Real time RT-PCR assay for detection of different serotypes of FMDV in Egypt

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Abstract

Aim: The present study indicated that rRT-PCR could be provided for the detection of FMDV in infected, contact and carrier cattle and also provide a rapid sensitive tool aiming to aid in rapid disease detection and control. Foot and Mouth disease virus serotypes O_1 and A still existing in Egypt. In January 2012, sever outbreaks struck the animal population in most Egyptian governorates. The causative virus was identified as FMDV SAT2.

Material and Methods: Five samples of tongue epithelium (ET) and five oesophageal-pharyngeal (OP) fluid samples were collected from FMD suspected cattle in infected farm at El-Fayoum and 20 OP samples from in-contact cattle at the same farm in addition to 30 OP samples from apparently healthy cattle at three different localities in El-Fayoum governorate (12 from Fayoum; 9 from Sinoras and 9 from Edsa) in order to detect carrier cattle. All of these samples were collected during November and December 2011 and January 2012.

Results: All the ET and OP samples were inoculated on BHK cell culture and baby mice. The obtained results were identified using complement fixation test in addition to real-time reverse transcriptase polymerase chain reaction (rRT-PCR). In the infected farm at El-Fayoum FMDV type SAT2 was detected in cattle which are considered as the first introduction of this type while FMDV type O and SAT2 were detected in the in-contact cattle in the same farm. The sensitivity of rRT-PCR was cleared in the in-contact cattle as 13 out of 20 OP samples were positive to FMDV by rRT-PCR while 11 out of 20 OP samples were positive to FMDV by CFT. The OP samples collected from apparently healthy cattle from Fayoum, Sinoras and Edsa localities in Fayoum governorate demonstrate the circulation of the FMDV type A, O and the recent SAT2 in carrier cattle which threaten cattle population in Fayoum governorate. Also the sensitivity of real time RT-PCR over the CFT in detection of FMDV carrier cattle was clearly noticed in these localities as 19 out of 30 OP samples were positive by rRT-PCR while in contrast there were only 16 out of 30 samples positive by CFT.

Conclusion: In conclusion, this 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 especially in-contact and carrier cases. The rRT-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 CFT and virus isolation. This is an important feature when definitive diagnostic results are required in a short timescale during emergencies. Also this study demonstrates the current situation of FMDV circulating in EL-Fayoum governorate and the introduction of new SAT2 serotype beside type A and O.

Key words: carrier, cattle, FMDV, Isolation, rRT-PCR, SAT-2, typing

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Introduction

Foot and mouth disease causes serious economic losses due to reduction in productivity and mortality of young animals. Control of outbreaks is depend upon a system of monitoring and early detection, which requires basic familiarity with clinical signs and the ability to characterize the strain of virus responsible by laboratory tests [1].

A critical issue in this respect is the occurrence of carrier animals and the risk that pose in transmitting the virus [2,3]. Asymptomatic persistent infection is a common after-effect following infection of ruminants with FMD virus [4,5]. A carrier is defined as an animal from which live viruses can be recovered for longer than 28 days after exposure [2,6,7]. These carrier animals can precipitate new outbreaks of disease [3, 8,9].

Identification of FMD carrier ruminants mainly depends on isolation of the virus from oesophageal / pharyngeal fluid samples (probang samples) on primary

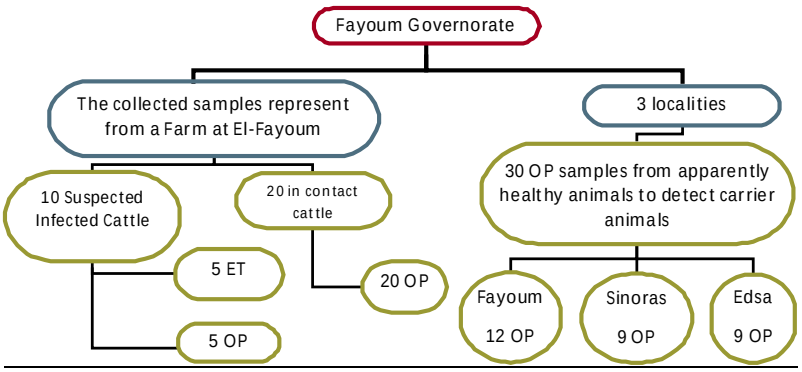

Figure-1. Schedule of collected samples for detection of FMDV in cattle

calf thyroid cells (BTY) cells [10] and definitive diagnosis of FMD requires the detection of virus, antigen or genome in clinical material. For almost twenty years, the WRL for FMD has used an indirect, sandwich enzyme-linked immunosorbent assay (ELISA) [11,12] to identify FMDV, and however the ELISA is not 100% sensitive. Consequently, suspensions of each specimen are also propagated in sensitive cell cultures [11] and the specificity of any isolated virus confirmed by the ELISA. Whilst such virus isolation (VI) methods are highly sensitive, they require four days before a negative result can be concluded and reported as 'no virus detected' (NVD).

A number of reverse transcription polymerase chain reaction (RT-PCR) assays have been developed as an alternative to viral culture [13-18], but these assays require other laboratory techniques to detect the amplified product, which is associated with considerable hands-on time, poses a serious hazard for amplification product carryover, and limits the number of specimens that can be processed simultaneously.

Recently, the development of a real-time reverse transcription polymerase chain reaction (rRT-PCR) procedure has provided an additional tool which can be used for FMD diagnosis [19]. Furthermore, this real-time RT-PCR method can be automated allowing increased through put samples with fewer user-dependent steps [20].

Also Real-time RT-PCR has several advantages over conventional RT-PCR where it is more rapid and sensitive and performed in a closed one-tube system and avoids potential cross contamination during sample preparation for post-PCR analysis [21].

Real-time RT-PCR or quantitative PCR is a variation of the standard PCR technique used to quantify DNA or messenger RNA in sample using sequence specific primers, the relative number of copies of a particular DNA or RNA sequence can be determined. The quantification arises by measuring the amount of amplified product at each stage during the PCR cycles. Quantification of amplified product is obtained using fluorescent SYBR green. SYBR green is a dye bind to double stranded DNA. The intensity of fluorescent emissions increase as more double stranded amplicon is produced with the dye signal increase. The dye will bind to any double strand DNA molecule, while the 5' nuclease probe assay is specific to a pre-determined target [22].

The present study was planned to investigate the efficacy of a fully automated real-time RT-PCR with virus isolation (VI) and CFT for the detection of FMDV in infected and apparently healthy cattle (carrier) to provide a rapid sensitive tool aiming to aid in disease control.

Materials and Methods

Samples: Epithelial tongue (ET) and Oesophageal/ pharyngeal fluid samples (OP) were collected according to [6] from El- Fayoum governorate during November and December 2011 and January 2012.

Five ET and five OP samples were collected from 10 suspected infected cattle and 20 OP samples were collected from in-contact cattle from the same farm. In addition to 12, 9 and 9 OP samples were received from Fayom, Seniores and Edsa localities respectively in El-Fayoum governorate (fig-1). These samples were examined by tissue culture and baby mice, then typed using complement fixation test (CFT) and SYBR green real time RT-PCR.

Virus assay:

Virus isolation:

A- On Tissue culture: The infectivity of ET and OP samples were determined by inoculation in monolayers of baby hamster kidney cells (BHK), as described by [10] through three successive passages. The cells were

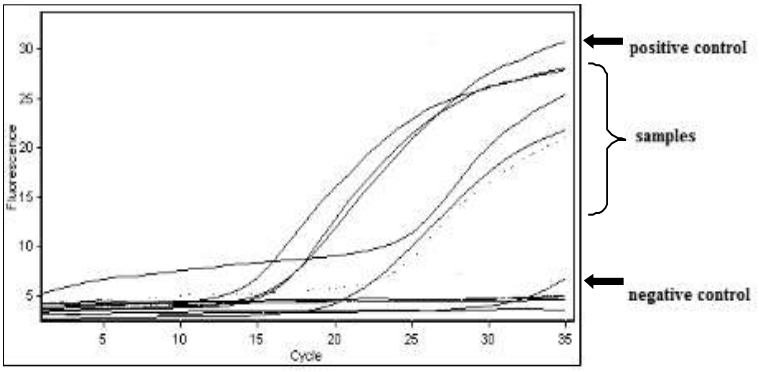

Figure-2. rRT-PCR result of FMDV isolates

examined daily for the presence of viral cytopathic effect (CPE) for 3 days according to [23].

B- In Baby mice inoculation: Suckling Swiss Albino mice of 2-3 days old were used to detect FMD virus in ET and OP samples inoculated in 4-baby mice I/P in a dose of 0.1ml / mice and deaths were recorded from 48 hours to the 7th day post inoculation.

Typing of obtained suspected isolates using CFT: CFT was carried out according to [24] for typing of FMDV 7 serotypes using known hyperimmune sera against 7 serotypes.

Real time RT-PCR technique:

A- RNA extraction: RNA extraction was carried out using the QIAamp® Viral RNA kit (Qiagen, Germany) according to the manufacturer's protocol to all samples in a final volume of 50 ml according to the manufacturer's instruction and stored at -80°C until used.

B-Primers: Primer pair (PoR/PoF) for real time RT-PCR was synthesized by BioBasic, Canada. PoF (5´-CCT ATG AGA ACAAGC GCA TC -3´) and PoR (5´-CAA CTT CTCCTG TAT GGT CC -3´) were derived from FMDV 3D polymerase for detection of FMDV and have no cross reaction with swine vesicular disease (Universal primer for FMDV).

C- Real-time RT-PCR (rRT-PCR): rRT-PCR was performed using QuantiTect® SYBR® Green RT-PCR Kit (Qiagen, Germany) as manufacturer's instructions. The cycling parameters were 50 °C for 30 min and 95 °C for 15min; then 30 cycles consisting of 94 °C for 15 s, 55°C for 30 s and 72 °C for 30 s. Negative control specimen was involved. Each sample was tested in duplicate. PCR amplification was carried out in the Thermocycler Rotor-Gene Q (Qiagen, Germany)

Results and Discussion

The occurrence of persistently infected (carrier) of FMD virus in ruminants represents further complications to disease control. So the detection of FMD virus in persistently infected carriers among exposed cattle is of great importance that needs a suitable sensitive and rapid tool and accordingly the present study was planned to evaluate rRT-PCR assay for detection of FMD virus either in naturally infected; in contact or apparently health cattle.

VI has been the recommended laboratory procedures for FMD diagnosis, based on their suitability to detect the presence of FMDV antigen in tissue samples. If one considers that VI procedures actually measure then it is evident that its effectiveness for diagnostic use is inherently compromised. Virus isolation depends on the presence of infectious virus in sample submissions. It depends upon the antigen being present in sufficient concentration to work [25].Complement fixation test and rRT-PCR were used for detection of FMDV. CFT had a reasonably high specificity but the sensitivity was moderate and for this reason, the technique would not be used for quantitative measurement of FMDV [26] while 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 FMD [23,25].

The obtained results showed that all of ET and OP obtained from suspected cattle induced characteristic cytopathic effect of FMD virus on BHK cell culture and specific signs of FMD in baby mice indicating the presence of FMD virus as shown in table (1). Such methods for detection of FMD virus were recommended by [10,25] they showed that FMD virus isolation depend on the presence of infectious virus in sample submission. Regarding the identification of the detected FMD virus, CFT and rRT-PCR; using specific primers; confirmed that the obtained virus isolate is SAT2 (Table-1). These findings indicates that the results of CFT and rRT-PCR came in a parallel

Table-1. Detection of FMD virus in suspected infected and in contact cattle in farm (1) in El-Fayoum governorate

Tested samples	Samples obtained from suspected infected cattle (10 Case)								Samples obtained from in contact cattle (20 case)							
	No.	TC*	BM**	CFT***				rRT-PCR	NO.	TC	BM	CFT				rRT-PCR
				A	O	SAT2	T					A	O	SAT2	T	
ET	5	5	5	0	0	5	5	5	0	0	0	0	0	0	0	0
OP	5	3	5	0	0	5	5	5	20	8	11	0	6	5	11	13
Total	10	8	10	0	0	10	10	10	20	8	11	0	6	5	11	13

*TC= Tissue Culture, **BM= Baby Mice, ***CFT= Complement Fixation Test, T= Total no. of positive typed FMDV serotypes

Table-2. Detection of FMD virus in OP samples in apparently healthy cattle at 3 localities in El-Fayoum governorate

Localities	No. of samples	TC*	BM**	CFT***				rRT-PCR
				A	O	SAT2	T	
Fayoum	12	4	6	0	6	0	6	6
Sinoras	9	2	2	0	2	0	2	4
Edsa	9	5	7	1	5	2	8	9
Total	30	11	15	1	13	2	16	19

*TC= Tissue Culture, **BM= Baby Mice, ***CFT= Complement Fixation Test

manner confirming each other and supported by [23, 25,27] they showed that rRT-PCR is more rapid and sensitive technique suitable for detection and identification of FMD virus. Also these observations support the suggestion that rRT-PCR has a sensitivity of 100% compared with the virus isolation test as demonstrated by [27,28].

Table-1 clarified that OP samples obtained from the same in-contact cattle induced positive results in both of BHK cell culture and baby mice inoculation tests (8 out of 20 and 11 out of 20 respectively) with positive CFT and rRT-PCR which identified the same FMD virus strain (SAT2) which was detected in 5 out of 20 OP samples indicating that these in contact cattle are carriers to the same virus strain isolated from infected cattle and clarify the idea about FMD carrier cattle. However; 6 OP samples; from in-contact cattle were found to be positive to FMDV type O. The presence of carrier cattle having the same FMDV type SAT2 in contact cattle to infected ones appears to be logic while the presence of type O could be attributed to a mixed infection or a recent sub-clinical infection. Such findings come in agreement with [4,5] they stated that asymptomatic persistent infection is a common after-effect following infection of ruminants with FMD virus and [2,6,7] they identified animal carrier as an animal from which live virus can be recovered for several days after exposure. In addition the sensitivity of rRT-PCR assay for detection of carrier cattle was 100% compared with virus isolation suggesting its suitability for screening FMDV carrier animals as reported by [28] who also stated that cell culture technique detects virus infection while the rRT-PCR detects a small segment of virus RNA which is noticed clearly in Table-1 that 2 OP samples in the in-contact cattle were positive in rRT-PCR while negative in CFT.

Table-2 demonstrated that by using the same techniques; 6 samples out of 12 at Fayoum and 2 out of 9 samples at Sinoras were positive to FMDV type O while in Edsa types A, O and SAT2 were detected in 1; 5 and 2 out of 9 samples respectively. These findings showed that the three types of FMDV are circulating in Fayoum Governorate threaten cattle population. Also the sensitivity of real time RT-PCR over the CFT in detection of FMDV carrier cattle was clearly noticed in Sinoras and Edsa as in Sinoras where 2 OP samples were positive by rRT-PCR and in contrast they were negative by CFT while in Edsa one OP sample was positive by rRT-PCR while it was negative by CFT. These findings agree with those of [22,27] who stated that the real-time RT-PCR has proven to be highly sensitive and specific under laboratory condition.

Conclusion

This 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 especially in-contact and carrier cases. The rRT-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 CFT and virus isolation. This is an important feature when definitive diagnostic results are required in a short timescale during emergencies. Also this study demon-

strates the current situation of FMDV circulating in EL-Fayoum governorate and the introduction of new SAT2 serotype beside type A and O.

Author's contribution

LE drafted and revised the manuscript, AMHA supervised the study and tabulate the result, WM collected tongue epitlelium and OP samples from El-Fayoum Governorate, inoculated samples in baby mice, done RT-PCR and help in drafting of manuscript, EE collected samples from 3 localities in El-Fayoum Governorate, prepared BHK cell line, inoculated samples, done RT-PCR and help in drafting of manuscript, corresponding with Journal, AI prepared tongue epitlelium and OP samples, applied CFT and RT-PCR and help in drafting of manuscript, WD done RT-PCR, helped in manuscript preparation, drafted and revised

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Competing interests

Authors declare that they have no competing interests.

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