Laboratory diagnosis of FMD using real-time RT-PCR in Egypt

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Abstract: Definitive diagnosis of foot-and-mouth disease (FMD) requires the detection of virus antigen or genome in clinical material. The aim was performance of real-time RT-PCR (rRT-PCR) procedures for this purpose. Twenty nine cattle samples of vesicular epithelial and nasal swabs from four localities of Ismailia governorates were examined by ELISA, VN, RT-PCR and rRT-PCR. The results showed that 11 samples were positive by ELISA and virus isolation, 8 of serotype O and 3 for type A. Fourteen samples out of 29 were positive by RT-PCR and rRT-PCR. The features that influence sample quality appear to be less important for the rRT-PCR and RT-PCR as they can detect a small fragment of FMDV genomic RNA. Real-time RT-PCR provided an extremely sensitizer and rapid procedures that contributes to improved laboratory diagnosis of FMD.

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Introduction

Control of outbreaks of foot-and-mouth disease (FMD) is dependant upon a system of monitoring and early detection, which requires basic familiarities with clinical signs and the ability to characterize the strains of virus responsible by laboratory tests. Definitive diagnosis of FMD requires the detection of virus, antigen or genome in clinical material. Ideally, the samples of choice should vesicular epithelium from clinically affected animals since, during the acute stage of the disease, it is rich in virus **(1-3)**. Consequently suspension of samples is propagated in sensitive cell culture **(4)** and the specificity of any isolated virus is confirmed by ELISA. Virus isolation methods are highly sensitive, they require four days before a negative result can be concluded. In emergence, speed of diagnosis (clinical and laboratory confirmation) is of paramount importance to control spread and eradicate the disease.

The development of real-time reverse transcription polymerase chain reaction (rRT-PCR) procedure has provided an additional tool, which can be used for FMD diagnosis **(3, 6-7)**. 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.

2. Material and Methods

2.1. Samples

Twenty nine cattle samples, a vesicular epithelium (ep) and twenty nasal swabs (ns) were received from Fayed, Tal El-Keber, Kassasen and Kantara of Ismailia governorate during January, February, March and April 2011. Due to the contagious nature and economic importance of FMD, the laboratory diagnosis and serotype identification of the virus should be done in a facility that meets the requirements for containment group 4 pathogens with bio-safety and bio-security in the laboratory.

2.2. Virus isolation (VI) of the samples

The samples were filtered using 0.2µm filter, and then were inoculated onto monolayer BHK-21 cell line with three passages. The cultures were checked for specific cytopathic effect (CPE) every 24 hrs for 72 hrs. Cultures were stored at -70 ^{o}C until processing for ELISA **(8)**. Indirect sandwich ELISA was performed for the detection and identification of viral serotypes **(3)**.

2.3. RT-PCR

It was used to amplify genome fragment of FMDV in the samples. One-step RT-PCR was carried out as described by the manufacture's protocol to perform the reverse transcription and subsequent PCR by One-step RT-PCR (Qiagen, Germany) **(9-10)**. The method amplifies a serotype specific segments of FMDV VP1 (1D) gene of type A and another for type O. The primer sequences were as listed in **Table 1**. Amplified products were analyzed on agarose gel. Negative control specimen and DNA ladder were involved in agarose gel electrophoresis.

Table 1. FMDV specific primer sequences

Primer	Orientation	Sequence (5' to 3')	Serotype Specificity	Genomic Location	bp
PH9	Forward	TAC CAA ATT ACA CAC GGG AA	A	1C	863-866
PH10	Reverse	GAC ATG TCC TCC TGC ATC TG	All serotypes	2B	863-866
PH2	Forward	GCT GCC TAC CTC CTT CAA	O	1D	402
PH1	Reverse	AGC TTG TAC CAG GGT TTG GC	All serotypes	2B	402

2.4. rRT-PCR

RNA extraction was carried out using the QIAamp® Viral RNA kit (Qiagen, Germany) according to the manufacturer's protocol. Primer pair (PoR/PoF) for real time RT-PCR was synthesized by BioBasic, Canada. PoF (5´- CCT ATG AGA ACA AGC GCA TC -3´) and PoR (5´- CAA CTT CTC CTG TAT GGT CC -3´) were derived from FMDV 3D polymerase **(11)**. RT-PCR was performed using QuantiTect® SYBR® Green RT-PCR Kit (Qiagen, Germany) as manufacturer's instructions. The cycling parameters were 50 ^{o}C for 30 min and 95 ^{o}C for 15 min; then 30 cycles consisting of 94 ^{o}C for 15 s, 55 ^{o}C for 30 s and 72 ^{o}C for 30 s. Negative control specimen was involved. Thermocycler Rotor-Gene Q (Qiagen, Germany) was used for real time detection of FMDV by RT-PCR.

3. Results and Discussion

The results achieved by ELISA, VI in cell culture, RT-PCR and real-time RT-PCR are summarized in **Table 2** for the comparison of the performance of four assays. FMDV was detected in 11 samples by VI and antigen ELISA, nine of tongue epithelial from Fayed, Tal El-Keber, Kassasen and Kantara while two nasal swabs from Kantara. These viruses represented 8 of serotype O and 3 of serotype A. There were broad agreement between RT-PCR and rRT-PCR, where 9 positive epithelial samples and 5 positive nasal swabs out of 20 samples from Fayed, Kassasen and Kantara were detected. All samples assigned negative by RT-PCR and rRT-PCR were also negative by virus isolation and ELISA **(Figs. 1 and 2)**

ELISA and VI have 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 and ELISA procedures actually measure then it is evident that their effectiveness for diagnostic use is inherently compromised. Virus isolation is dependant upon the presence of infectious virus in sample submissions. While ELISA can detect both infectious and non-infectious FMD viral antigen, it is dependent upon the antigen being present in sufficient concentration to work **(1)**. 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 **(1, 3)**. The aim of the study was to use real-time RT-PCR to detect FMDV in suspected samples. Five positive nasal swabs. Samples 1, 3 and 4 were identified by rRT-PCR, whereas only two of these samples were positive by VI and ELISA indicating comparable sensitivity between these diagnostic methods that was in agreement with previous authors **(2, 12-13)**. The negative results were likely to occur in samples in which cattle recovered from clinical lesions, since virus isolation was extremely reduced with more than 7-10 days after the appearance of gross lesions **(5)**.

In conclusion, the real-time RT-PCR method used in this study has proven to be highly sensitive and specific under laboratory condition.

Table 2 Positive samples from four localities of Ismailia governorate by VI and ELISA RT-PCR and rRT-PCR during 2011

Locality	Sample	No	VI	ELISA		RT-PCR		rRT-PCR
				A	O	A	O	
Fayed	ep	3	3	-	3	-	3	3
	ns	4	-	-	-	-	1	1
Tal El-Keber	ep	2	2	-	2	-	2	2
	ns	5	-	-	-	-	-	-
Kassasen	ep	2	2	2	-	2	-	2
	ns	6	-	-	-	-	1	1
Kantara	ep	2	2	1	1	1	1	2
	ns	5	2	-	2	-	3	3
Total		29	11	3	8	3	11	14

. ep= tongue epithelium, ns= nasal swab

Fig. 1 RT-PCR for detection of FMDV serotype A and O. M: 100 bp ladder, lanes 1-3: serotype A (863 bp), lanes 4-6: negative samples, lane 5: serotype O (402 bp)

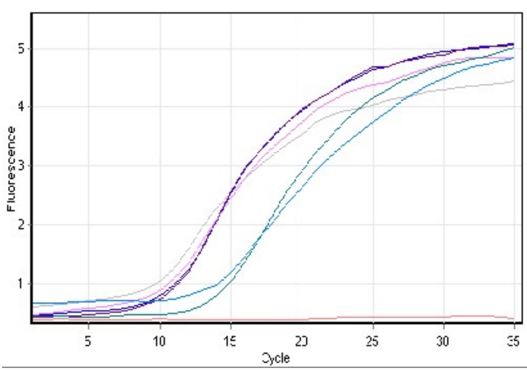

Fig. 2 Real Time RT-PCR result of FMDV isolates. Grey is positive control, red baseline is negative control, other curves are positive viral samples

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