTCTTG 400
BAN_NA_Ha_156_2013 ACACAAACAAAAGATTGTGGCACCCGTGAAACAGCTGTTG 400
AF292107_1        ACACAAACAGAAATAGTGGCGCCTGTGAAACAACTCCTG 400
O_India_R2_75     ACACAAACAGAAATAGTGGCACCTGTGAAGCAGCTCCTG 400
Manisa            ACACAAGCAGAAATAGTGGCACCGGTGAAACAGCTTCTA 400
HKN15_2010       ACACAAACAGAAAATAGTGGCACCTGTAAAGCAGTCCCTG 400
NC_004004_1      ACACAAGCAGAGGATTGTGGCACCCGCAAACAGCTTCTG 400
AF308157_1       ACACAAGCAGAGGATTGTGGCACCCGCAAACAGCTTCTG 400
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Fig.1(a-d): Multiple alignment of the nucleotides sequences of the VP1 proteins of the FMD-O/Eg/Mans/14 isolate and some reference isolates

**Phylogenetic analysis based on the partial VP1-protein sequences:** The selected sequenced samples were identified by BLAST and found to be of serotype O, FMD-O/Mans/Eg/14 (accession number: KP121442) and SAT2 (Table 3). After having shown that the new FMD isolate shares the highest sequence identify-on both (nt) and (aa) levels-with the Sudan isolate SUD\_6\_2008 and O\_SUD\_1\_99, further analyses of the phylogenetic relationship of the isolate (FMD-O/Mans/Eg/14) to the different reference viruses were performed based on the (aa) composition of the partial VP1-protein sequences using the Phylogeny. fr tool, the constructs phylogenetic tree was designed based on maximum likelihood analyses. By the construction of phylogenetic tree (Fig. 3), we could show that the new Egyptian isolate (FMD-O/Mans/Eg/14) form a distinct phylogenetic cluster together with the Sudan isolate SUD\_1\_2008 and O\_SUD\_1\_99.

Table 3: Analysis of the field samples by standard and high-speed RT-qPCR protocols using two primers (3D-OIE and IRES1) and VP1 Sequencing for some selected samples

Sample	Routine RT-qPCR 3D-OIE	Routine RT-qPCR IRES1	High-speed RT-qPCR 3D-OIE	High-speed RT-qPCR IRES1	VP1 sequencing
Bovine gum epithelium	29.85	31.91	32.20	32.76	N/S
Cattle gum epithelium	31.64	33.05	42.13	32.20	N/S
Sheep gum epithelium	20.12	25.37	22.33	23.92	Serotype O
Buffalo gum epithelium	20.05	24.17	21.34	25.89	FMD-O/Eg/Mans/14
Buffalo saliva	32.21	36.31	34.18	N/S	SAT2
Sheep buccal swab	28.46	35.73	27.98	N/S	N/S
Sheep buccal swab	21.31	29.09	25.71	29.13	Serotype O
Sheep buccal swab	24.80	27.15	26.10	24.31	Serotype O
Buffalo buccal swab	31.50	34.89	33.12	35.08	FMD-O/Eg/Mans/14
Buffalo gum swab	33.99	35.12	43.66	34.96	N/S
Buffalo gum swab	31.93	33.11	32.83	38.07	N/S
Buffalo gum swab	27.95	30.72	30.21	29.52	N/S
Cattle saliva	20.92	25.78	22.71	24.85	Serotype O
Buffalo saliva	33.29	33.34	36.84	34.32	EMD-O/Eg/Mans/14
Cattle tongue epithelium	31.33	34.27	32.46	31.66	N/S
Cattle vesicular fluid	27.73	29.33	28.98	28.21	N/S

(a)

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FMD-O/Eg/Mans/14      KHEGNLTWVPNGAPETALDNTTNPAYHKAPLTRLALPYTAPHRVLATVYNGNCKYGGAQ 60
O/ETH/30/94          .....R..E.F
O/SUD/1/99           .....R..E.F
ERI/2/96              .....A.....R..E.F
SUD/6/2008            .....E.P
SOM/1/80              .....S.....K.R..E.S
FMDV (NC_004004.1)    .....D.....E.....SS...DTS
FMDV (AF308157.1)    .....D.....E.....SS...DTS
BFS/89/68             .....D.....K.....E.R.SRNA
BFS/1860/B5/9D/V     .....D.....K.....E.R.SRNA
HKN15/2010            .....D.....A.....A.GS
O/India/R2/75        .Y.....N.....Q.....DGS
ISR/2/2011            .....E...SESR
BUL/30/2011           .....D...SESH
O/K/117/1999         .....M.....TP
UAE/7/99              ..K.D.....VESP
O/ISL/PAK/L1573/2009 .....D.....ESS
FMDV (AF292107.1)    .....R...EST
Israel/07/6380        .....ESH
KEN/7/98 (K30/98)    .....S.....E.S
IRN/073/2001          .....ESP
Tibet/CHA/99          .....ESP
O/SKR/2000            .....EGP
clskr iso85           .....EGP
Manisa                .....A.....DGT
BAN/NA/Ha-156/2013   .....A.....E.....EGA
BHU/1/2013            .....A.....ESD
SAU/3/2013            .....A..A.....ENN
LIB/2/2013            .....A.....ESN
Kumi District         .....A.....Y.....S.SS
OUGA2009LIRA         T.....A.....SDSS
FMDV (EF611987.1)    .....A.....V.....S.SS
.:*:*****:****:****:* *****:*****:****:.*

FMD-O/Eg/Mans/14      VTNVRGDLQVLAQKAARTLPSTFNYGAIKATRVTELLYRMKRAETYCPRPLLAHPTEAR 120
O/ETH/30/94          .....V.....
O/SUD/1/99           .....
ERI/2/96              .....
SUD/6/2008            .....
SOM/1/80              A.....V.W.....SD..
FMDV (NC_004004.1)    TN.....E.....F.....Q.SD..
FMDV (AF308157.1)    TN.....E.....F.....Q.SD..
BFS/89/68             .P.L.....V.....
BFS/1860/B5/9D/V     .P.L.....V.....
HKN15/2010            LP.....P.....V..SA..
O/India/R2/75        ..I.....N.....
ISR/2/2011            T.....N.....N.....
BUL/30/2011           T.....N.....N.....
O/K/117/1999         .....SD..
UAE/7/99              ..AI.....S.....
O/ISL/PAK/L1573/2009T.....V.....S.....
FMDV (AF292107.1)    .....I.....N.....
Israel/07/6380        T.....I.....D.....
KEN/7/98 (K30/98)    .....S.....
IRN/073/2001          .....A.....S.....
    
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Fig. 2(a-b): Continue

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(b)
Tibet/CHA/99 .....S...
O/SKR/2000 .....S...
olskr iso85 .....S...
Manisa .A.....A.....DQ..
BAN/NA/Ha-156/2013 .....T.....SQ..
BHU/1/2013 .....P.....SQ..
SAU/3/2013 .....P.....D...
LIB/2/2013 E.....P.....N...
Kumi District .....A.....V.....SD..
OUGA2009LIRA A.....SD..
FMDV (EF611987.1) A.....R.....S...
* *****;. *****;***** *****;*****; **

FMD-O/Eg/Mans/14   HKQKIVAPVKQLL 133
O/ETH/30/94 .....
O/SUD/1/99 .....
ERI/2/96 ..T.....
SUD/6/2008 .....
BH57/14/3 .....
KEN/7/98 .....
SCM/1/80 .....
FMDV (NC_004004.1) ..R...A...
FMDV (AF308157.1) ..R...A...
BFS/89/68 .....T...
BFS/1860/B5/9D/V .....T...
HKN15/2010 .....S...
O/India/R2/75 .....
ISR/2/2011 .....
BUL/30/2011 .....
O/K/117/1999 .....A...
UAE/7/99 .....
O/ISL/PAK/L1573/2009 .....I...
FMDV (AF292107.1) .....
Israel/07/6380 .....
KEN/7/98 (K30/98) .....
IRN/073/2001 .....
Tibet/CHA/99 .....
O/SKR/2000 .....
olskr iso85 .....
Manisa .....
BAN/NA/Ha-156/2013 .....
BHU/1/2013 .....
SAU/3/2013 .....
LIB/2/2013 .....
Kumi District .....
OUGA2009LIRA .....
FMDV (EF611987.1) .....
** :****.** *

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Fig. 2: Multiple sequences alignment of the amino acid residues of the partialVP1 protein sequences for the FMD-O/Eg/Mans/14 isolate and some reference isolates

## DISCUSSION

The FMD is a highly contagious, world-wide distributed disease affecting cloven footed animals causing vesicular lesions in the mouth and foot epithelial tissues. The development of an accurate and sensitive method for detection of FMDV required the recognition of conserved sequence in FMDV genome, located in all serotypes of FMDV and less affected by mutations such as 2B, 3D, 5UTR (IRES) regions (Vangrysterre and de Clercq, 1996; King *et al.*, 2006). In this study, we used IRES and 3D regions as targets to perform an assay of sensitive, fast and accurate detection of FMDV. The exploitation of the two RT-qPCR techniques either routine or high speed protocol could assess the sensitivity and accuracy of FMD diagnosis of the field samples

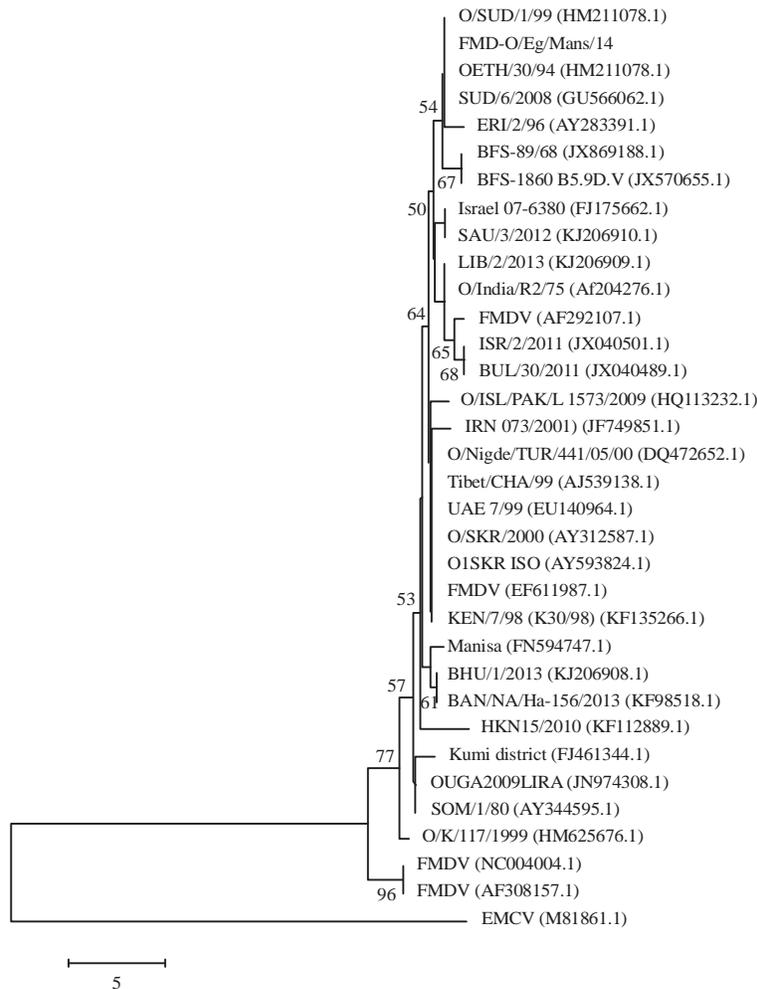


Fig. 3: Phylogenetic tree analysis of the new Egyptian FMD isolate and selected reference isolates: based on the partial VP1 gene sequencing

from different animal species as cattle, buffalo and sheep. Furthermore, this study describes a molecular phylogenetic tree of a new serotype of FMDV circulating in Egypt during the outbreak of 2012.

Using of variety of samples and tissues isolated from different animal species in Egypt was considered an important need and good tool for detection of a real-life situation of FMDV observed in areas of endemic infections (Hole *et al.*, 2010). Suitable laboratory methods are so-called rapid-cycle or high-speed PCR assays; results are provided in less than 1 h (Wittwer *et al.*, 2001), those PCR systems have been described recently for various pathogens such as *Vibrio cholera*, group B *Streptococcus* bacteria, Influenza virus or adenoviruses (Fujimoto *et al.*, 2010; Wilson *et al.*, 2010; Sakurai *et al.*, 2011; Koskela *et al.*, 2009; Molsa *et al.*, 2012). Modifications are processing to improve the routinely used machines of RT-PCR for achieving rapid processing of the samples, like our used machine (Eco™ Real-Time PCR system) which showed an excellent high-speed PCR results using a short thermal profile in the test confirming the TM possibility for an effective speeding up of PCR protocols in general. The obtained results clarified the sensitivity

of the two RT-PCR methods in detecting the viral genome in the analyzed samples. The used primers against 3D-OIE and the IRES1 in RT-qPCR assays showed nearly identical results independent if a routine or a high-speed RT-qPCR temperature profile. In contrast, the high speed RT-qPCR needed only 30 min for amplification of both 3D and IRES1 which indicated a rapid method of detection of FMDV. From this point, it could be claimed that the use of fast and reliable method of minor risk of cross-contamination and rapid reaction time is suitable for diagnosis of FMD outbreaks (Wernike *et al.*, 2013).

In this study, a new variant type of serotype O (FMD-O/Eg/Mans/14) has been detected and molecularly analyzed during the outbreak of 2012 in different animal species. The new virus could be introduced to Egypt through live animal's importation causing severe clinical signs occurred among cattle, sheep and buffaloes indicated that this virus does not circulate in Egypt before (Abd El-Moety *et al.*, 2013).

For highly contagious animal diseases rapid diagnosis is essential to prevent the spread into further animal populations, the use of advanced rapid real time RT-qPCR is a valuable method for rapid diagnosis of FMD virus with high sensitivity and specificity for different samples collected from different susceptible animal species. New technologies like the Peltier element of the ECO™ Real-Time PCR system for a very fast ramping combined with intuitive soft-ware package will open up the pen-side diagnostic market also for real-time PCR technology.

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