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By Hany I Abu-Elnag

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*Keywords:* *foot-and-mouth disease virus; rRT-PCR; MGB probe.*

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*Strictly as per the compliance and regulations of:*

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# Minor Groove Binder Probe Real-Time RT-PCR for Detection of Foot-and-Mouth Disease Virus in Egypt

Hany I Abu-Elnaga

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## I. Introduction

Foot-and-mouth disease (FMD) is the most economically significant animal viral disease worldwide affecting cloven-hoofed animals and caused by Foot-and-mouth disease virus (FMDV). The virus is a picornavirus (genus *Aphthovirus*, family *Picornaviridae*). The virus has a linear single-stranded RNA genome [1]. Seven antigenically distinct forms of the virus are known, called serotypes, but serotype C has not been detected anywhere for many years and may now be extinct. The serotypes have been further divided into topotypes (except for serotype Asia-1 viruses, which comprise a single topotype), genotypes, lineages, and sublineages, which are usually restricted to specific geographical regions [2].

The rapid and precise detection of FMD virus is a prerequisite. Conventional reverse transcriptase polymerase chain reaction (RT-PCR) [3-6] and real-time RT-PCR assays [7-10] have been developed to complement primary diagnostic techniques for the detection of FMDV. Real-time RT-PCR recommended by the World Organization for Animal Health (Office International des Epizooties, OIE) for detection of FMDV incorporate universal primers and fluorescent-labeled probes that recognize conserved regions within the 5-UTR & 3D polymerase [11].

The usage of a panel of rRT-PCR assays is imperative, as RNA, viruses are prone to mutation. If one assay is rendered ineffective due to a catastrophic mutation in the primer or probe binding regions or as a result of the event of contamination problems, results from a other assay will still be valid especially when using assays targeting different areas of the genome [12]. The MGB molecule involved in the detector probe design increase the Tm of the probe. This shortens the probe sequence and enables it to be designed strictly to in the variant region. Moreover, the dynamic range of MGB-NFQ probes is larger because of its increased fluorophore quenching, efficiency and resulting low fluorescent background compared to FAM-TAMRA probes [9, 13].

FMDV serotype O is the most ancient well-identified worldwide type [14]. Also, in Egypt it the classical enzootic and the most prevalent serotype pose many outbreaks [15, 16]. Therefore, application and assessing of a new trend for viral detection in Egypt depending on minor groove binder probe (MGB) real-time RT-PCR (rRT-PCR) was applied on local FMDV serotype O, A, and SAT2 with special handling of FMDV O serotype specific primer sets in SYBR Green real-time RT-PCR for the rapid and precise detection of the virus

## II. Materials and Methods

### a) Viruses

Strains designated O/EGY/2009 iso1 (cell culture grew virus), A/EGY/2009 iso-Cai (clinical isolate) and SAT2/EGY/H1Ghb/2012 (bovine tongue epithelium suspension from Gharbia, Egypt), local strains of serotypes O, A and SAT2, respectively [17-19] used. They were initially employed in the validity of Minor groove binder probe real-time RT-PCR to detect different Egyptian FMDV serotypes. Moreover, Other FMD viruses representing FMDV serotypes found in Egypt, previously type discriminated by RT-PCR assay and nucleotide sequence used as unknown samples in the performance of the probe rRT-PCR assays. These FMD viruses were clinical and culture viruses, O ($n$=10), A ($n$=4) and SAT2 ($n$=2). The context of that, a related vesicular viral disease that cause mucosal lesion with excessive salivation accompanied by lameness in

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chronic infection also incorporated in the assays. The virus belongs to the family Flaviviridae, causative agent is bovine viral diarrhea virus (BVDV). The genome consists of a positive-sense, single-stranded RNA molecule of approximately 12.3 kb; two BVDV genotypes are recognized, its strains divided into distinct biotypes (cytopathic or non-cytopathic) according to their effects on tissue cell culture.

*b) RNA purification and analytic sensitivity*

QIAamp® Viral RNA kit (Qiagen, Germany) for RNA extraction used according to the manufacturer's instruction. Extracted RNAs from three FMDV O were quantitated by ultraviolet (UV) spectrophotometry and used as; in-house Standard (a candidate culture propagated virus in 2017 with titer ~7.3 $TCID_{50}$/ml on BHK), Positive control 1 (semi-purified concentrated culture grown virus in 2012) and Positive control 2 (cell adapted FMDV isolated in 2009). The standard was imperative to obtain the standard curve. Bulks of extracted RNAs from the standard and positive control two were divided in two aliquots for each. From one aliquot of the standard RNA, seven times, serial 10-fold dilutions in RNAse-free water were performed to obtain the Standard RNA Dilutions (SRD) to have values that were used to construct a standard curve to calculate unknown sample concentrations.

An archived Stock virus (SV) RNA [20], kept for a complete 6-years in an ordinary kitchen fridge and previously assessed by SYBR Green rRT-PCR, was examined in the current article for assessment rRT-PCR assays on RNAs that suffered storage for a long duration. Briefly, 10-fold serial dilutions of stock virus (SV) in minimum essential medium (MEM) with Hank's salts in the range of $10^{-1}$-$10^{-8}$ performed. Each dilution was exposed to RNA isolation procedures to prepare SV RNAs. The previous different RNAs preparation formats viz. SRD and SV RNA was used in analytic sensitivity. Negative controls included: no template control, NTC, which was RNAse free water; Negative control one that was RNA from healthy BHK cells; and Negative control 2 that was RNA from non-infected BHK cells showed contamination. For quantification the mass concentration of RNA, two spectrophotometer instruments utilized, one was the conventional spectrophotometer (Milton Roy 601 Spectronic 335104, USA) and other was the modern spectrophotometer (Nano Drop 2000c Spectrophotometer, Thermo Fisher Scientific, USA).

Repeatability assay of MGB probe rRT-PCR sensitivity was performed after one-month interval on FMDV O RNA standard, its 1st dilution ($10^{-1}$) and positive control two, which considered as an old exhausted aliquot RNA after using for one-month. Where the standard and control two had been exposed to repetitive freezing and thawing for ten and six times, respectively.

Using samples and depending on the current primers flanked the MGB probe, each MGB and SYBR green rRT-PCR were extra re-assessed with the previous old aliquot RNA, but also, new aliquots of the standard, positive control two and primers/probe set were additionally involved in the assay.

*c) Real-time RT-PCR (rRT-PCR)*

All the extracted RNAs tested on the real-time PCR system Rotor-Gene Q 2 (Corbett Life Science, a QIAGEN Company, Germany) using either QuantiTect Probe RT-PCR Kit or QuantiTect SYBR Green RT-PCR Kit (Qiagen, Germany). Primer set: PF-5-GTT TTG TTC TTG GTC ACT CCA T-3'; PR-5'-ACG GAG ATC AAC TTC TCC TGT A-3' and a labeled FAM, 5' conjugated minor groove binder (MGB) probe CTC TCC TTT GCA CGC C, 5'- FAM _ 3'-NFQ/MGB, were employed in MGB rRT-PCR investigations with approximately 163 bp target genome fragment amplification [12]. The primers were purchased from either Metabion, Germany or Bioneer, Korea; while, the Probe designed by Applied Biosystems, Life Technologies, Thermo Fisher Scientific, USA. The serotype O specific primer pair (our lab termed PH1/PH2), designed from the 1D and 2AB regions of the viral genome as described previously [4] to give 402 bp expected band sizes used in SYBR Green rRT-PCR assay for further MGB comparative assessment. Also, for auxiliary performance evaluation, the previously [21] documented oligos O-1C124 (ARS4)/NK61 of an expected amplicon of approximately 1126 bp were implemented by SYBR rRT-PCR method. For TaqMan MGB rRT-PCR, cycling conditions were: 1 cycle at 45°C for 30min, 1 cycle at 95°C for 15min and 45 cycles at 95°C for 30s, 51°C for 30s, and 72°C for 30s. For the SYBR Green rRT-PCR methods, the optimized reaction contained 5% RNA template. The cycling parameters were as described previously [4, 21] and for 45 cycles. At least one of the positive controls and negative controls was involved in every assay.

## III. Results

MGB rRT-PCR assay were valid to detect the specific target genome fragment of different Egyptian FMDV serotypes strains. Besides, various negative controls and the tested BVDV produced neither threshold cycle ($C_T$) values nor the expected amplification sizes in agar gel electrophoresis. Using MGB amplification, RNA sensitive detection of the standard and all its dilution extended to the theoretical mass quantification of six hundred attograms (ag) RNA per microliter (μl).Also, sensitivity were attained in examining the archived stock virus (SV) RNA, MGB fluorogenic signals were attained in dilutions from $10^{-1}$ to $10^{-4}$ and $10^{-6}$. On the other hand, MGB probe and primers failed to hybridize to the respective templates in dilutions $10^{-5}$, $10^{-7}$ and $10^{-8}$ (Fig. 1 and 2).

SYBR green rRT-PCR method using primer pair for FMDV serotype O (Fig. 3), showed specific sensitivity reached to sixty femtogram RNA per microliter utilizing PH1/PH2 primer set. Furthermore, quantification of six



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hundred picograms (might barely reach 60 pg in repeatability) of RNA per microliter were obtained when employing alternative oligos for FMDV O, 1C124 (ARS4)/NK61 (Fig. 4).

Likewise, Repeatability of MGB rRT-PCR assay revealed variability in the sensitivity between aliquots and replicates with a highlighted effect of RNA degradation and an approximately negligible impact of the primers /probe hybridization regression. $C_T$ values variability using MGB probe between the replicates for each sample in comparison with the mean was ±3.5 with values difference reached 6.5, 5.7, and 2.9 for Positive Control 2, Standard and Standard $10^{-1}$, respectively (Fig. 5 and 6) and Table 1. Accordingly, it was suggested to be the cut off ≤ 45 cycles for MGB probe due to its high sensitive and specific detection.

Using the primer set that flanked the MGB probe in either Probe or SYBR rRT-PCR methods, successively at the same day, clearly showed the performance of the standard and its dilution were promising in Probe in comparison to SYBR assays, where the signals were linear in the exponential phase. The standard curve efficiency for the MGB probe was 0.89, while for the SYBR PH1/PH2 primers was 0.79. Using Taqman MGB Probe, the standard virus and its serially 10-fold dilutions showed a 2.8-4.4 increment increasing of $C_T$ values between undiluted virus until its $10^{-6}$ dilution (undiluted & 6 dilution series). Whereas, there were no prominent changes in $C_T$ value at the $10^{-7}$ dilution. Furthermore, using SYBR Green PH1/PH2 oligos protocol, the standard virus and its serially 10-fold dilutions gave $C_T$ values of 3.3-4.6 differences between undiluted virus until its $10^{-6}$ dilution (undiluted & 6 dilution series). Melt curve peak (*T*m) showed that specific amplification giving the expected peak, affirmed by yielding the anticipated fragment size on agarose based electrophoresis. The negative samples controls either did not exhibit the anticipated specific former *T*m of the primers or showed the expected peak, but they all had higher $C_T$ values above the last positive standard dilation.

## IV. Discussion

Shorter, more specific minor groove binders (MGBs) probes are dsDNA-binding agents attached to the 3' end of TaqMan probes to increase the Tm value (by stabilization of hybridization) and to design shorter probes. Shorter probes make it easier to use short conserved or unique sequences for hybridization. MGBs also reduce background fluorescence and increase dynamic range due to increased efficiency of reporter quenching due to shorter distances between the reporter and quencher and the use of non-fluorescent (dark) quenchers (NFQ) at the 3' end instead of fluorescence dyes like TAMRA. MGB probes have more sequence specificity for better mismatch recognition.

The format of standard and its dilutions (SRD) in the current article was selected according to what comprehend from the thermal cycler manufacture's recommendation, which mentioned that the DNA used in the standard curve should be derived from similar DNA in the samples being measured. It was recommended that the concentration of at least one DNA sample be determined using ultraviolet spectrophotometry and that this sample be used as the standard. The minimum number of standards used should be three (with replicates). Importantly, DNA standards used in fluorescence detection are only linear within the range of 100 nano-grams per micro-litre to 1 nano-gram per micro-litre. That is, within this range, if the concentration of DNA is halved, so is the fluorescent reading. The confidence intervals for any concentration outside this range are very broad due to non-linearity in the chemistry. In addition, differences have been observed in the measurement of various forms of DNA. For example, genomic DNA compared with plasmid DNA. Therefore, it is recommended that only alike DNA are measured together, and the use of plasmid DNA as a standard be avoided when measuring genomic DNA.

The oligonucleotide probe with a 5' conjugated minor groove binder (MGB) ligand as a reporter in real-time PCR. The hybridization of the probe triggered fluorescent. MGB probe rRT-PCR was the best specificity and sensitivity than the other two primer pairs used in SYBR Green RT-PCR protocols. Relevant amplification of the homologous templates were implemented, whereas, the heterologous templates were mismatched. Consequently, the specificity of this fluorogenic probe was very satisfactory for FMDV investigation and quantification. The fluorogenic MGB probes were more specific for single base mismatches and fluorescence quenching was more efficient, giving increased sensitivity [13]. Result revealed lower $C_T$ values, in addition to, higher detection specificity and sensitivity when using FMDV O specific primers that produce smaller amplification size, in comparison to, O specific oligos amplifying larger fragment. In real-time PCR with TaqMan probes, the amplicon size directly influenced detection: the larger the amplicons, the later the detection. Earlier detection and a higher fluorescence level (plateau phase) were generally observed for shorter amplicons [22]. On the other side in SYBR Green methods, melting curve analysis was considered as a tool to verify the specificity of the amplified product, although it is a common indicator used in fluorescence rather than flurorophore-based RT-PCR assays. Besides, agar gel electrophoresis support the amplicon specificity. In contrary to MGB probe, the serotype specific oligos, 1C124 (ARS4)/ NK61, trial in SYBR rRT-PCR format gave the poorest analytical specificity and sensitivity.

Analytical sensitivity was a trial to detect variable genome of FMDV of different serotype to overcome the possibility of false negative result due to serotype unspecificity. In the absence of a target molecule, the MGB probe does not fluoresce, as there is



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sufficient interaction between the reporter fluorophore and the quencher to prevent a fluorescent signal. Hybridisation to a complementary target molecule triggers an increase in fluorescence due to the separation of the fluorophore and quencher [12]. Probe with minor grove binder (MGB) form stable, higher melting temperature interactions with their target sequences. The positive result of rRT-PCR in this article are mainly $C_T$ values. Where there is general correlation between them and quantity of input nucleic acid. The more target template is present in the reaction, the fewer cycles it requires to reach logarithmic growth and end point of RT-PCR (i.e. lower $C_T$ values) [9].

After one month of 1st assessment of the performance and the analytic sensitivity of MGB rRT-PCR, an anticipated detection limit decreased by 10-fold, with 2.6-5.5 increment difference in $C_T$ values between undiluted virus until its $10^{-6}$ dilution, where higher increment values in lower dilutions and lower increment values in higher dilution. The operator for faulty cost saving did not carry out re-assessment of dilution $10^{-7}$. However, result was satisfactory by ending the re-assessment by dilution $10^{-6}$ and not exceed to dilution $10^{-7}$, because the signal curve of dilution $10^{-6}$ was at the border of the threshold ($C_T = 43$) that predict if dilution $10^{-7}$ was done, the $C_T$ value would be weak positive or negative.

Six-years before the current assay, SYBR Green rRT-PCR had detection limit for the stored Stock virus (SV) RNA that was extended to $10^{-7}$ dilutions using 0.2 RNA template/rxn volume (Azab et al., 2012). In context, the current MGB probe was still cable to detect RNA in the archived RNA across approximately 6-log range of input template with 1 $\log_{10}$ regression, taking in consideration that the RNA template input in this paper was 4x lesser. Also, the detection signals produced as a result of SYBR Green DNA incorporation were generally more earlier as revealed in repeatability assay when MGB probe complementary primers used in SYBR Green investigation. This result was satisfactory when investigation a long period storage of RNA template.

The nucleic acid amplification detection on the real-time PCR platform was verified by agarose gel electrophoresis that revealed the expected positive band. Serial dilution of the virus RNA control could be used as one of the viable reference for relative FMDV quantification. The virus RNA control sample was of cultured derived virus, not a wild virus to minimize the possibility of contamination by non-specific fragment. Furthermore, in future, we hope to use in-vitro synthetic FMD RNA fragment of the primer/probe target sequence as a positive amplification control.

The drawback of the current MGB probe (FAM dye–labelled, with NFQ), in comparison to the non-MGB assay (FAM dye–labeled, with BHQ) in the poor developing country is the relative comparative higher cost of MGB probe. That might be reached to 1.7x the price of the non-MGB probe that will be translated to thousands of EGP (or hundred of USD), in consequence of that, rising the finance of the quantity detection assay of the unknown samples.

Finally, MGB RT-PCR assay provided a rapid, sensitive, specific and less labor for detection of FMDV with subsequent early planning for a control strategy in case of an outbreak with liberating FMDV free animals from quarantine measure.

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*Conflict of Interest*

The authors declare that they have no conflict of interest.

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*Table 1:* $C_T$ values of three samples exposed to different conditions

| Conditions 1 Time; Probe or SYBR | 1st day probe same run | | 2nd day probe same run | | | | 2nd day SYBR same run | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Conditions 2 rxn mixture content | old primers /probe aliquot | | old primers /probe aliquot | | new primers /probe aliquot | | old primers aliquot | | new primers aliquot | | |
| Conditions 3 rxn mixture content | old RNA aliquot | new RNA aliquot | old RNA aliquot | new RNA aliquot | old RNA aliquot | new RNA aliquot | old RNA aliquot | new RNA aliquot | old RNA aliquot | new RNA aliquot | |
| Replicate no | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Mean using Probe |
| Positive Control 2 | 29.6 | 27.7 | 27 | 23.1 | 27 | 23.3 | 20.4 | 18.8 | 19.6 | 17.3 | 26.2 |
| Standard | 25 | 25.2 | 25.9 | 20.2 | 22.1 | 23.4 | 16.2 | 13.5 | 15.3 | 13.8 | 23.6 |
| Standard, $10^{-1}$ | 28.5 | ND | 26.5 | ND | 25.6 | ND | 20 | ND | 19.7 | ND | 26.8 |
| NTC | - | - | - | - | - | - | 42.4 | 42.4 | 42.4 | 42.4 | - |

.ND=not done, Reading was CT values recorded in various conditions



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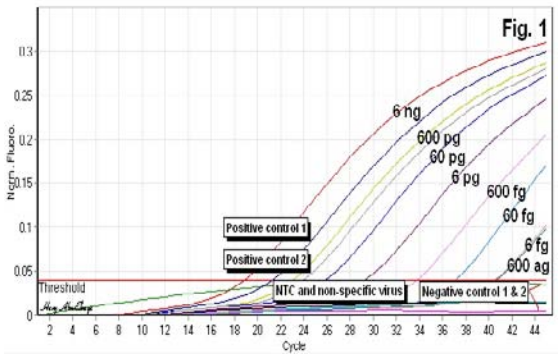



Fig. 1A

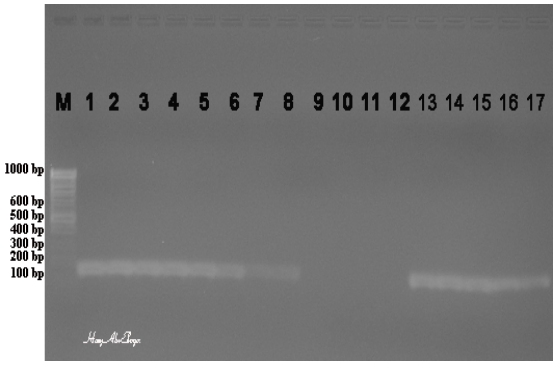



Fig. 1B

*Fig. 1:* Amplification sensitivity of MGB probe rRT-PCR for FMDV O RNA pre-quantified, 6 ng/µl, and serially 10-fold diluted until theoretically reach 6 ag/µl. The former virus was used as standard. Nomenclature on the real-time figure is representing curves either by intersecting the 1st digit (i.e. 6) in the quantity number of the RNA masses or by having an annotation with a black shadow pointed to it. The threshold is the horizontal red line intersecting the curves. (A) Quantitation data of the standard and its dilutions. Two positive controls, two negative controls, no template control (NTC) and non-specific virus (BVD) were involved in the assay run. Positive $C_T$ values were curves peaks above the threshold, while negative values were peaks at the borders or below the threshold (B) Agar-based electrophoresis of the amplified products by the MGB probe. M: 100 bp ladder. Expected fragment size were approximately 163 bp of FMDV 3D gene. Lanes 1-8: The standard dilutions from 6 ng/µl to 6 ag/µl, Lanes 9-10: negative control 1 & 2, Lanes 11: BVD, Lane 12: NTC, Lanes 13-17: Unknown samples, that were skipped in Quantitation data figure to not hide the illustrated standard curves. Positive controls were not shown.

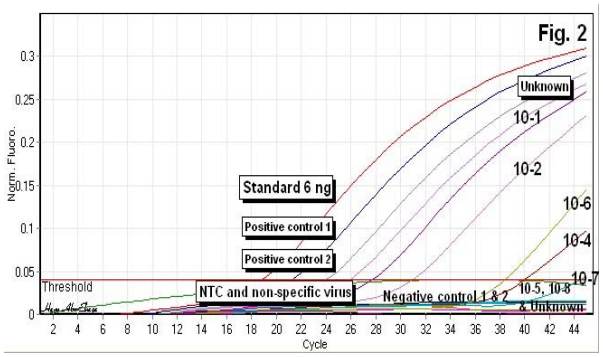



Fig. 2A

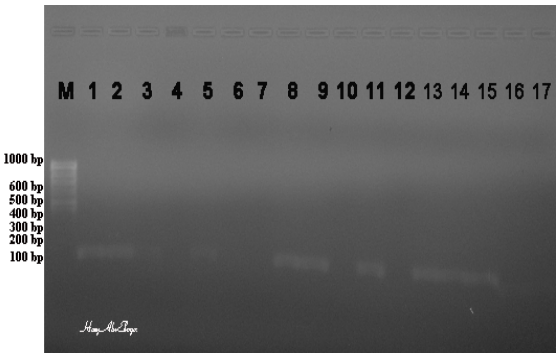



Fig. 2B

*Fig. 2:* (A) Detection of an archived FMDV stock virus (SV) RNA by MGB probe rRT-PCR. SV dilutions $10^{-1}$-$10^{-8}$, except $10^{-3}$, showed various $C_T$ values with amplification cycles. Two positive and negative controls, NTC, non-specific virus (BVD) and unknown samples were involved in the test. (B) Detection of stock virus (SV) RNA with anticipated 163 bp. M: 100 bp ladder. Lanes: 1-7 SV dilutions $10^{-1}$-$10^{-8}$, except the not done dilution $10^{-3}$. Lanes 8 & 13 were positive control 1& 2. Other lanes were unknown samples, Lanes 16-17: showed a very faint bands of an archived unknown samples, which were amplified by another TaqMan probe of an expected size of 107 bp and were used as a control of electrophoresis.



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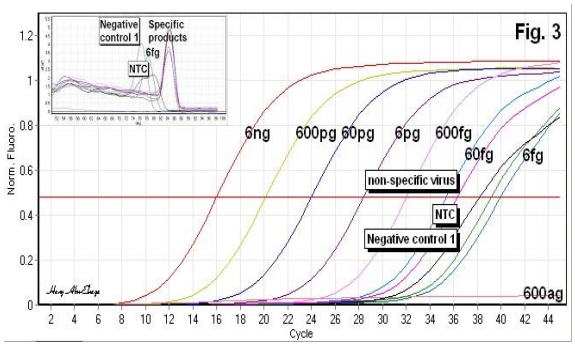



Fig. 3A

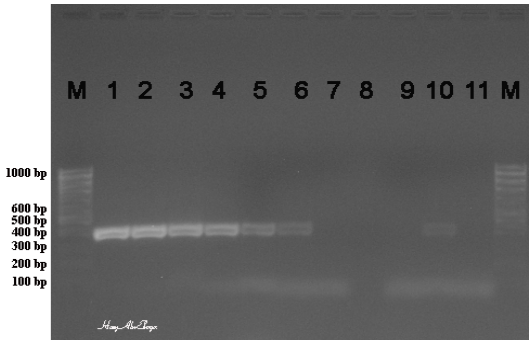



Fig. 3B

*Fig. 3:* Implementation of SYBR Green rRT-PCR using oligos targeting FMDV O 1D gene of an expected 402 bp for minimum detecting of FMDV O RNA pre-quantified as 6 ng/μl, serially 10-fold diluted and theoretically extended to 6 ag/μl. The former virus was used as standard. (A) Graphical representation of real time one-step RT-PCR data of the standard and its dilutions. Negative control, NTC and non-specific virus, BVD, were involved in the assay run. Positive $C_T$ values were curves peaks above the threshold, while negative values were peaks at the borders or below the threshold (B) Electrophoresis on agarose gel of the rRT-PCR assay samples using SYBR Green. M: 100 bp ladder. Positive bands were approximately 402 bp of FMDV O 1D gene. Lanes 1-8: The standard dilutions from 6 ng/μl to 6 ag/μl, Lane 9: negative control 2, Lane 10: BVD, Lane 11: NTC, Insets show melting curve analysis.

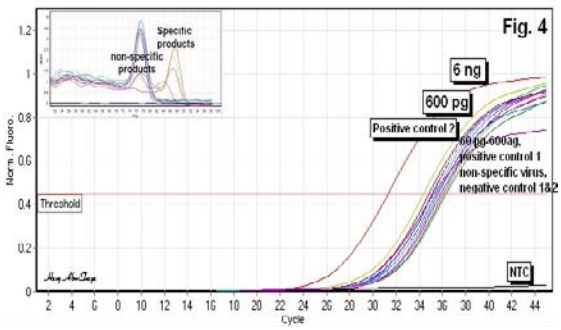



Fig. 4A

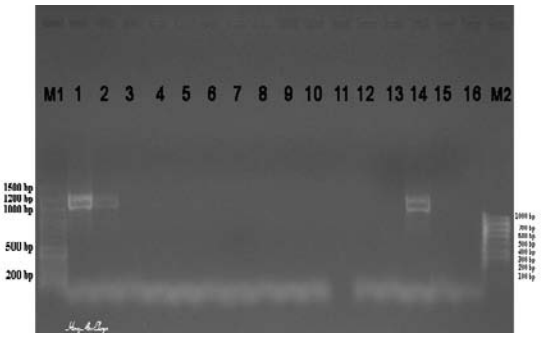



Fig. 4B

*Fig. 4:* SYBR Green rRT-PCR amplification of an expected 1301 bp embarrassing FMDV O 1D gene of for assesment the detection limit of FMDV O RNA pre-quantified as 6 ng/μl, serially 10-fold diluted and theoretically extended to 6 ag/μl. The former virus was used as standard. (A) Graphical representation of real time one-step RT-PCR data of the standard and its dilutions. Two positive controls, two negative controls, no template control (NTC) and non-specific virus (BVD) were involved in the assay run.Positive $C_T$ values were curves peaks above the threshold, while negative values were peaks at the borders or below the threshold (B) Electrophoresis on agarose gel of the rRT-PCR assay samples using SYBR Green. M: 100 bp ladder. Positive bands were approximately 1301 bp of FMDV O 1D gene. Lanes 1-8: The standard dilutions from 6 ng/μl to 6 ag/μl, Lane 9-10: negative control 1 &2, Lane 11: NTC, Lane 12: BVD, Lanes 13-16: unknown samples, Melting curve analysis (insets) revealed amplification specificity.



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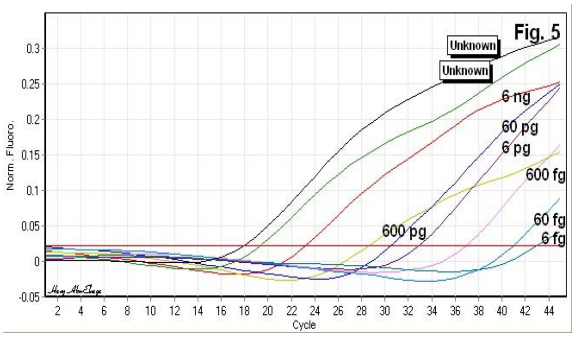



Fig. 5A

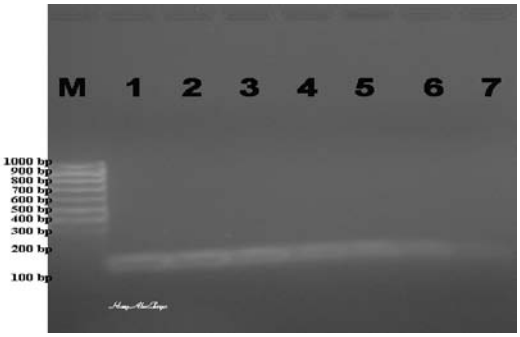



Fig. 5B

*Fig. 5:* Re-assessment (1-month later from previous evaluation) of the sensitivity of MGB probe rRT-PCR for FMDV O RNA standard (6 ng/μl, and serially 10-fold diluted). (A) Quantitation data of the standard and its dilutions. Unknown samples were involved in the assay run. (B) Agar-based electrophoresis of the amplified products by the MGB probe. M: 100 bp ladder. Expected fragment size were approximately 163 bp of FMDV 3D gene. Lanes 1-7: The standard dilutions from 6 ng/μl to 6 fg/μl. Unknown samples were not shown.

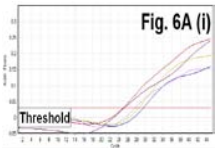



Fig. 6A (i)

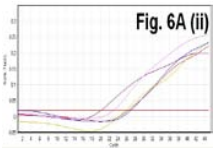



Fig. 6A (ii)

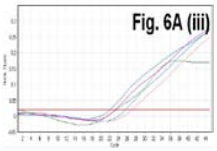



Fig. 6A (iii)

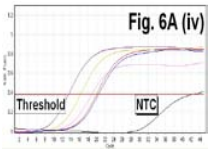



Fig. 6A (iv)

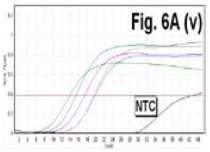



Fig. 6A (v)

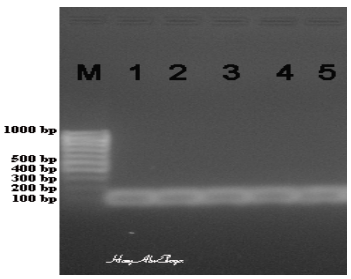



Fig. 6B (i)

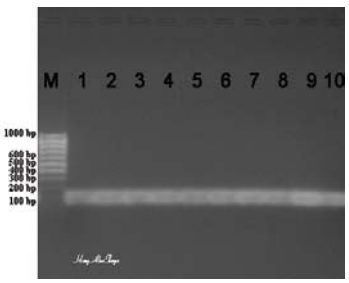



Fig. 6B (ii)

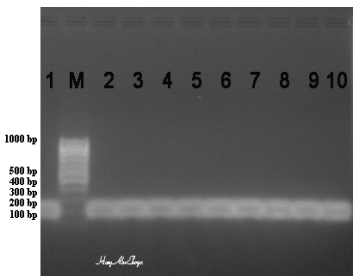



Fig. 6B (iii)

*Fig. 6:* Re-testing, 1-month later from previous current article evaluation, of a standard & its 1st RNA dilution for investigate the efficiency of a MGB probe rRT-PCR under variable factors. Positive control 2 using the primers either with MGB probe rRT-PCR Fig 6A (i)-(iii), Fig 6B (i) or with SYBR Green rRT-PCR Fig 6A (iv)-(v), Fig 6B (iii) depending on an old & new RNA aliquot of the standard and positive control 2, also an old & new aliquots of primers & probe. (A) Real-time detected signals curve (B) Agar gel based electrophoresis.



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