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Molecular Characterization of Foot-and-Mouth Disease Virus In Sheep and Goats in some Egyptian Governorates

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Abstract

Eighty seven samples from (Buffy coat, saliva, nasal swabs, tongue epith. Buckel swab, pharyngeal fluid and rectal swabs) were collected and identified using Indirect sandwich ELISA for Antigen detection and serotyping of foot and mouth disease virus, serotyps (A, O, SAT2, C, Asia).

The result revealed 33 samples out of 87 were positive (16) serotype A, (10) serotype O, (7) serotype SAT2.

By using (RT-PCR) the results demonstrated that all serologically positive samples were positive. Sequencing and phylogenetic analysis of the amplicons obtained from positive RT-PCR samples were done.

Keywords: *FMDV, ELISA, PCR2.*

Introduction

Foot and mouth disease (FMD) is the most important disease of the international organization epizooties (OIE), and one of the most contagious disease among domestic animals **(Carrol *et al.,* 198 4; OIE/FAO/WHO, 1996; Saiz *et al.,* 2002 and Michael *et al.,* 2007 and Kasambula *et al.,* 2012**).

FMD is caused by that's belongs to MDV Family *picornaviridae* virus, of genus *A phtoho virus*. it contains a single stranded RNA molecule. The virus has seven major serotypes: A, O, C, SAT1, SAT2, SAT3 and Asia, 1, infection with one serotype doesn't confer immunity against another. The virus is easily spread by several means, the most important arebeing recovered animals or products from such animals **(Anthony and Werner, 1992).**

The disease is characterized by the formation of vesicles in the mucosa of the mouth, external nares and in coronary band of claws, other areas including udder and teats. Lameness is seen reduced lactation mastitis and abortion are common clinical signs range from a mild or in apparent infection to one that is sever. Death may result in some cases, mortality from a myocarditis is most common seen in young animals myositis may also occur in other sites **(FAO, 1984)**.

Methods for the diagnosis of foot and mout disease consistent with office International des Epizooties (OIE) standards for FMD diagnosis include: antigen-capture ELISA for viral antigen typing, several molecular diagnostic methods have also been developed for detection of frament of FMD genome within viral samples such as multiplex RT-PCR, typing RT-PCR and real time RT-PCR **(Lu *et al.,* 2008).**

The aim of this study

Trials for isolation and identification of the

FMD virus by inoculation on BHK cell line" and virus identification by antigen capture ELISA, real time PCR and conventional RT-PCR sequencing for new field isolates and matching on Gene Bank.

Materials and Methods

Samples: for Virus isolation

A total number of (87) samples wear collected from clinically infected sheep and goats from different localities (Cairo, Dakahlia, Menia, Gharbia, Mounofya, Kalubia, Suez, Alexandria, Al-Fayoum and Ismailia) (8) samples (Buffy coat), (15) samples (Saliva), (31) samples (Nasal swabs), (4) samples tongue epith., (16) samples (Buccle swabs), (3) samples (Or pharyngeal fluid) and (10) samples (Rectal swab). were collected as a source of virological specimens, samples of tongue epithelium and buckle cavity and three vesicular fluid samples were collected from clinically suspected sheep and goats showed oral lesions by sterile scissor, and then were placed in sterile bottles containing 50% glycerin buffer saline PH 7.2, It was used for FMDV identification by, ELISA, trials of FMDV isolation on BHK-21 cell line and detection of FMDV RNA by polymerase chain reaction (RT-PCR) as shown in **table (1).**

Table (1). Number & types of Samples collected from Egyptian governorates for FMD virus Detection and Isolation:

Gov.	Buffy coat	Saliva	Nasal swabs	Tongue epith.	Buckle swabs	Oro-pharyngeal fluid	Rectal swabs	Total
Cairo	0	0	10	0	0	0	0	10
Dakahlia	0	6	10	0	0	0	10	26
Ismailia	0	0	6	0	3	0	0	9
Gharbia	6	0	0	0	9	0	0	15
Mounofya	1	0	5	0	3	0	0	9
Kalubia	0	8	0	0	0	0	0	8
Suez	1	1	0	1	0	0	0	3
Alexandria	0	0	0	0	0	3	0	3
Al-Fayoum	0	0	0	3	0	0	0	3
El-Menia	0	0	0	0	1	0	0	1
Total	8	15	31	4	16	3	10	87

Materials used for FMD virus isolation on BHK-21 cell line:

Tissue culture media and Solutions

Minimum Essential Medium (MEM Cell culture media).

Baby hamster kidney cell line **(BHK-21)** was obtained from Animal Health Research Institute, Dokki, Giza (Virology Department) was used for viru isolation **According to Adamowicz *et al.*, (1974) Clarke and Spier (1980).**

Detection FMD virus antigens by Antigen-Capture ELISA and serotyping A, O, C, ASIA, SAT 1, 2)

Supplied by Izsler- Itali

The assay is a sandwich ELISA performed with selected combinations of anti-FMDV monoclonal antibodies (MAbs), used as coated and conjugated antibodies.

The test can be applied for detection and typ-

ing of FMD viruses in homogenates of epithelium vesicles and vesicles fluid. Only in these clinical samples the FMD virus usually achieves the concentration required to provide a positive signal in ELISA assays.

The kit is designed for detection and typing of viruses of type O, A, C, Asia1, SAT1 and SAT2. A pan-FMDV test, detecting any isolates of type O, A, C, and Asia1 and addition some of SAT serotypes is also included in the kit to complement the specific typing and to detect FMD viruses which might escape binding to the selected type-specific MAb.

Microplates are supplied pre-coated with catching MAbs and positive inactivated controls are already incorporated onto plates.

Criteria for test validity

The positive navigated controls are expected to give OD values of **≥ 1.0** unit.

The negative controls for serotypes O, A, C, Asia1 and pan-FMDV are expected to give OD values **< 0.1.**

The negative controls for serotypes SAT1 and SAT2 are expected to give OD values **≤ 0.2.**

Interpretation of results

Results for the samples examined are interpreted as indicated in the table blew. After subtraction of the OD value of each negative control from the OD value measured for samples with the corresponding catching Mab.

Negative for FMDV	OD < 0.1.
FMDV positive type O	OD ≥ 0.1 with the type O MAb and with the pan-FMDV MAb. Some samples may cross-react with the 1st MAb type A, but OD values with MAb O are higher.
FMDV positive type A	OD ≥ 0.1with at least one of the two type A MAbs and with the pan-FMDV MAb
FMDV positive type Asia1	OD≥0.1with the type Asia1 MAb and with the pan-FMDV MAb
FMDV positive type C	OD ≥ 0.1with the type C MAb and with the pan-FMDV MAb
FMDV positive type SAT1	OD ≥ 0.1with the type SAT1 catching MAb Some samples could be positive also with the pan-FMDV MAb
FMDV positive type SAT2	OD≥0.1. 1with the type SAT2 catching MAb Some samples could be positive also with the pan-FMDV MAb
FMDV positive type (untyped)	OD ≥ 0.1with the pan-FMDV catching MAb and <0.1 with the type specific MAbs

Note: OD values **≥ 0.05** and **< 0.1** should be considered suspect and should be retested. New samples or further investigations by other diagnostic tests be required. An example of results

Identification of FMD virus by using RT PCR

Extraction of RNA from collected samples

Method for Extraction of RNA:

RNA extraction was carried out using the Patho Gene-spinTM DNA/RNA Extraction Kit (iNtRon biotechnology) according to manufacturer's instructions of the kit.

Agarose gel electrophoresis procedure

Using AmpliTaq Gold® 360 Master Mix. The PCR reaction mixture was adjusted to 25 µl as recommended by kit

Sequencing of the PCR products

Primers

FMD serotype	Primer	Sequences 5'-3'	Location	Amplified product
A	Short A			750 bp
	NK61	GAC ATG TCC TCC TGC ATC TG	2B	

Primers

FMD serotype	Primer	Sequences 5'-3'	Location	Amplified product
O	Short O			283 bp
	NK61	GAC ATG TCC TCC TGC ATC TG	2B	

Primers

FMD serotype	Primer	Sequences 5'-3'	Location	Amplified product
SAT2	SAT2 Fcl	GTAACCCGCTTTGCCATC	1D	288 bp
	SAT2 Rcl	CGCGTCGAATCTGTCTCTG	1D	

Data was applied by MARS (MARS is a new version of BMG LABTECH,S
Methods used for sequence reaction BigDye® Terminator v3.1 Cycle Sequencing Kit:
Thermal Profile (Fast PCR Machine) for Staphylococcus aureus enterotoxin (B,C)

Results

Eighty seven (87) samples from Buffy coat, saliva, nasal swabs, tongue pith., buckle swab, or pharyngeal fluid and rectal swabs were collected from Egyptian governorates (Alexandria, Dakahlia, Gharbia, mounofya, Kalubia, Cairo, Suez, Ismailia, Al-Fayoum and El-Menia) **Table (1)** were identified and serotypes using **indirect sandwich ELISA**. It was observed out of 87 samples (16) were +ve serotype A, (10) were +ve serotype O and (7) samples were Serotype sat2 as shown in **Tables (2).**

Table (2). Detection of FMDV antigen in samples of sheep and goat by using: FMD virus antigens detection ELISA serotyping (A, O, C, ASIA, SAT 1, 2)

Gov.	Total samples	Serotype A	Serotype O	Serotype SAT2	Total +ve
Cairo	10	2	0	0	2
Dakahlia	26	4	8	1	13
Ismailia	9	3	1	0	4
Gharbia	15	0	0	1	1
Mounofya	9	0	0	0	-
Kalubia	8	4	0	3	7
Suez	3	0	0	1	1
Alexandria	3	3	0	1	4
Al-Fayoum	3	0	1	0	1
El-Menia	1	0	0	0	-
Total	87	16	10	7	33

out of 87 samples (16) were +ve serotype A, (10) were +ve serotype O , (7) samples were Serotype sat2 ,

Isolation and Identification of FMD virus:
Isolation of FMD virus from samples collected from sheep and goats in Egyptian governorates:
From **33** positive FMDV samples by **ELISA**, were inoculated and adapted on BHK21cell line. **(16 +ve** samples) was observed that the CPE was developed early (within 24-48h) with rapid progression by two serial successive passages and the infected cell culture was characterized by cell rounding, cell aggregation and clusters and partially sheet detachment.. **Tables (3).**

Table (3). Virus isolation and adapted on BHK21 cell culture from +ve samples with FMDV antigen detection ELISA

Gov.	Total sample	Buffy coat	Saliva	Nasal swab	Tongue epith.	Buccle swabs	Oro-pha. fluid	Rectal swab	Total +ve
Cairo	2	0	0	1	0	0	0	0	1
Dakahlia	13	0	0	4	0	0	0	0	4
Ismailia	4	0	0	1	0	1	0	0	2
Gharbia	1	1	0	0	0	0	0	0	1
Mounofya	0	0	0	0	0	0	0	0	0
Kalubia	7	0	3	0	0	0	0	0	3
Suez	1	0	0	0	1	0	0	0	1
Alexandria	4	0	0	0	0	0	3	0	3
Al-Fayoum	1	0	0	0	1	0	0	0	1
El-Menia	0	0	0	0	0	0	0	0	0
Total	33	2	3	31	4	2	3	0	16

Table (3). Showed (16) out of (33) were isolated on T.C. from different samples

Characterization and Identification FMDV isolates:
Detection of FMDV antigen in isolates samples of sheep and goat by using: FMD virus antigens detection ELISA and serotyping (A, O, C, ASIA, SAT 1, 2):
Serological identification of **16** FMDV isolates by antigens detection ELISA and serotyping (A, O, C, ASIA, SAT 1, 2): revealed that FMDV is serotype A, O and SAT2 isolates was identified in 7, 4 and 1 samples respectively. **Tables (4).**

Tables (4). Identification of 16 FMDV isolates by antigens detection ELISA and serotyping (A, O, C, ASIA, SAT 1, 2)

Total samples	Serotype A	Serotype O	Serotype SAT2	Total +ve
16	**7**	**4**	**1**	**12**

Molecular diagnosis:

Results of reverse transcription PCR (RT – PCR) on extracted viral RNA:

RT-PCR was performed on the extracted RNA of FMDV isolates by using specific primer for O, A and SAT2 serotypes to amplify the VP1 coding region fragment of FMDV. All serologically ELISA positive isolates for FMDV serotypes O and Agave positive results with RT-PCR. While serotypes SAT2 gave negative. The FMDV type O resulted in a positive PCR signal at 283bP while FMDV type A gave a band at 750 bP with variable intenisity on ethidium bromide gel **(fig 1, 2)**

The results of PCR amplification revealed the 2 isolates from sheep samples for serotype O were positive as shown in (Fig1).

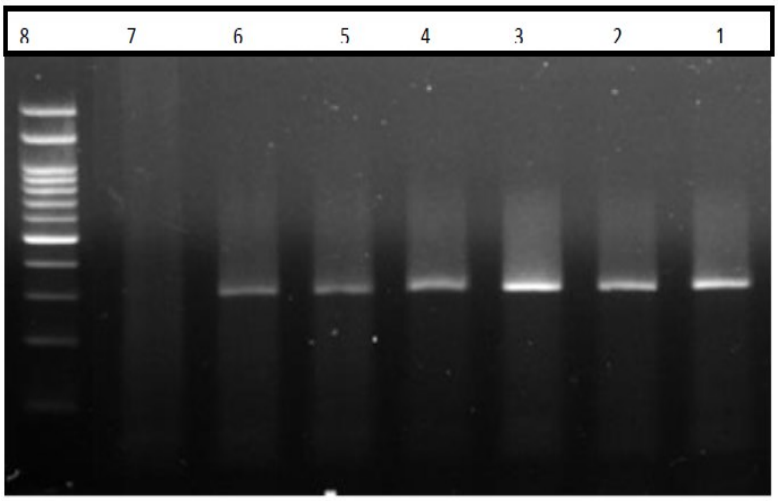


Fig. (1): GEL electrophoresis of FMD type O
5 positive samples
6 positive control
7 negative control
8 Marker 283bP

The results of PCR amplification revealed the 2 isolates from goat samples for serotype A were positive as shown in (Fig2).

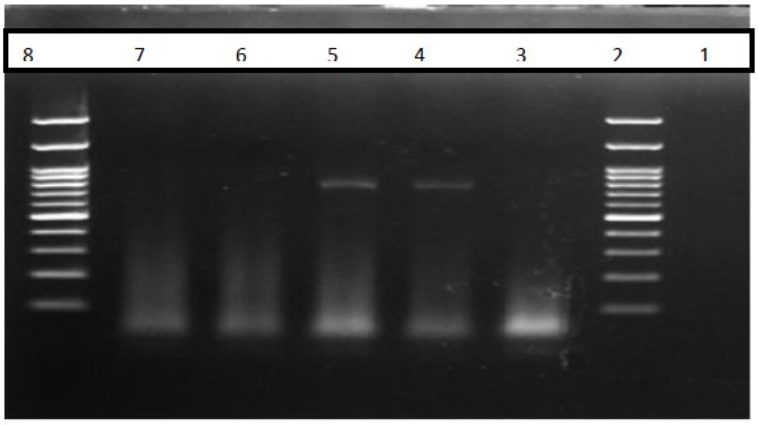


Fig. (2): GEL electrophoresis of FMD type A PCR
4 positive samples
5 positive control
6 negative control
7 Marker 750 BP

Results of phylogenetic tree of FMD local isolates with different known isolates

Three nucleotide sequences of the **2** isolates were aligned using Bio Edit Bioinformatics program version 7.0.8 after sequence trimming. The aligned fragment were utilized using different global FMDv isolates for construction of phylogenetic tree using MEGA (Molecular Evolution Genetic Rectalysis) program version 4.0 by neigh hour – Joining method.

Fig. (3): Sequencing and phylogenetic rectalysis of serotype O:-

O-EAST AFRICA 3
ATTCTCGACAGATTTGTGAAGGTAACACCACAACCCCAAACAAGCGTGTTGGAC-CTGATGCAGACCCCCCCACACACGCTGGTCGGGGCGCTCCTCCGCTCTGCTACCT ACTACTTTGCAGACCTTGAAGTGGCAGTGAAGCACGAGGGGAACCTCACGTGGGT CCCCAACGGGGCGCCAGAAACAGCTCTGGACAACACAACCAACCCAACAGCCTAC CACAAGGCACCACTTACTCGGCTTGCCCTGCCATACACGGCGCCACACCGCGTGC TTGCAACTGTCTACAACGGGGGTTGCAAGTACGGTGAGGCTCGGGAGACCAATGT GAGAGGTGACCTCCAAGTCTTGGCCCAGAAGGCAGCCCGAACAC

Fig. (4): Results of phylogenetic tree for serotype O

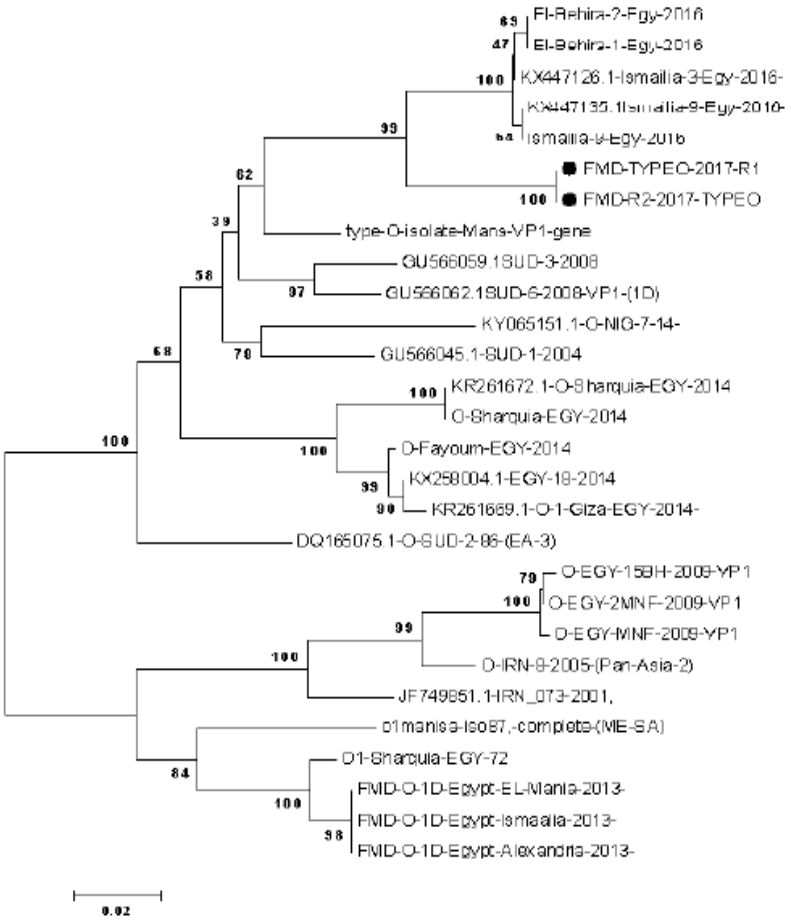
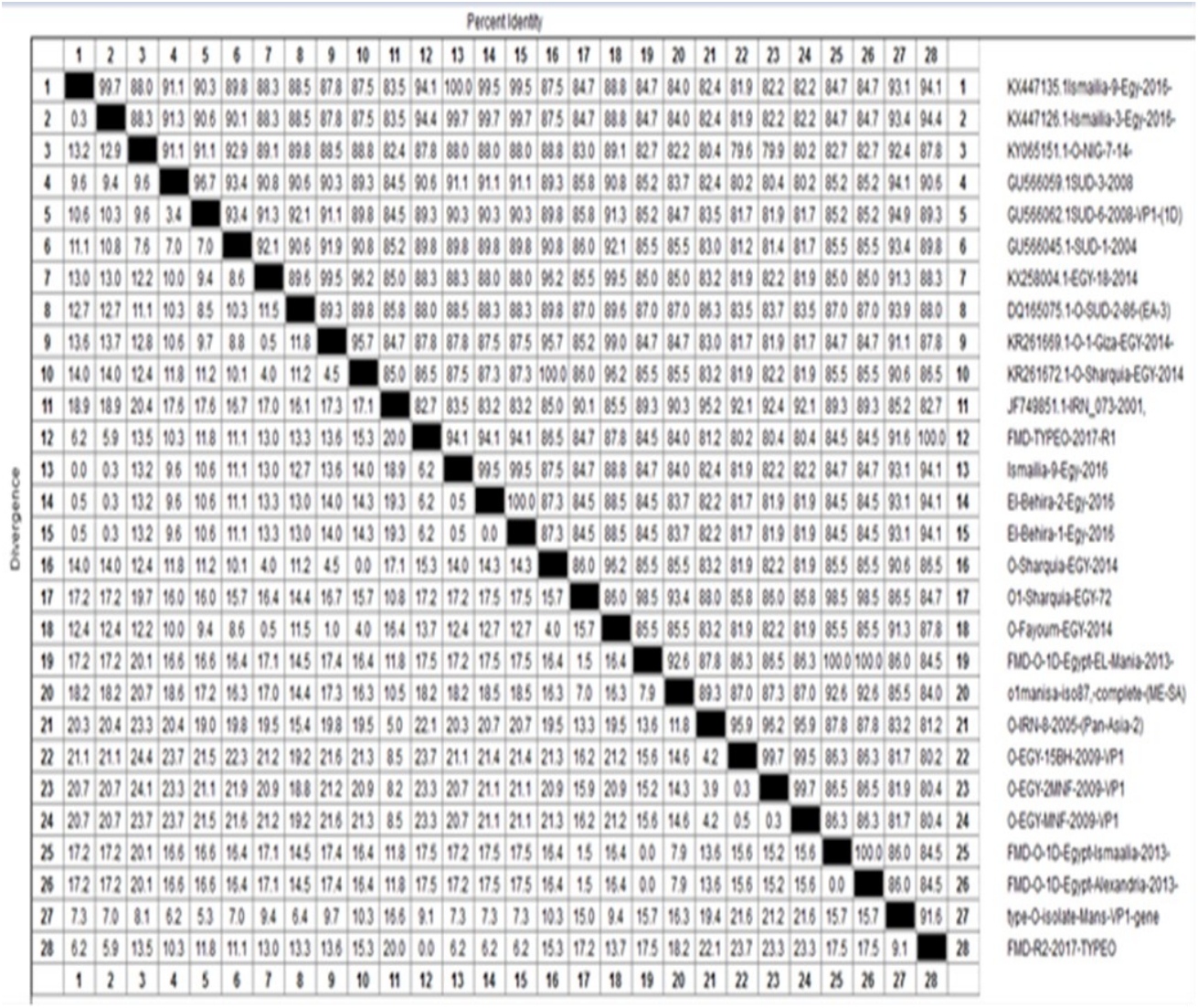


Figure (4). Phylogenetic tree for partial VP1 sequence of our FMDV type O local strains compared with other FMDV type O reference sequence obtained from Greenback database. The scale bar represents the number of substitutions per nucleotide. our FMDV type O local strains from the present study is indicated by black angular

Fig. (5): Identity and divergence % of type o



Percentage of nucleotides identity between FMD serotype O Egypt Strains with the published reference strains on Gene bank based on VP1

Results of nucleotide sequence of three representive positive samples for FMD serotype A

Three positive viral samples were selected for sequencing Sequencing, Editing and trimming all sequences was reanalyzed using Bio edit program version 7.0.8 for alignment. Phylogenetic tree was constructed for obtained sequence with represented strains of FMD extracted from the Gene Bank using MEGA program version 5.05 the obtained sequence and constructed tree are shown in **fig (2).**

Fig. (6): Sequencing and phylogenetic rectalysis of serotypeA:-
Sample no 1--fmd type A

CTTGTGTCTGTCGTGCGACAACACTTCCACTGCCAGCAGAGGCCTGGGGCAG-TAGAGTTCGGCACGTTTCATGCGCACGAGAAGCTCGTGGATGGTCGTGGCCCGGATTGCACCAAAGTTGAAAGAGCTGGGTAACTGTGCGGCGACCCGCGCCGCAAGAGACCCCAGGTCACCCCTTCTACCACCACCAGTTGCAGAGTACTTGCTTACTCCGTTGTACACCGTTGCCAACACTCGGTGTGGCGCGGTGTAAGGGAGCGCGAGTCTGGTAAATGGCTGCTTGTGGTAGGCGGTGGGGTTGCTTGTGTTGTCCAAGGCTTGTTCAGGTGCTCCATTGGGTACCCACGTCAAGTTGCCATCGTGACGCACCACAATCTCC

Fig. (7): Phylogenetic rectalysis of type A :-

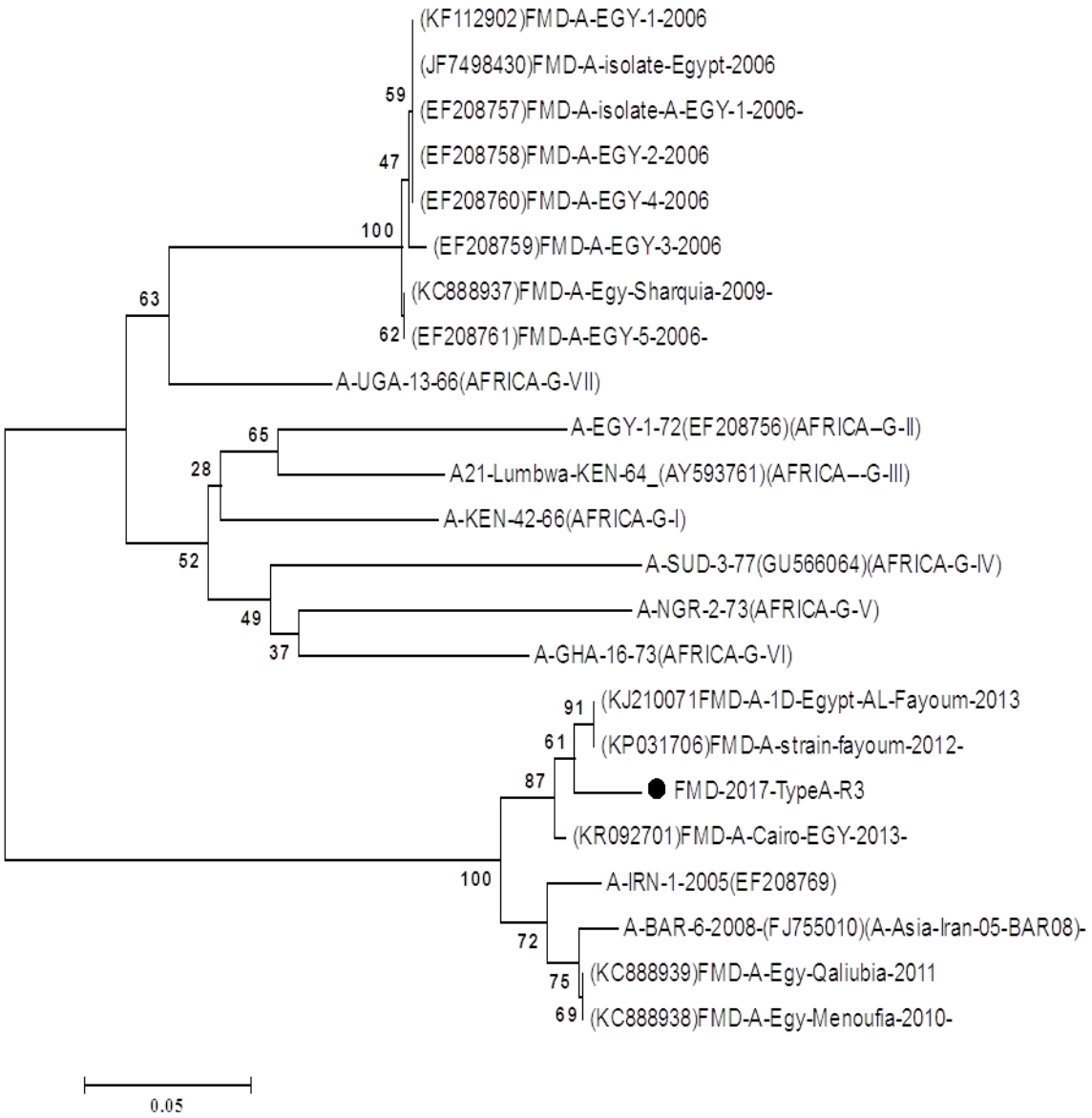


Figure (8). Phylogenetic tree for partial VP1 sequence of our FMDV type A local strains compared with other FMDV type A reference sequence obtained from GenBank database. The scale bar represents the number of substitutions per nucleotide. Our FMDV type A local strains from the present study is indicated by black rectangular.

Fig. (9): Identity and divergence %OF type A :--

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		
1		96.1	96.1	80.4	97.6	99.7	80.4	80.1	80.1	80.1	80.1	78.6	80.1	80.1	78.9	78.3	75.3	78.6	78.0	80.1	93.7	96.1	97.0	1	(KJ210071FMD-A-1D-Egypt-AL-Fayoum-2013
2	3.8		100.0	80.7	96.1	96.4	80.7	80.4	81.0	80.4	80.4	78.9	80.4	79.2	79.8	80.4	76.5	78.9	79.5	80.4	96.7	98.8	94.9	2	(KC888939)FMD-A-Egy-Qaliubia-2011
3	3.8	0.0		80.7	96.1	96.4	80.7	80.4	81.0	80.4	80.4	78.9	80.4	79.2	79.8	80.4	76.5	78.9	79.5	80.4	96.7	98.8	94.9	3	(KC888938)FMD-A-Egy-Menoufia-2010-
4	22.7	22.7	22.7		81.3	80.7	100.0	99.7	99.1	99.7	99.7	97.9	99.7	85.8	84.3	85.2	81.9	83.4	83.1	89.8	79.5	79.8	79.5	4	(KC888937)FMD-A-Egy-Sharquia-2009-
5	2.2	4.1	4.1	21.7		97.9	81.3	81.0	81.0	81.0	81.0	79.5	81.0	80.1	79.8	79.2	75.9	78.6	78.9	81.6	93.7	96.1	97.6	5	(KR092701)FMD-A-Cairo-EGY-2013-
6	0.0	3.7	3.7	22.7	2.1		80.7	80.4	80.4	80.4	80.4	78.9	80.4	80.1	79.2	78.6	75.6	78.9	78.3	80.4	94.0	96.4	97.3	6	(KP031706)FMD-A-strain-fayoum-2012-
7	22.7	22.7	22.7	0.0	21.7	22.7		99.7	99.1	99.7	99.7	97.9	99.7	85.8	84.3	85.2	81.9	83.4	83.1	89.8	79.5	79.8	79.5	7	(EF208761)FMD-A-EGY-5-2006-
8	23.2	23.1	23.1	0.3	22.2	23.1	0.3		99.4	100.0	100.0	98.2	100.0	85.5	84.0	84.9	82.2	83.7	82.8	89.5	79.8	79.5	79.2	8	(EF208760)FMD-A-EGY-4-2006
9	23.2	22.3	22.3	0.9	22.2	23.1	0.9	0.6		99.4	99.4	97.6	99.4	85.5	84.0	84.9	82.8	83.7	83.4	88.9	80.4	80.1	79.2	9	(EF208759)FMD-A-EGY-3-2006
10	23.2	23.1	23.1	0.3	22.2	23.1	0.3	0.0	0.6		100.0	98.2	100.0	85.5	84.0	84.9	82.2	83.7	82.8	89.5	79.8	79.5	79.2	10	(EF208758)FMD-A-EGY-2-2006
11	23.2	23.1	23.1	0.3	22.2	23.1	0.3	0.0	0.6	0.0		98.2	100.0	85.5	84.0	84.9	82.2	83.7	82.8	89.5	79.8	79.5	79.2	11	(EF208757)FMD-A-isolate-A-EGY-1-2006-
12	23.2	23.2	23.2	0.3	22.2	23.1	0.3	0.0	0.6	0.0	0.0		98.2	83.7	82.2	83.7	81.0	81.9	81.3	88.0	78.3	78.0	77.7	12	(KF112902)FMD-A-EGY-1-2006
13	23.2	23.1	23.1	0.3	22.2	23.1	0.3	0.0	0.6	0.0	0.0	0.0		85.5	84.0	84.9	82.2	83.7	82.8	89.5	79.8	79.5	79.2	13	(JF7498430)FMD-A-isolate-Egypt-2006
14	23.2	24.8	24.8	16.0	23.5	23.5	16.0	16.4	16.4	16.4	16.4	16.7	16.4		86.1	88.6	84.9	83.4	86.4	88.3	79.2	79.5	79.8	14	A-KEN-42-66(AFRICA-G-I)
15	24.9	23.8	23.8	17.9	23.8	24.8	17.9	18.3	18.3	18.3	18.3	18.7	18.3	15.7		88.3	81.9	83.1	84.0	85.2	79.2	79.5	78.9	15	A-EGY-1-72(EF208756)(AFRICA--G-II)
16	25.8	23.0	23.0	16.7	24.8	25.8	16.7	17.1	17.1	17.1	17.1	16.6	17.1	12.7	12.9		84.0	82.2	88.0	88.9	79.8	80.1	78.9	16	A21-Lumbwa-KEN-64_(AY593761)(AFRICA---G
17	30.2	28.6	28.6	21.1	29.5	30.1	21.1	20.7	19.8	20.7	20.7	20.3	20.7	17.1	21.1	18.3		82.2	85.2	85.5	76.2	76.8	75.0	17	A-SUD-3-77(GU566064)(AFRICA-G-IV)
18	25.5	25.4	25.4	19.2	25.9	25.5	19.2	18.8	18.8	18.8	18.8	19.2	18.8	19.3	19.8	20.9	20.8		85.5	82.8	79.2	78.3	78.3	18	A-NGR-2-73(AFRICA-G-V)
19	25.3	23.3	23.3	18.7	24.2	25.2	18.7	19.1	18.2	19.1	19.1	19.1	19.1	14.3	17.4	12.3	15.8	15.6		86.1	79.5	79.2	78.9	19	A-GHA-16-73(AFRICA-G-VI)
20	23.2	23.1	23.1	11.1	21.4	23.1	11.1	11.5	12.2	11.5	11.5	11.4	11.5	13.1	16.9	12.3	16.3	20.3	14.7		79.5	80.4	80.4	20	A-UGA-13-66(AFRICA-G-VII)
21	6.4	3.4	3.4	24.4	6.7	6.4	24.4	24.0	23.1	24.0	24.0	24.1	24.0	25.0	24.9	24.0	29.2	25.1	23.5	24.6		96.7	93.1	21	A-IRN-1-2005(EF208769)
22	3.7	1.2	1.2	23.9	4.0	3.7	23.9	24.3	23.4	24.3	24.3	24.4	24.3	24.4	24.3	23.5	28.3	26.4	23.8	23.1	3.4		94.9	22	A-BAR-6-2008-(FJ755010)(A-Asia-Iran-05-
23	2.8	5.4	5.4	24.3	2.5	2.8	24.3	24.8	24.8	24.8	24.8	24.9	24.8	23.9	25.1	25.2	31.0	26.3	24.2	23.0	7.4	5.3		23	FMD-2017-TypeA-R3
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		

Percentage of nucleotides identity between FMD serotype A Egypt Strains with the published reference strains on Gene bank based on VP1:

Discussion

Foot and mouth disease is a highly devastating and debilitating viral disease with highly contagious nature affecting cloven hoofed animals with an extremely wide host range including cattle, buffaloes, sheep, goats, pigs and camels and more than 70 wildlife species **(Alexanderson *et al.,* 2003; Jamal and Belsham, 2013).**

The disease is caused by 7 immunologically distinct serotypes, O, A, C, Asia 1, South African Territories (SAT) 1, SAT 2, and SAT 3, which belong to the species Foot-and-mouth disease virus (genus Aphthovirus, family Picornaviridae). Several of these serotypes circulate currently or periodically in the Middle East and North Africa **(Musser, 20004). (Knowles *et al.,*2003)** with no cross protection against others So it is important to take account of the characteristics of the different serotypes in controlling disease in endemic countries.

FMD virus has 4 structural proteins (SP) (VP1, VP2, VP3 and VP4). Different population of antibodies are elicited during viral replication in sheep directed against the non-structural proteins L, 2A, 2B, 2C, 3A, 3B, 3C and 3 D **(Brocchi *et al.,*1989; O I E, 2000; Kitching, R. P. 2002 and Pereda *et al.,* 2002),** regardless the sheep exhibited symptoms of disease or not **(Berger *et al.,* 1990).** (**Rweyemamu *et al.,* 2008).**

In Egypt, Three types of FMDV are endemic in Egypt and the numbers of outbreaks have increased in different provinces **(Ahmed *et al.,* 2012).** Between 1960 to 2005, only serotype O was reported in Egypt Where a routine prophylactic vaccination has been conducted with

A total number of (87) animals samples from different localities (Cairo, Dakahlia, Menia, Gharbia, Mounofya, Kalubia, Suez, Alexandria, Al-Fayoum and Ismailia.) as shown in **(Table 2).**

Samples were initial screened using antigen detection ELISA which is the preferred procedure for the detection of FMD viral antigen and characterization of viral serotype. ELISA preferred over Complement fixation test for detection and type differentiation of FMD viruses in epithelial samples, vesicular fluid and cell culture fluids because it is not affected by anticomplementary factors **(Hamblin *et al.,* 1984; Roeder and Le Blanc Smith, 1987; Ferris and Dawson, 1988)**

samples identified and serotypes using indirect sandwich ELISA. It was observed out of 87 samples (16) were +ve serotype A, (10) were +ve serotype O and (7) samples were Serotype sat2 as shown in **Tables (2).**

Data obtained in **Table (2)** in agreement with **Longjam *et al.,* (2011)** that demonstrate that ELISA could detect FMDV in (53.57%) and **Ibrahim *et al.,* (2015)** who stated that serotype O is predominant in Egypt and may be subjected to nucleotide sequence variability compared to the others so need continuous follow up. These findings were in agreement with the results of recent outbreaks occurred in Egypt where **Diab *et al.,* (2015)** stated that the outbreaks during 2013-2014 that occurred in some Egyptian governorates caused by the three FMDV serotypes O, A, SAT2 with high prevalence to serotype O with (6.8%). Also **Rady *et al.,* (2014)** who mentioned that the three FMDV serotypes O, A, SAT2 were responsible for 2012-2013 outbreaks that occurred in three northen Egyptian governorates where serotype O is the most predominant.

However, negative ELISA result not necessarily mean that the sample is truly negative as it may contain insufficient virus concentrations for the ELISA to detect. So, such suspensions are routinely inoculated into sensitive BHK cultures in attempts to amplify the virus and visualize CPE **(Reid *et al.,* 2001).**

From 33 positive FMDV samples by ELISA, were inoculated and adapted on BHK21cell line. (16 +ve samples) was observed that the CPE was developed early (within 24-48h) with rapid progression by two serial successive passages and the infected cell culture was characterized by cell rounding, cell aggregation and clusters and partially sheet detachment. **Tables (4).** Trails for propagation on BHK-21 cell line from 33 positive samples (by ELISA) that ex-

amined daily for 3 successive days for CPE **(Shawky *et al.,* 2001).** According to **Paixão *et al.* (2008**) viral isolation from vesicular fluid and oral epithelium on BHK-21 is the most reliable diagnostic method, So This result wasn't predicted. Negative results may be due to delayed reporting of disease and late sample collection, Secondary bacterial infection, physico-chemical stresses (elevated Temperatures, pH changes) which lead to a reduction in FMDV infectivity.

These results agree with agree with **Anderson (1969); Baranowski *et al.,* (2000); Mackay *et al.,* (2001) and Airaksinen *et al.,* (2003)**. Who reported that Buffy coat, OPF and Epithelial tissue were samples of choice for FMD virus isolation and BHK21 cells were always more sensitive than sheep tongue to infection with any of the strains of FMD but not in agree with **Marquardt *et al.,* (1995)** who reported that the nasal discharge is a suitable material to detect FMD by RT-PCR even at an easily stage of infection, when clinical signs have not yet developed.

Serological identification of **16 FMDV isolates** by antigens detection ELISA and serotyping (A, O, C, ASIA, SAT 1, 2) :revealed that FMDV is serotype A, O and SAT2 isolates was identified in 7, 4 and 1 samples respectively. **Tables (4).**

ELISA (enzyme-linked immune sorbent assay) and Virus isolation and are the gold standard tests for diagnosis of FMD based on their suitability to detect the presence of FMDV antigen in tissue samples but these methods are time consuming and their effectiveness for diagnosis is compromised as Virus isolation depend on the presence of infectious virus in sample while the ELISA can detect both infectious and non-infectious FMD viral antigen but in sufficient concentration (1-2 ng/ml) If neither of these two conditions is met then FMDV will not be recognized. Consequently, assays based on viral nucleic acid amplification have been developed where the Real Time-PCR procedures have been evaluated at the world reference laboratory (WRL) for the routine diagnosis of FMD virus where it can detect a small fragment of FMDV genomic RNA, not just live virus in low concentration with high sensitivity and in short time (one day compared to four days in ELISA/VI) **(Shaw *et al.,* 2004, Reid *et al.,* 2001) while Reid *et al.,* (2002),** stated that Real Time-PCR may have the greater sensitivity over the conventional RT-PCR procedure, ELISA and virus isolation for the diagnosis of samples containing low concentrations of virus which are neither detected by the ELISA nor produce a CPE in cell cultures with fast and quantitative assessment of the virus. Diagnosis of FMDV.

The Conventional reverse-transcriptase PCR RT-PCR is reliable, simple to perform method. However, it is not sufficiently superior to replace ELISA and virus isolation for the diagnosis of FMD virus but could be used in combination with them **(Reid *et al.,* 1999, 2000).** While (**Locher *et al.,* 1995)**, Stated that reverse transcription PCR followed by nucleotide sequence analysis of the PCR products is useful for the rapid detection and differentiation of foot-and-mouth disease virus.

In the present study RT-PCR was performed on the extracted RNA of FMDV isolates by using specific primer for O, A and SAT2 serotypes to amplify the VP1 coding region fragment of FMDV. All serologically ELISA positive isolates for FMDV serotypes O andA gave positive results with RT-PCR. While serotypes SAT2 gave negative **(fig 1 & 2).**

These results were in accordance with **Reid *et al.,* (2014) and** who used antigen detection ELISA, Virus isolation, Real time RT-PCR and Conventional PCR for FMDV detection in animal clinical samples.

In the current study three nucleotide sequences of the six isolates were aligned using **Bio Edit Bioinformatics program version 7.0.8** after sequence trimming. The aligned fragment were utilized using different global FMDv isolates for construction of phylogenetic tree using **MEGA** (Molecular Evolution Genetic Rectalysis) program **version 4.0** by neigh hour – Joining method.

Phylogenetic tree for partial VP1 sequence of our FMDV type O local strains compared with other FMDV type O belong to Topotype EA-3 (East Africa-3) reference sequence obtained from Greenback database. The scale bar represents the number of substitutions per movement and presence of area not covered by control programs with 98% nucleotide identity between each other and 86.4%, 89% identity respectively with O/sud/8/2008 (Kj831705.1) while (81-85%) nucleotide identity with vaccinal strains used O/EGY/3/93 (EU553840), O1/ Manisa/Turkey/69 (AJ251477), O1/ Sharquia/ EGY/72 (DQ164871). This result is in agreement with **(Rady *et al.,* 2014)** who detect the O -EA-3 topotype first appearance in Egypt at the end of 2013 and the beginning of 2014 that differ from the vaccinal strain used and recommend to include this Topotype in the vaccine for maximum protection also confirm it's introduction through un controlled trans boundary animals movement.

Consequently, as type O field strains (O-EA3 topotype) has high nucleotide divergence from the vaccinal strains used O/EGY/3/93 (EU5-53840), O1/Manisa/Turkey/69 (AJ251477), O1/ Sharquia/EGY/72 (DQ164871) with nucleotide identity (81-85%) and according to **(Samuel and Knowles, 2001)** the cut off value for the FMDV topotypes division is 15% and **(Jamal and Belsham, 2011)** there may be no complete cross protection between subtypes of the same serotype. So, there is a need to investigate the cross protection between detected strains and the currently used vaccine.

For the VP1 sequencing of the detected serotype A (3)Three positive viral samples were selected for Sequencing, Editing and trimming all sequences was rectalyzed using Bioedit program version 7.0.8 for alignment. Phylogenetic tree was constructed for obtained sequence with representive strains of FMD extracted from the Gene Bank using MEGA program version 5.05 the obtained sequence and constructed tree are shown in **fig (5 and 6).**

Phylogenetic tree for partial VP1 sequence of our FMDV type A local strains compared with other FMDV type A results revealed that it belongs to Asia topotype lineage A-Iran-05 reference sequence obtained from GenBank database. The scale bar represents the number of substitutions per nucleotide. Our FMDV type A local strains from the present study is indicated by black rectangular. Where it was closely related to the strains that cause outbreaks between 2009 and 2013 and the vaccine strain used in Egypt A/EGY/1/2012 (KC440882) by 96%. While it was different from historical serotype A strains identified in Egypt from A (African topotype) A/EGY/1/72 (EF208756) and A/EGY/1/2006 (KF112902) and A/EGY/1/ Sharquia/2009 (JF749843) that caused outbreaks in 1972 and between 2006 and 2009 . The Asian strain originated in Iran in 2005 and then spread to the nearby countries Pakistan, Turkey and then Bahrain 2008 **(Knowles *et al.,* 2009);** FMDV serotype A Asian topotype (Iran - 05 lineage) was the cause for different Egypt governorates outbreaks between the years 2009, 2010 and isolated in Egypt during 2012 outbreaks .

Conclusion

The continuous monitoring of FMDV genetic changes with establishment of national data base of the origin and FMDV genetic changes to control the disease and all-over country surveillance of the FMDV strains to study FMDV spread is a necessary issue **(EL-Shehawy *et al.,* 2011)**. Moreover, strict quarantine measures on imported live animal and animal products is of great necessity to prevent other FMDV serotypes incursion **(Balinda *et al.,* 2010).** With frequently conducted molecular epidemiology to FMDV to detect the virus origin of the emerged outbreaks and the mutation and evolution occurred to the virus (about 1% per year changes in the VP1 genes of the FMDV serotypes) **(Abd EL hamed *et al.,* 2011)** so effictive and appropriate FMD vaccines should be continuously changed after cross protection studies due to continuous introduction or **mutation** of new strains to protect against the disease. Also country has to

implement an effective vaccine strategy including financial supports and vaccination in remote area.

References

Anderson, E.C. (1969). Some observation of the serological response of pigstoemulsified EMD vaccine. European commission control FMD, Mtg. Res. P. stand. Tech. comm.. Brescia, Italy, 5: 177-190.

Anthony, E. and Wermer, P. (1992). Veterinary diagnostic virology. A practitioner's guide.

Alexandersen, S.; Zhang, Z.; Donaldson, A.I. and Garland, A.J. (2003). The pathogenesis and diagnosis of FMD. Journal of comparative pathology, 129, 1-36.

Ahmed, H.A.; Salem, S.A.H; Habashi, A.R.; Arafa, A.A.; Aggour, M.G.; Salem, G.H.; Gaber, A.S.; Selem, O.; Abdelkader, S.H.; Knowles, N.J.; Madi, M.; Valdazo- González, B.; Wadsworth, J.; Hutchings, G.H.; Mioulet, V.; Hammond, J.M. and King, D.P. (2012). Emergence of foot-and-mouth disease virus SAT 2 in Egypt during 2012. Transbound. Emerg. Dis. 59: 476–481.

Baranowski, E.; Rwiz – Jarbo, C.M.; Sevilla, N.; Andrew, D.; Beck, E. and Domingo, E. (2000). Cell recognization by foot and mouth disease virus that lacks the RGD integrin-binding motsf: flexibility in Aphthovirus receptor usage. J. Virol. 47, 1641-1647**.**

Berger, H.G.; Straub, O.C.; Ahl, R.; Tesar, M. and Marquardt, T.O. (1990). Identification of foot and Mouth Disease virus replication in vaccinated cattle by antibodies to non-structural virus proteins. Vaccine, 8 (3): 213-219.

Bronsvoort, B.M. dec; Sorensen, K.J.; Anderson, J.; corteyn, A.; Tanya, V.N.; Kitching, R.P. and Morgan, K.L. (2002). Comparison of two 3 ABC ELISAs for diagnosis of multiple serotype foot and mouth disease in a cattle population in an area of endemicity. J. CliMicrobiol., 42: 2108-2114.

Brocchi, E.; De Diego, M.T.; Berlinzni, A.; Gamba, D. and Desimone, F. (1998). Diagnostic potential of mab-based ELISAS for antibodies for antibodies to non-structural proteins of foot and Mouth Disease virus to differentiate infection from vaccination. Vet. Q., 20: 520-524.

Carroll, A.R.; Rowlands, D.J. and Clark, B.E. (1984). Nucleic acids Res-, 12; 2461-2472.

FAO (Food and Agricultural organization of the United Nations) (2002). Preparation of FMD contingency plans, ISBN 92-5-104867-3.

Ghoneim, N.H.; A.K. Abdel-Karim; L. El-Shehawy and K.A. Abdel-Moein, (2010). Foot and mouth disease in animals in Sharkia governorate Egypt. Transboud Emerg. Dis., 57: 19-21.

Jamal, S.M. and Belsham F.J. (2013). FMD: past, present and future. Veterinary Research 2013, 44:116. http://www.veterinaryresearch.org/content/44/1/116.

Knowles, N.J. and Samuel, A.R. (2003). Molecular epidemiology of foot and mouth disease virus. Virus Res. 91: 65-80.

Knowles, N.J.; Samuel, A.R.; Davies, P.R.; Midgley, R.J. and Valarcher, J.F. (2005). Evolution and spread of a pandemic strain of FMDV serotype O emerging infectious diseases 11: 1887-1893.

Kasambula, L; Belsham, G.J.; Siegismund, H.R; Muwanika, V.B; Andemunokurut. A.R. and Masembe, C. (2012). Serotype identification and VP1 coding sequence anal-

ysis of FMDV from outbreaks in eastern and Northern Uganda in 2008/9: transboundemerg. Dis 2012; 59: 323-30

Kitching, R.P. (2002). Problems of diagnosis of foot and mouth disease in domestic animals. In foot and mouth disease, control strategies, symposium proceedings, 2-5 June 2002, Lyon, France: 353-359.

Locher, F.; Suryanarayana, V.V.S. and Tratschin, J.D. (1995). Rapid detection and characterization of FMDV by restriction enzyme and nucleotide sequence analysis of PCR products Journal of clinical Microbiological, 33: 440 – 444.

Loeffler, F.; LU, Z.; Cao, Y.; Bao, H.; Qis, Guo, J.; Shang, Y.; Jiang, T.; Zhang, Q.; Ma, J.; Liu, Z.; Liu, X.; Yin, H. and Xie, Q. (2008). Techniques developed in china for foot and – mouth disease diagnosis, Transbound Emerg Dis. Aug. 55 (5-6): 196-9.

Mackay, D.K.; Vorsytha, M.A.; Davis, P.R.; Belizan, A.; Belsham, G.J.; Flint, M. and Ryan, M.D. (1998a). Differentiation infection from vaccination in foot and Mouth Disease using a panel recombinant, non-structural protein in ELISA. Institute for Animal Hlth, Pirbright Laboratory, Working, Survey, UK Davio. Vaccine, 16 95): 446-459.

Mackay, D.K.J.; Forsyth, M.A.; Davies, P.R. and Salt, J.S. (1998b). Antibody to non -structural proteins of foot and Mouth Disease virus in vaccinated animals exposed to infection. Vet. Q, 20: 9-11.

Mackay, B.; Pattinaik, B., Toch, C.; Sanyal, A.; Hemadri, D.; Patil, S.S. and Venkatarramanam, R. (2001b). Genetic and antigenic analysis of type A foot and mouth disease viruses isolated in India during 1987-1996 Acta. Virol. 45, 13-21.

Marquardt, O.; Straub, O.C., Ahi, R. and Hass. B. (1995). Detection of FMDV in nasal swabs of asymptomatic cattle by RT-PCR within 24 hours. J. of virol. Methods, 53; 255 -261.

Michael, P.; Ward, Shawn W.; Laffan and Lina D. High field (2007). The potential role of wild and feral animals as reservoirs of foot and mouth disease. Preventive. Veterinary, medicine. 80, 9-23.

Musser, J.M. (2004). A practitioner's primer on FMD J. Am. Vet. Med. Assoc.; Apr. 15, 224 (8): 1264-1268.

FAO (Food and Agricultural organization of the United Nations) (1984). Emerging disease of livestock. Vol1 the disease and their diagnosis and Geering W.A., ed. FAO, Rome, Italy. 43-51.

Reid, S.M.; Hutchings, G.H.; Ferris, N.P. and De Clercq, K. (1999). Diagnosis of foot -and-mouth disease by RT-PCR: evaluation of primers for serotype characterization of viral RNA in clinical samples. Journal of Virological Methods, 83, 113 – 123.

Reid, S.M.; Ferris, N.P.; Hutchings, G.H.; Samuel, Ar. and Knowles, N.J. (2000). Primary diagnosis of foot – and – mouth disease by reverse transcription polymerase chain reaction. J. Virol Methods. 89 (1-2): 167-67.

Reid, S.M.; Ferris, N.P; Hutching, G.H.; Clercq, R.D; Newman, B.J; Knowles. N.J; and Samuel, A.R. (2001). Diagnosis of foot and mouth disease by RT-RCR use of phylogetic data to evaluate primers for typing of viral RNA in clinical samples archives of virology 146; 2421-2434.

Reid, S.; N.J. Brownie and D.P. King (2014). Development of tailored real-time RT-PCR assays for the detection and differentiation of serotype O, A, Asia1 FMDV lineages circu-

lating in the Middle East. Virological Methods, 207: 146 –153.

OIE/FAO / WHO, (1996). Manual of standards for diagnostic tests and vaccnes.

Rodriguez, A.; Numez, J.I.; Nolasco, G.; Ponz, F.; Sobrino F. and DE Blas, C. (1994). Direct PCR detection of foot and Mouth disease (Virus Jornal of virological Methods 47 (345-349).

Paixao, T.A.; Neta, A.V.; Paiva, No.; Reis, J.R.; Barbosa, M.S.; Serra, C.V.; Silva, R.R.; Beckham, T.R.; Martin, B.M.; Clarke, N.P.; Adams, L.G. and Santos, R.L. (2008). Diagnosis of foot – and mouth disease by real time reverse transcription polymerase chain reaction under field conditions in Brazil BMC Vet. Res. Dec. 31; 4:53.

Pereda, A.J.; Konig, G.A.; ChimenoZoth, S.A.; Borca, M.; Palma, E.L. and Piccone, M.E. (2002). Full length nucleotide sequence of foot and mouth disease virus strain O1 compos/bra/ 58.

Rweyemamu, M.; Roeder, P.; Mackay, D.; Sumption, K.; Brownlie, J.; Leforban, Y.; Valarcher, J.F.; Knowles, N.J. and Saraiva, V. (2008). Epidemological pathogens of FMDV selects viruses that binds to heparin and are attenuated in cattle J. Virol. 71: 5115 – 5123.

Saiz, M.; Nunez, J. and Jimenez – Clavero, Metal (2002). FMD; biology and prospects for disease control. Microbes Infect., 4 (11); 1183-1192.

Samuel, A.R. and Knowles, N.J. (2001a). Foot and mouth disease virus cause of the recent crisis for the UK Lives tock industry.

Samuel, A.R. and Knowles, N.J. (2001b). Foot and mouth disease type O virus exhibit genetically and geographically distinct evolutionary lineages (topotype) J. General virology 82, 609-621.

Shawky, M.; Ismail, I.; Abd El-Aty, M.M.; Hassan, N.M. and Daoud, A.M. (2001). Isolation and idertification of FMD virus in clinical samples from Ismailia and Menofia governorates in Egypt during year 2000. J. Egypt. Vet. Med. Ass., 61: 251-257.