Serological and Molecular diagnosis of FMD virus in Sharkia and Fayoum governorates 2014

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ABSTRACT

Foot and mouth disease (FMD) is the most contagious disease of mammals and has a great potential for causing severe economic loss in susceptible cloven-hoofed animals. There are seven serotypes of FMD virus (FMDV), namely O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. Tests for antibodies of FMDV nonstructural proteins (NSPs) are useful in providing evidence of previous or current viral replication in the host, irrespective of vaccination status. NSPs, unlike structural proteins are highly conserved and therefore are not serotype specific and as a consequence, the detection of these antibodies is not serotype restricted. The current study aimed on detection of early NSPs in apparently healthy cattle, and detects FMDV by real-time RT-PCR and Indirect sandwich ELISA in suspected samples. The serum samples were collected from Sharkia and Fayoum governorates (30 and 40 sera respectively) submitted to laboratory examination by Priochek for NSPs (3ABC) 32 out of 70 were positive (12 sera samples from Sharkia and 20 sera samples from Fayoum), while 22 out of 70 were positive by real time RT-PCR (10 sera samples from Sharkia and 12 sera samples from Fayoum). Definitive diagnosis of foot and mouth disease requires the detection of virus antigen or genome. So, the vesicular epithelial tissues collected from infected cattle from Sharkia (7) and Fayoum (5) governorates were subjected to antigen ELISA and RT-PCR. 8 epithelial tissues were identified by Indirect sandwich ELISA. The results were 5 and 3 positive from Sharkia and Fayoum respectively, while 12 samples were positive by RT-PCR. In conclusion, this study demonstrates that real-time RT-PCR currently used at the World Reference Laboratory (WRL) for FMD provides an extremely sensitive and rapid additional procedure for improved laboratory diagnosis of FMDV. Moreover, Real time PCR is an important feature when definitive diagnostic results are required in a short timescale during emergencies. Current study on serum samples and epithelial tissues demonstrate the circulation of the FMDV type A and O in Sharkia and Fayoum Governorates.

INTRODUCTION

Foot and mouth disease is one of the most contagious animal diseases caused by FMDV which belongs to the genus *Aphthovirus* of the family *Picornaviridae* (*Pereira, 1981)* and has a great potential for causing severe economic losses in susceptible cloven-hoofed domesticated (cattle, pigs, sheep, goats and water buffalo) and wild animals *(Alexandersen et al.; 2003).* FMD is widespread throughout the world, particularly in Africa, Asia, and the Middle East. There are seven serotypes of FMDV, namely O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. *(Grubman and Baxt, 2004).* Clinical signs can vary from mild to severe, and fatalities may occur especially in young animals in high rate due to severe lesion in the myocardium *(Domingo et al.; 1990)* FMD cannot be differentiated clinically from other vesicular diseases, such as swine vesicular disease,

vesicular stomatitis and vesicular exanthema *(Rémond et al.;2002).*

Diagnosis of FMD is depending on virus isolation or by the demonstration of FMD viral antigen or nucleic acid in suspected samples of tissue or fluid. Detection of virus-specific antibody can also be used for diagnosis, and antibodies to viral nonstructural proteins (NSPs) can be used as indicators of infection, and irrespective of vaccination status.

Over the past several decades, a series of diagnostic methods have developed that can provide rapid and accurate isolation and identification of serotype or genotype of FMDV and are suitable for large-scale serological surveys, which will be presented in detail. Thus, diagnostic methods play an even more important role than vaccination for FMD control and securing (*Verma et al., 2012*).

MATERIAL AND METHODS:

1- Serum samples:

A total of 70 serum samples were collected from Sharkia and Fayoum governorates (30 and 40 sera respectively) from apparently healthy and infected cattle for detection of NSPs of FMDV antibodies by ELISA and FMDV antigen by Real time-PCR. The commercial PrioCHEK®FMDV- NS supplied by Prionic Sweden for in vitro detection of antibodies against FMDV in serum of cattle, sheep, goat and pigs. The kit is performed according to (*Sorensen et al., 1998)* instructors. Samples give percent of inhibition PI <50% considered negative (antibodies against the NS protein of FMDV are absent in the serum sample) and that give PI =>50% considered positive (antibodies against the NS protein of FMDV are present in the serum sample).

2- Epithelial tissue suspension:

Epithelial tissue samples were preserved in equal volume of glycerol – buffer saline and transported in ice box at 4ºC to Virology Department, Animal Health Research Institute - Dokki. The epithelial tissue samples were prepared by grinding and centrifugation to obtain 0.2ml of the supernatant for virus antigen detection and serotyping by Indirect sandwich ELISA and Real time-PCR according to (Kitching and Doanldson, 1987 and Reid *et al.*, 2002).

3-Serotyping of serum antibodies:

The typing of serum antibodies against FMD three serotypes (A, O, and SAT2) which were done by liquid phase blocking ELISA (LPBE) supplied by BDSL-Pirbright Laboratory (WRL) UK according to *(Hamblin et al. 1986 a, b).*

4-Indirect sandwich ELISA for FMDV antigen:

The typing of FMDV antigen types A, O, SAT_2 according the protocol of OIE/FAO WRL for FMD, Pirbright Laboratory, UK according to ***(Peter and Elizabeth. 1985).***

5- RNA extraction

RNA extraction was performed on 70 serum samples (30 and 40 sera from Sharkia and Fayoum respectively) and 12 vesicular epithelial tissues (7 and 5 from Sharkia and Fayoum respectively) using GeneJET nucleic acid purification kits (Thermo Scientific) according to the manufacturer's protocol. After extraction, RNA was stored at -20°C.

6- RNA amplification in real time PCR:

At first, the qRT-PCR were performed on all samples by using common primer and probe used for detecting FMDV fragment 3D region in all of the FMDV serotypes ***(OIE, 2012).***

The positive samples were subjected to serotyping by real time PCR using three sets of primers and probes specific for A, O and SAT2 serotypes of FMDV (Table 1).

Real-time RT-PCR probe and primers.

Quantitative real time PCR was performed on each sample using Precision OneStepTM qRT-PCR Mastermix with ROX (Primerdesign. co. Uk, cat. No. OneStep -R) as manufacturer's instructions. Reaction mixture was composed of 2ul of RNA template and 18ul of a master mix in a tube. The master mix was composed of 10ul of 2x Precision OneStepTM qRT-PCR Mastermix, 10 PM of each primer, 5 PM of probe, and sterile distilled water till reach 20ul. The optimized cycle program of denaturation, annealing and extension temperature was as follows: initial denaturation the cycling parameters were 55°C for 10 min. 1 cycle, 95°C for 10 min. 1 cycle; then 50 cycles consisting of 95°C for 15 sec. and 60°C for 1 min.. Negative control specimen was involved. Thermocycler applied biosystem 7000 was used for real time detection of FMDV *(OIE 2012).*

Table 1: Oligonucleotide sequences of used primers and probe specific for FMDV

	Sequences
3D F	ACT GGG TTT TAC AAA CCT GTG A
3D R	GCG AGT CCT GCC ACG GA
3D P	FAM- TCC TTT GCA CGC CGT GGG AC -TAMRA
A F	ACG ACC ATC CAC GAG CTY
A R	RCA GAG GCC TGG GAC AGT
A P	FAM-CGT GCG CAT GAA ACG TGC-TAMRA
O F	CCG AGA CAG CGT TGG ATA ACA
O R	CCA TAC TTG CAG TTC CCG TTG T
O P	FAM-CCG ACT TGC ACT GCC TTA CAC GGC-TAMRA
SAT2-F	TGA AGA GGG CTG AGC TGT ACT G
SAT2-R	CTC AAC GTC TCC TGC CAG TTT
SAT2 P	FAM- ACA GAT TCG ACG CGC CCA TCG-TAMRA

RESULTS

The results in (Table 2) showed that 12 out of 30 sera samples from Sharkia and 20 out of 40 sera samples from Fayoum were positive for 3 ABC nonstructural proteins in parallel with RT-PCR where 10 out of 30 sera samples from Sharkia and 12 out of 40 sera samples from Fayoum were positive. The results of detection of FMDV antigen by ELISA were 4 type A and 1 type O from Sharkia and 2 type A and 1 type O from Fayoum, while detected viruses by RT-PCR were 5 type A and 2 type O from Sharkia and 3 type A and 2 type O from Fayoum.

The results in (table 3) showed that the highest level of FMDV antibodies serotype A (27) out of 70 sera samples (12 from Sharkia and 15 from Fayoum), while FMDV antibodies serotype O (36) out of 70 sera samples (11 from Sharkia and 25 from Fayoum) and FMDV antibodies serotype SAT2 (13) out of 70 sera (3 from Sharkia and 10 from Fayoum).

Table (2): detection of FMDV in different samples by different methods and NSPs in sera.

Type of samples / governorates		**No. of samples**	**Indirect sandwich ELISA**			**NSP-3ABC**	**Real time-PCR**			
							common primer and probe	**primer and probe specific for each serotypes**		
			A	**O**	**SAT2**			**A**	**O**	**SAT**
			No. of +ve samples							
Serum samples	**Sharkia**	30	N.	N.D	N.	12	10	7	3	-
	Fayou	40	N.	N.D	N.	20	12	5	7	-
Total	-	70	-	-	-	32	22	12	10	-
Epithelial tissue	**Sharkia**	7	4	1	-	N.D	7	5	2	-
	Fayou	5	2	1	-	N.D	5	3	2	-
Total	-	12	6	2	-	-	12	8	4	-

Table (3): serological investigation of FMDV antibodies serotypes A, O, SAT2 by blocking Elisa (LPBE)

Governorate	**No of Sample**	**Titer**								
		A			**O**			**SAT2**		
		8	**16**	**32**	**8**	**16**	**32**	**8**	**16**	**32**
Sharkia	**30**	9	9	12	-	19	11	8	19	3
Fayoum	**40**	6	19	15	-	15	25	10	20	10
Total	**70**	15	28	27	-	34	36	18	39	13

As shown in figures 1 and 2; the results showed that 22 out of 70 serum samples (10/30 from Sharkia and 12/40 from Fayoum) were identified by real time RT-PCR, whereas 32 of those samples were positive by NSP-3ABC, indicating comparable sensitivity between these diagnostic methods. While all 12 epithelial tissues (7 from Sharkia and 5 from Fayoum) were identified by real time RT-PCR, whereas only 8 of those samples were positive by Ag ELISA. Serotype A and O were confirmed by qRT-PCR in both serum (22) and epithelial tissue (12) samples (Table 2 and Fig. 1 & 2).

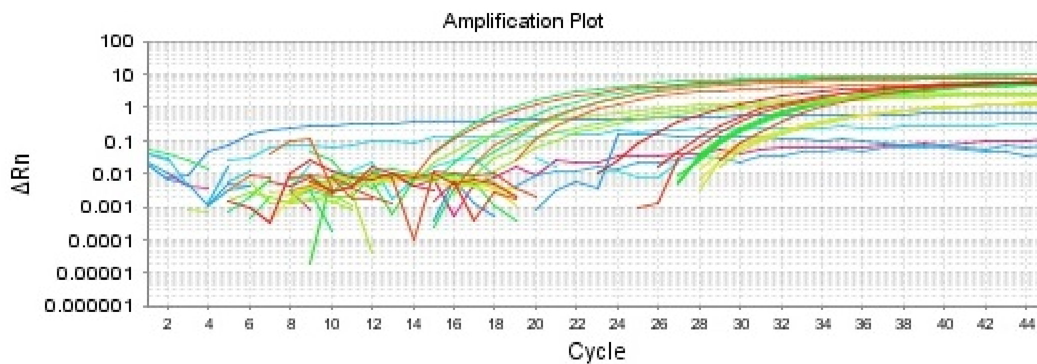

Fig. (1): Amplification plot of real time RT-PCR of FMDV samples by using common 3D primer and probe.

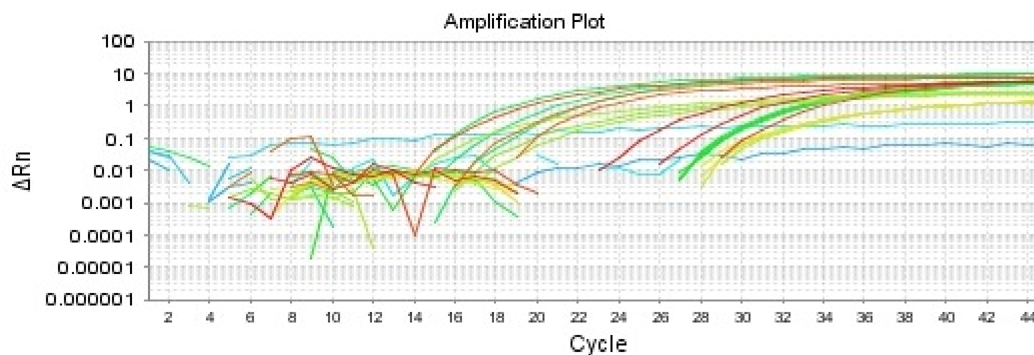

Fig. (2): Amplification plot of real time RT-PCR of FMDV samples by using primer and probe specific to A, O and SAT2 FMDV.

DISCUSSION

ELISA has been recommended laboratory procedures for FMD diagnosis for nearly twenty years based on their suitability to detect the presence of FMDV antigen in tissue samples. Recently, the development of a real-time reverse transcription polymerase chain reaction (RT-PCR) procedure has provided an additional tool which can be used for FMD diagnosis *(Reid et al., 2002).*

Indirect sandwich ELISA is more sensitive, specific in both detection and serotyping of FMDV as demonstrated by (*Veerasami et al., 2008).* Conventional serological tests cannot differentiate FMDV vaccinated from infected animals, so a blocking ELISA that differentiated FMDV infected animals from vaccinated animals was developed which uses baculovirus expressed FMDV 3ABC non-structural protein as antigen and monoclonal antibody against FMDV 3ABC non-structural protein as capture and detector antibody. Sera from vaccinated and infected cattle, sheep and pigs were examined. The specificity of the test was high *(Sorensen et al., 2005).* Detection of FMD virus nonstructural proteins (NSPs) antibodies could be taken as a potential indicator for positive cases that exposed to natural infection with FMD virus while negative cases

means that these animals were uninfected *(Bronsvoort et al., 2004).*

The results achieved by different types of ELISA and real time RT-PCR are summarized in Table (2) for the comparison of the two assays. A total of 32 out 70 serum samples (12 out of 30 sera samples from Sharkia and 20 out of 40 sera samples from Fayoum) were positive for 3 ABC nonstructural proteins in parallel with RT-PCR where 22 out of 70 serum samples (10 out of 30 from Sharkia and 12 out of 40 from Fayoum) were identified by real time RT-PCR.

Definitive diagnosis of foot-and-mouth disease requires the detection of virus antigen or genome in clinical material. So, the vesicular epithelial tissues which collected from infected cattle from Sharkia (7) and Fayoum (5) governorates were subjected to Indirect sandwich ELISA and RT-PCR. 8 out of 12 epithelial tissues were identified by Indirect sandwich ELISA. The results were 5 and 3 positive from Sharkia and Fayoum respectively, while 12 samples were positive by RT-PCR. These detected viruses by Indirect sandwich ELISA were 4 type A and 1 type O from Sharkia and 2 type A and 1 type O from Fayoum, while detected viruses by RT-PCR were 5 type A and 2 type O from Sharkia and 3 type A and 2 type O from Fayoum. All samples assigned positive by RT-PCR, where 4 samples were negative by antigen ELISA (Table 2). ELISA can detect both infectious and non-infectious FMD viral antigen, The difference between the two techniques due to ELISA is dependent upon the antigen being present in sufficient concentration (1 ng/ml to 2 ng/ml of antigen or 5 log^{10}/ml to 6 log^{10}/ml of live virus) to work. If neither of these two conditions is met then FMDV will not be recognized by ELISA. Ideally, vesicular epithelium should be collected from an animal during the acute stage of FMD when the concentration of virus associated with the sample is high *(Shaw et al., 2004).*

Table (3) showed the titer of FMDV antibodies serotypes O, A and SAT2 by Liquid Phase Blocking ELISA (LPBE). The results showed the highest level of serotype O (36), A (27) and SAT2 (13) out of 70 sera respectively. Vaccination against FMD is obligatory by inactivated local trivalent vaccine (produce by VSVRI) in Egypt. The sero-diagnosis results of serotypes O, A and SAT2 may be due to vaccination or due to the exposure to one or more of endemic serotypes in Egypt *Grubman and Baxt (2004).*

Real time RT-PCR can detect a small fragment of FMDV genome RNA, not just live virus. Real-time RT-PCR provides an extremely sensitive and rapid procedure that contributes to improve laboratory diagnosis of FMDV *(Bernd, et al 2009).*The positive samples which identified by real time RT-PCR, importantly, showed no false-negative results since none of the samples was positive by ELISA and negative by real time RT-PCR.

Regarding the identification of the detected FMD virus, ELISA and qRT-PCR; using specific primers; confirmed that the obtained virus isolate is A and O. These findings indicate that the results of qRT-PCR is more sensitive and supported by *(Andrew et al. 2007)*, they showed that qRT-PCR is more rapid and sensitive technique suitable for detection and identification of FMDV. *Callahan et al. 2002* reported that a method based on real-time RT-PCR amplification and a fluorescent probe demonstrated high specificity and

sensitivity for the diagnosis of FMD under field conditions in Brazil.

In conclusion, the study demonstrates that real-time RT-PCR currently used at the WRL for FMD provides an extremely sensitive and rapid additional procedure for improved laboratory diagnosis of FMD. The RT-PCR generated results in less than one day from test commencement, in contrast to up to four days to define some positive and all negative samples by combined use of classical method for virus isolation. This is an important feature when definitive diagnostic results are required in a short timescale during emergencies. Also this study demonstrates that the most circulating FMDV in Sharkia and Fayoum Governorates from type A and O.

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