Acta Scientific Veterinary Sciences (ISSN: 2582-3183)

Volume 3 Issue 2 February 2021

Research Article

Utility of Vaccine Extracted FMDV RNA as a Positive Control in Real-time PCR for the Detection of FMDV Carrier Animals

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Received: October 20, 2020

Published: November 21, 2020

Abstract

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals and wild ruminants. It is caused by the foot-and-mouth disease virus (FMDV). Due to the contagiousness and aerosol transfer of FMDV, not all labs can handle FMDV. Only labs equipped with proper biocontainment facility (BSL3) can handle the work related to live FMDV. This may create hindrance in the expedition of sample testing by virus isolation. Thus, it is imperative to make the existing BSL-2 labs competent for testing the FMDV infected samples. Real-time PCR is one of the rapid ways of testing FMDV infected samples. Positive control of FMDV RNA is required for performing this assay. However, due to biosecurity reasons live FMDV cannot be used in BSL-2 labs. Therefore, one of the ways of obtaining FMDV RNA is extracting FMDV RNA from FMDV inactivated vaccine for its subsequent use in real-time PCR. In this work, we have extracted the FMDV RNA from inactivated FMDV vaccine and have subsequently used it as a positive control for screening oro-pharyngeal fluid from field animals (buffaloes) to confirm the FMDV carrier state. This work explores new opportunities to strengthen existing BSL -2 labs for testing the FMDV infected samples without virus isolation.

Keywords: Foot-and-mouth Disease Virus; Vaccine; qRT-PCR; Positive Control; FMDV RNA

Introduction

In the present world, due to an imbalance between nature and human activities, there is a frequent occurrence of emerging and transboundary diseases as exhibited by the recent ongoing COVID-19 pandemic. It has become the need of the hour for any country to increase its diagnostic capacity to handle the bulk of samples. Moreover, many animal diseases are there which we aim to eradicate from the globe by the end of the year 2030. Foot and mouth disease (FMD) is one of the diseases which is an important transboundary disease of cloven-hoofed animals including wild ruminants and is caused by foot-and-mouth disease virus (FMDV).

FMDV exists as seven distinct serotypes [O, A, C, Asia1, and South African Territories (SAT-1, SAT-2 and SAT-3)] and each serotype contains multiple subtypes. During an FMD outbreak, the spread of FMDV can occur at a large scale, simultaneously affecting several farms/animals within a short period [1]. These outbreaks are controlled by using an inactivated FMD vaccine which is a polyvalent vaccine comprising of vaccine antigen from FMDV O, A, Asia1, SAT serotypes.

Antigen ELISA, real-time PCR/RT-LAMP, and virus isolation are used to detect FMDV antigen, FMDV genome and live FMDV in infected samples, respectively [2,3]. FMDV infected animal can turn into a carrier if a live FMDV/FMDV genome is detected in the oropharyngeal fluid (OPF) after 28 days post-infection [4-6]. These carriers can be confirmed in the OPF either by real-time PCR or virus isolation [7]. Out of these, the most rapid and sensitive test is real-time PCR [8]. However, to run this test a positive control (RNA) is required. This needs virus isolation and subsequent extraction of viral RNA from the isolate. Labs lacking the facility of BSL3 cannot handle live FMDV due to biosafety issues. In such cases, a secondary source for the extraction of FMDV RNA can serve the purpose of positive control.

The method introduced in this work can be used in the labs which lack the BSL3 facility and are restricted not to handle the live FMDV. The control (RNA) can be used in real-time PCR for the detection of carriers from OPF of FMDV infected animals. Hence, addressing this issue, in this study we have successfully isolated FMDV RNA from commercially available FMDV vaccine which was further used as a positive control in qRT-PCR to detect FMDV carriers from OPF of suspected FMDV infected animals.

Materials and Methods

RNA extraction from vaccine sample

One commercial oil emulsion trivalent FMD vaccine (Indian Immunologicals, Hyderabad, India) comprising of antigens for FMDV serotypes O, A and Asia 1 was used for isolation of FMDV RNA. The extraction of FMDV RNA from the vaccine sample was done as per method [9] suggested earlier with slight modifications. Since, the vaccine was in an oil emulsion, before RNA extraction, 1 ml of FMD vaccine suspension was centrifuged at 10000 rpm for 20 minutes and the pellet was subjected for FMDV RNA extraction by using TRI reagent (Sigma-Aldrich, USA). Briefly, the pellet obtained was re-suspended in 400 µl of DEPC treated distilled water followed by the addition of 600 µl of TRI reagent and vortexing. This was followed by the addition of 120 µl chloroform and vortexing the mixture, to separate it into an aqueous and organic phase. The mixture was then allowed to stand in ice for 5 min. This was followed by centrifugation at 12,000g for 15 minutes at 2 - 8°C which separates the mixture into 3 phases: an organic phase (containing protein), interphase (containing DNA), and an upper aqueous phase (containing RNA). The aqueous phase was transferred to another tube followed by the addition of 0.5 ml of isopropanol per ml of TRI Reagent and mixed by inverting the tube upside down. The sample was then allowed to stand for 5 - 10 minutes at room temperature followed by centrifugation at 12,000g for 10 minutes at 2 - 8°C. The RNA was obtained as a pellet on the side bottom of the tube. The supernatant was removed and RNA pellet was washed with 1 ml of 75% ethanol. This was followed by vortexing and centrifugation at 7,500 *g* for 5 min at 2-8°C. The RNA pellet was dried and dissolved in 50 µl Tris-EDTA (TE) buffer, pH 8.0 for its subsequent use in simple PCR and real-time PCR (qRT-PCR). To optimize the standard procedure for RNA extraction from the vaccine, four small modifications in the above protocol were done which were as follows:

- Method 1: Vaccine sample was not centrifuged. Incubation with isopropanol at room temperature. Rest protocol followed as above.
- Method 2: Vaccine sample was not centrifuged. Incubation with isopropanol at -20°C overnight. Rest protocol followed as above.
- Method 3: Vaccine sample was centrifuged at 10000 rpm for 20 minutes and then pellet subjected for RNA extraction. Incubation with isopropanol at room temperature. Rest protocol followed as above.
- Method 4: Vaccine sample was centrifuged at 10000 rpm for 20 minutes and then pellet subjected for RNA extraction. Incubation with isopropanol at -20°C overnight. Rest protocol followed as above.

Quantification of RNA by nanodrop

The extracted RNA was quantified by NanoDrop™ (Thermo Scientific™). Briefly, 1 µl of the extracted RNA was subjected to RNA quantification and the reading obtained was taken as quantified RNA.

Reverse transcription-polymerase chain reaction (RT-PCR) for FMDV RNA confirmation

Extracted RNA was confirmed in RT-PCR which was performed as mentioned earlier [10]**.** It was accomplished in two steps. In the first step, cDNA was synthesized from extracted RNA using reverse transcriptase and in the second step, the cDNA was amplified by a polymerase chain reaction.

cDNA synthesis

Reverse transcription was carried out in a 25 µl reaction mixture using Reverse Primer NK 61. Components used for reverse transcription in a total volume of 25 µl were as follows**:** Primer NK 61 (10 pmols/µl) 2 µl, Template RNA 8 µl, 10 mM deoxynucleotide

triphosphates (dNTPs) 2 µl, 5X Reaction Buffer 5.0 µl, RevertAidTM M-MuLV Reverse Transcriptase (200 units/ µl) 0.5 µl, Ribonuclease Inhibitor (40 units/µl) 0.2 µl and Nuclease Free water 7.3 µl. This mixture was then put in the water bath at 60°C for 42 minutes. Finally, cDNA was placed on ice and used as the template for PCR amplification.

Polymerase chain reaction amplification of cDNA

The PCR was performed as mentioned earlier [10]. The amplification cycles were preceded by heating the mixture tube for 2 minutes at 94°C and followed by heating for 5 min. at 72°C. The cDNA of FMDV was amplified using gene-specific primer pairs, targeting the 1D (VP1) gene of FMDV belonging to serotype O. The initial denaturation step was done at 95ºC for 3 minutes for all primers, three-step cyclic denaturation at 94ºC for 30 seconds, extension step was at 72ºC for one minute and a final extension at 72ºC for 8 minutes for all primers. Different gradients of temperature were used to decide the annealing temperature of primers (Table 1). The final volume of the master mix was 25 µl which consisted of 2 µl 10X PCR buffer, 1.5 µl cDNA, 1 mM forward primer, 1 mM reverse primer, 25 mM $MgCl_2$, 10 mM dNTPs, 2.5 Units Taq DNA polymerase and 16.1 µl nuclease-free water. The primer sequence and the annealing temperature used for the standardization of PCR are given in table 1.

Primers	Sequences	Annealing temperature	Product size
FMD-$2B_{58}$ (NK61) (-ve sense primer)	GACATGTCCTCCTGCATCTG		
O-$1C_{124}$ (ARS4) (+ve sense primer)	ACCAACCTCCTTGATGTGGCT	54°C 56°C 58°C 60°C 62°C	1301 bp
O-$1C_{609}$ (Ovp3) (+ve sense primer)	TAGTGCTGGTAAAGACTTTGAGCT	54°C 56°C 58°C 60°C 62°C	816 bp

Table 1: Primers, their annealing temperature tested for gradient PCR and expected product size for the amplification of FMDV cDNA.

Agarose gel electrophoresis (AGE) for PCR product of VP1 gene

The PCR products were resolved in 1% agarose (LifeTech) gel containing 0.5 µg ethidium bromide (Sigma) per ml in tris-acetate-EDTA (TAE) buffer along with 1 kbp DNA ladder (Thermo Scientific Gene Ruler 1kb ladder). The 5 µl of PCR product was mixed with 1 µl of 6X loading dye and was loaded in the wells. The electrophoresis was carried out at 12 V/cm of gel in 1X TAE running buffer in a horizontal electrophoresis unit (Biometra, USA) till the indicator 6X loading dye reached the last third of the gel. The gels were visualized under UV transilluminator (Biovis) and photographed. The expected size of PCR products was estimated by comparison with that of the standard DNA ladder.

Test samples for real-time PCR

OPF samples were collected from field buffaloes to detect the FMDV carriers circulating in field conditions. For this purpose, thirty OPF samples from zones previously infected with FMDV were randomly selected from buffaloes for testing of FMDV RNA in real-time PCR.

RNA extraction from OPF samples for its subsequent use in qRT-PCR

RNA extraction from OPF samples was performed as suggested above by Chomczynski and Sacchi, 1987.

Application of extracted RNA in real-time PCR (qRT-PCR) for the detection of FMDV carriers in OPF samples

qRT-PCR was carried out in 7500 Standard Real-time PCR (Applied Biosystems, USA) by using Titan One Tube RT-PCR kit and TaqMan probe purchased from Sigma-Aldrich, USA. Reactions were performed in a 20 µl volume reaction mixture. A No Template Control (NTC) was also used which comprised of all other components except the RNA template. RNA extracted from commercially available FMD vaccine was used as a positive control. Primer sequences and conditions used to amplify the 3D region of FMDV were as described earlier [8].

The template was subjected to following cycling conditions- 50°C for 2 minutes: 1 cycle; 95°C for 10 minutes: 1 cycle; 95°C for 15 seconds, 60°C for 1 minute: 50 cycles.

The primer and probe sequence was as follow Forward primer 5' ACTGG GTTTT ACAAA CCTGT GA 3', reverse primer 5' GCGAG TCCTGCCACG GA 3' TaqMan® probe: TCCTT TGCAC GCCGT GGGAC.

The result of one-step RT-PCR was analyzed according to the standard of OIE [7] that is Ct value of 40 - 50 is considered as borderline, Ct value of less than 40 is considered positive and Ct value less than 20 is considered strong positive.

Results

Extraction of RNA from vaccine sample, RNA quantification, and PCR

The vaccine sample was centrifuged first at 10000 rpm for 20 minutes. The pellet was subjected to routine TRI reagent RNA isolation protocol. The RNA concentration was then measured by NanoDrop™ (Thermo Scientific™). No RNA yield was obtained by Method 1 and Method 2. The yield of 50.72 ng/µl (Table 2) was obtained when the vaccine sample was centrifuged at 10000 rpm for 20 minutes and then pellet subjected for RNA extraction along with overnight incubation with isopropanol at -20°C as given in Method 4. The yield of 11.68 ng/µl (Table 2) was obtained when the vaccine sample was centrifuged at 10000 rpm for 20 minutes and then pellet subjected for RNA extraction along with incubation with isopropanol at room temperature as given in Method 3.

Amplification of extracted RNA by RT-PCR

The extracted RNA from the vaccine were subjected to reverse transcription using NK61 primer to form cDNA. The cDNA was subjected to PCR amplification using primer pairs NK61/Ovp3 and NK61/ARS4. An 816 bp band was obtained with primer pair NK61/Ovp3 at annealing temperature 56°C (Figure 1). PCR with NK61/Ovp3 gave bands in vaccine samples using Method 3 and 4. No band was observed using Method 1 and Method 2 (Figure 1). No amplification was observed in any of the four methods using primer pair NK61/ARS4 (Figure 1).

Method	RNA concentration (ng/µl)
Vaccine sample was centrifuged at 10000 rpm for 20 minutes and then pellet subjected for RNA extraction. Incubation with isopropanol at room temperature (Method 3).	11.68
Vaccine sample was centrifuged at 10000 rpm for 20 minutes and then pellet subjected for RNA extraction. Overnight incubation with isopropanol at -20°C (Method 4)	50.72

Table 2: Concentrations of RNA obtained by various methods of RNA extraction.

Figure 1: Amplification of vaccine extracted FMDV RNA using primer pairs NK61/Ovp3 (left hand side to ladder) and NK61/ARS4 (right hand side to ladder). No amplification was obtained by using NK61/ARS4.
1 - Vaccine sample was not centrifuged. RNA extraction using TRI reagent. Incubation with isopropanol at room temperature. (Method 1).
3 - Vaccine sample was not centrifuged. RNA extraction using TRI reagent method. Overnight incubation with isopropanol at -20°C. (Method 2).
2, 4 - RNA isolated from vaccine in duplicate. Vaccine sample was centrifuged at 10000 rpm for 20 minutes and then pellet subjected for RNA extraction by TRI reagent. Incubation with isopropanol at room temperature. (Method 3) Vertical arrow shows 816 bp band.
5 - Vaccine sample was centrifuged at 10000 rpm for 20 minutes and then pellet subjected for RNA extraction by TRI reagent. Incubation with isopropanol at -20°C overnight. (Method 4) Vertical arrow shows 816 bp band.
NTC - No Template Control.
M - Thermo Scientific gene ruler 1kb ladder, catalogue. no. #SM0313.

Detection of FMDV carrier buffalo by qRT-PCR

The real-time PCR was performed to demonstrate the successful use of extracted FMDV RNA as a positive control for the detection of carriers from OPF samples. All three copies of extracted RNA samples found positive in simple PCR (Figure 1) were also used in qRT-PCR. All three extracted RNA were strongly positive with Ct value 18 and negative controls were under acceptable range (Figure 2). Along with the above three extracted RNA from the vaccine, extracted RNA from 30 OPF samples of field buffaloes was also subjected to qRT-PCR. Out of 30 OPF samples, one OPF sample was found positive with the Ct value of 20 (Figure 2), which confirms that out of 30 animals, one animal turned to be FMDV carrier.

Figure 2: qRT-PCR amplification curve for detection of FMDV carrier animals.

A, B and C are positive controls showing Ct value 18 and D positive OPF sample with Ct value 20, E negative samples.

Discussion and Conclusion

In the current global scenario, viral diseases have become the major transboundary, emerging and re-emerging diseases. In livestock, foot-and-mouth disease is one of the most devastating diseases, which, if become uncontrolled, can cause heavy losses to a country's economy. Foot-and-mouth disease (FMD) is a highly contagious and economically important disease of domestic and wild cloven-hoofed animals. The disease is caused by foot-and-mouth disease virus (FMDV) which belongs to the genus *Aphthovirus* of the family *Picornaviridae* [11]. Extremely contagious nature of FMDV, its aerosol transfer and transboundary migration through various modes restrict it's handling only in BSL-3 containment. To cope up with the growing need for the development of diagnostics it is paramount to strengthen the available resources so that in case of sudden outbreaks we can meet the requirement of sample testing on at large scale. It will lead to early disease diagnosis followed by early application of preventive measures. Increased diagnostic capacity also strengthens the eradication of the infectious disease from endemic countries.

To control FMD outbreaks in endemic countries, an inactivated/killed vaccine is used all over the world. In endemic countries, the FMD vaccine used is a polyvalent vaccine which is a combination of FMDV serotypes inactivated by binary ethyleneimine (BEI). The virus inactivation by using a low concentration of BEI occurs because BEI enters through the capsid, alkylates the genome and prevents genome reading [12,13]. Thus, the integrity of the neutralizing viral epitopes of the capsid remains intact after treatment with a low concentration of BEI [14]. BEI is also used for the inactivation of other viruses like Ross river virus (RRV) [15], Human immunodeficiency virus (HIV) [16], Hematopoietic necrosis virus (HNV) [17], Nipah virus [18], Sheep pox virus [19] and Porcine reproductive and respiratory syndrome virus (PRRSV) [20]. In the absence of positive control/FMDV RNA, the FMDV vaccine can be used as a source for isolation of FMDV RNA and can be used subsequently for PCR amplification [21,22]. Thus, the inactivated vaccine can serve a dual purpose, that is, it can be used to control the FMD outbreaks as well as it can serve the purpose of positive control for PCR amplification by extracting viral RNA from it.

In our study, no RNA was obtained by following Method 1 and Method 2 which exclude the centrifugation step. This could be because RNA extraction from an oily base is cumbersome. Therefore, centrifugation is required to obtain the viral antigen pellet devoid of oil. RNA yield of 11.68 ng/µl was obtained after centrifuging the vaccine sample followed by RNA extraction by TRI reagent with isopropanol incubation at room temperature (Method 3). In our study, a yield of 50.72 ng/µl FMDV RNA was obtained after centrifuging the vaccine sample followed by RNA extraction by TRI reagent with isopropanol overnight incubation at -20°C (Method 4). Higher RNA yield obtained after overnight incubation with isopropanol at -20°C could be because nucleic acids are less soluble or insoluble in isopropanol and at lower temperature gives better precipitation**.** Other commercial kit protocols suggest room temperature incubation with isopropanol. This is because longer incubation with isopropanol may lead to precipitation of salts**.** However, since our sample didn't have any source of salt so we took advantage of long incubation time (overnight) at low temperature (-20°C) with isopropanol to get a better yield.

The extracted RNA was confirmed by using sequencing primers as mentioned earlier [10]. The annealing temperature of PCR for amplification of vaccine extracted FMDV RNA was standardized using a gradient of annealing temperatures. At annealing temperature of 56°C, the 816 bp was obtained by using the primer pair NK61/Ovp3. This confirmed that the FMDV RNA was present in the vaccine sample. The amplification of RNA from an inactivated vaccine antigen could be attributed to the fact that after inactivation of the virus by BEI, a short stretch of intact RNA may be present [23,24] which can be used for PCR amplification. No amplification was obtained with primer pair NK61/ARS4. The forward primer ARS4 starts from position $1C_{124}$ and forward primer Ovp3 starts from $1C_{609}$. The RNA segment before nucleotide position $1C_{609}$ in the reverse complementary strand of RNA used for amplification, must have been degraded or partially degraded due to which primer ARS4 could not amplify the sequence [23,24]. Due to lack of funds, we could not sequence the extracted RNA. In the future, we aim to perform this task to corroborate our findings with another set of experiments.

Subsequently, the extracted RNA was used as a positive control in real-time PCR which was performed as per the guidelines of OIE [7]. All the three positive controls showed a very strong positive response with a Ct value of 18. It was found that out of thirty oropharyngeal samples only one sample showed a strong positive response with a Ct value of 20. The negative control/No-template control (NTC) did not show any signal-noise ratio and their amplification cycle was below the baseline. In a study [22] FMDV RNA has been extracted by using QIAamp RNA Mini Kit (QIAGEN, 52304). Our study demonstrates RNA extraction using the conventional method which most of the labs follow for RNA extraction from clinical samples or cell culture. In endemic countries due to economic constraints where it won't be possible to test the bulk of samples using commercial kits, the conventional method may be useful to extract RNA in BSL 2 labs equipped with limited resources. This study will open new insights to strengthen the existing diagnostic capacity by testing the samples through real-time PCR and acquiring FMDV RNA through the alternate source (vaccine sample) other than virus isolation.

We would like to emphasize that as the viral inactivation affect RNA genome hence it could have an impact on the primer and probe binding in qRT-PCR. Hence, from future perspective we would like to add that RNA extraction will be performed from several batches of inactivated FMD vaccine in order to rule out the reproducibility of Ct values when uniform quantity of viral RNA is added for cDNA synthesis and uniform volume of cDNA is added to qRT-PCR. This will help to ascertain any batch to batch variation in RNA sequence.

The above study reveals that after centrifuging the vaccine sample followed by subjecting the pellet for RNA extraction by TRI reagent and subjecting it to overnight isopropanol incubation is the most efficient way to isolate the RNA from the inactivated vaccine. Subsequently, this extracted RNA can be used as a positive control in real-time PCR assay for the FMD diagnostic purposes in the labs equipped with BSL-2 facility without undergoing virus isolation.

Acknowledgments and Funding Information

This study was not funded by any research grant. The work conducted is a part of Ph.D. research work conducted by Dr. Beenu Jain and funded by University fund (Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, India) granted to students for conducting research.

Conflict of Interest

All authors declare no conflict of interest.

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