



Detection of foot-and-mouth disease virus in semen of infected cattle bulls

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ABSTRACT

Foot-and-mouth disease is a highly contagious disease of all cloven hoofed animals caused by FMD virus (FMDV) and is mainly transmitted by contact, aerosols and fomites. Reports of FMDV dissemination in the cattle population via frozen semen straw are scanty, but virus may spread to susceptible population through artificial breeding programme. FMD carrier or recovered animals may secrete virus in semen without manifesting clinical symptoms that is why biological security of semen used for artificial insemination should be ensured by regular screening for FMDV. However, routine FMD diagnostic methods such as virus isolation and immunoassays have low sensitivity for detection of FMDV in the semen. Hence the study was undertaken to optimize a multiplex PCR assay for rapid screening of FMDV in cattle bull semen. Different RNA extraction procedures from semen were modified and evaluated for *in vitro* amplification of FMDV target gene. Analytical sensitivity of optimized mPCR assay was determined by experimental spiking of FMDV in semen samples. Detection limit of the optimized RNA procedure for mPCR amplification was determined to 0.5 TCID₅₀/ml in FMDV serotypes O, A and Asia1. The optimized mPCR assay was used for screening of a cattle bull farm having recent history of FMD outbreak. Clinically infected animals were found positive for the amplification of FMDV gene in semen samples collected after more than 5 months of infections but not after 8 months post infection.

Key words: Foot-and-mouth disease, Multiplex PCR, Semen, Virus detection

Foot-and-mouth disease (FMD), caused by foot-and-mouth disease virus (FMDV), is a highly contagious disease of all cloven hoofed animals. Major modes of virus transmission are contact, aerosol, iatrogenic, and fomites. Though, reports of FMDV dissemination via semen are scanty but virus may spread to extensive geographical area through artificial insemination as viruses can survive for longer duration in the frozen semen (van Rijn *et al.* 2004, Guérin and Pozzi 2005). Control of FMD in endemic countries like India is mainly relied on routine preventive vaccination and sero-surveillance. However in such a scenario, FMD carrier animals are difficult to identify and continue to secrete viruses in body fluids which act as source of infection. There is a probability that semen of these infected or carrier animals may transmit FMDV to the cattle

population via artificial insemination. To ensure biological security of cattle bull semen, screening of FMDV in semen may be performed with rapid and sensitive assay. However, diagnostic methods such as virus isolation and antigen ELISA are less sensitive and require special approach for making diagnosis in the semen samples (Shao *et al.* 2008). More sensitive multiplex PCR (mPCR) assay for simultaneous detection and identification of FMDV serotypes also gives false negative results for semen samples. The major reason behind PCR failure are presence of high load of PCR inhibitors present in the semen (Shin *et al.* 1997, Vangrýsperre and De Clercq 1996), hence, viral RNA extraction methods have to be improvised for optimization of multiplex PCR for the screening of FMDV in cattle bull semen.

In this study, experimentally spiked and naturally infected semen samples were used for optimization and standardization of multiplex polymerase chain reaction (PCR) assay for effective detection of FMDV in cattle bull semen. Different RNA extraction methods from neat and extended semen samples (frozen semen straws) were evaluated with objective of reduction in the level of PCR inhibitors and spermatozoal DNA for successful PCR amplification.

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MATERIALS AND METHODS

Semen and serum samples: Neat semen and serum samples from 4 healthy bulls were collected from a farm having no previous history of FMD outbreak in last 10 years. Serum samples from these animals were subjected to liquid phase blocking ELISA (LPBE), for detection of FMDV structural proteins and r3AB3 (Mohapatra *et al.* 2011) and 3ABC indirect DIVA ELISA for detection of FMDV non-structural (NSP) proteins. The semen samples were used as negative control for optimization of the mPCR assay.

FMD virus isolates: FMD virus isolates of serotypes O (IND R2/1975), A (IND 40/2000) and Asia1 (IND 93/2008) were obtained from national FMD virus repository maintained at this laboratory. FMDV isolates were revived on BHK-21 cells monolayer and 50% tissue culture infective dose (TCID₅₀) was determined using Reed-Munch method. Serial 10-fold dilutions of viruses were prepared in maintenance media (2% FBS, IMDM) to obtained titer of 10⁵ to 10⁻¹ TCID₅₀/ml.

Virus spiking: Negative semen samples were mixed with virus of different titers to obtain final titers of 10⁵ to 10⁻¹ TCID₅₀/ml. Likewise, 7 semen standard controls in 3 replicates for each serotype were prepared. The spiked semen samples were then subjected to antigen detection ELISA and different viral RNA extraction procedures followed by multiplex PCR for detection of spiked virus.

Spiked semen samples were mixed with phosphate buffer saline (PBS) in 1: 1 ratio and thoroughly vortexed followed by centrifuged at 3000g for 15 min and supernatant was collected for testing in sandwich ELISA as per the protocol adopted by Project Directorate on Foot and Mouth Disease, Mukteshwar (Bhattacharya *et al.* 1996).

RNA extraction: RNA extraction procedures were evaluated on whole semen as well on seminal plasma obtained by centrifugation of whole semen at 8000g for 5 min. Different methods described in the literature and commercially available kits were employed for RNA extraction. Samples were subjected to RNA isolation using (i) acid guanidinium thiocyanate (Chomczynski and Sacchi 1987); (ii) phenol: chloroform: isoamyl alcohol (25: 24: 1, PCI) method (Sambrook and Russel 2001); (iii) TRIzol (iv) QIAamp viral RNA mini kit; (v) QIAshredder columns coupled with TRIzol, and (vi) QIAshredder columns coupled with QIAamp viral RNA mini kit. Further, a modified RNA extraction procedure involving pre-treatment (protocol vii) was standardized for semen. This involves pre-treatment of semen (300 µl) with equal volume of RLT lysis buffer. Sample was mixed by vortexing and lysate was loaded on to the QIAshredder column for homogenisation followed by centrifugation for 2 min at 8000g. Homogenised lysate thus obtained was subjected to further extraction using QIAamp viral RNA mini kit (protocol vii.a) and TRIzol method (protocol vii.b).

Reverse transcription polymerase chain reaction: Reverse transcription of extracted viral RNA was performed as described previously (Mohapatra *et al.* 2010). Synthesized cDNA was used as template for *in vitro* amplification using reverse primer pNK61 and forward primers DHP9, DHP13 and DHP15 (specific for serotypes Asia 1, O and A, respectively) as described by Giridharan *et al.* (2005). PCR amplified products were resolved on 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Serotype specific amplified products were then gel purified using gel extraction kit and subjected to nucleotide sequencing using pNK61 primer and Big dye terminator v3.1 cycle sequencing kit in genetic analyzer.

The optimized mPCR assay was analysed by testing field samples collected from clinically infected, recovered and uninfected cattle bulls from a farm having recent history of FMD outbreak. Initially, random 20 semen samples along with serum samples (1 month post outbreak) were collected from clinically infected cattle bulls and screened with optimized mPCR assay. Extended semen samples (frozen semen straws) of the same bulls were obtained from these farms which were stored 15 days before outbreak. On third and fifth month post infection all the animals at the farm (*n* = 77) were screened for FMDV in semen. Animals found positive for FMDV genome in semen at fifth month were again screened at 8 month post infection along with random semen samples (*n* = 24) from rest of the animals. Total of 198 semen samples were collected over period of time and examined in optimized mPCR assay. Serum samples were subjected to r3AB3 and 3ABC NSP indirect ELISA for retrospective diagnosis.

RESULTS AND DISCUSSION

The routine FMD diagnostic methods were found unsuitable for the semen samples. Virus could not be isolated following standard protocol due to cytotoxicity of the semen and requires careful dilution (Shao *et al.* 2008). Antigen trapping ELISA, although is a highly sensitive method for detection and serotyping of FMDV in the clinical samples, but the assay failed to detect any viruses in the spiked semen. The coated antibodies for capturing of serotype specific FMDV could not capture virus from spiked semen samples. The probable reason could be thickness of the semen samples which even on several dilutions with PBS remained the same.

mPCR has been validated in several places including in this laboratory for simultaneous detection and serotyping of FMDV from tongue epithelium, foot lesions and saliva and proven to be superior over virus typing ELISA (Giridharan *et al.* 2005, Vangrysterre and De Clercq 1996, Callens and De Clercq, 1997). However, mPCR assay using routine method of RNA extraction could not detect any virus even in spiked semen samples with high load of virus.

In this study, different RNA extraction methods from semen samples were modified to reduce the non-specific PCR

inhibitors for successful amplification of FMDV target gene in mPCR. Viral RNA extraction by protocols (i), (ii) and (iv) was unhelpful as sample became too viscous to handle even after several rounds of semen dilution in lysis buffer. RNA could be isolated and quantified in NanoDrop using protocol (iii) but failed to amplify in RT-PCR. RNA extracted using protocol (v) and (vi) were amplified into a very faint band in mPCR and that too at the titre of 5×10^5 TCID₅₀ only, reflecting its low sensitivity. All these protocols were found unsuitable for viral RNA extraction from semen and were thus discontinued (Table 1). Presence of non-specific PCR inhibitors such as lactoferrin, peroxidase, zinc residues hampers the action of polymerase enzyme reducing the sensitivity of the assay (Cohen *et al.* 1997). This problem was partially resolved by using seminal plasma for viral genome extraction (Gilbert *et al.* 1997) but in case of FMD, although, majority of the viruses are extracellular but present in tight association with spermatozoa (Cottral *et al.* 1968) which can only be removed by repeated washing, hence this portion cannot be excluded for RNA extraction. Different methods for RNA extraction were modified and evaluated to minimize PCR inhibitors contaminants (Christopher-Hennings *et al.* 2006). The modified RNA extraction procedure (protocols vii.a and vii.b) provided the most optimum results for *in vitro* amplification and 249, 376 and 537 bp amplified product was obtained for the FMDV serotype O, A and Asia 1, respectively, in the spiked semen samples. Dilution of semen with RLT lysis buffer and passing through QIAshredder column probably reduced the non-

Table 1. Viral RNA extraction methods used for RNA extraction from cattle bull semen followed by mPCR amplification

Method No.	RNA extraction method from semen	Result
(i)	Acid guanidinium thiocyanate	RNA could not be extracted
(ii)	Phenol: chloroform: isoamyl alcohol method (25: 24: 1, PCI)	RNA could not be extracted
(iii)	TRIzol	RNA extracted; no amplification in mPCR
(iv)	QIAamp Viral RNA Mini Kit	RNA could not be extracted
(v)	QIAshredder columns coupled with TRIzol	RNA extracted; faint amplification in mPCR
(vi)	QIAshredder columns coupled with QIAamp Viral RNA Mini Kit	RNA extracted; faint amplification in mPCR
(vii a)	Pre-treatment with RLT lysis buffer followed by protocol vi.	RNA extracted; good amplification
(vii b)	Pre-treatment with RLT lysis buffer followed by protocol v.	RNA extracted; good amplification

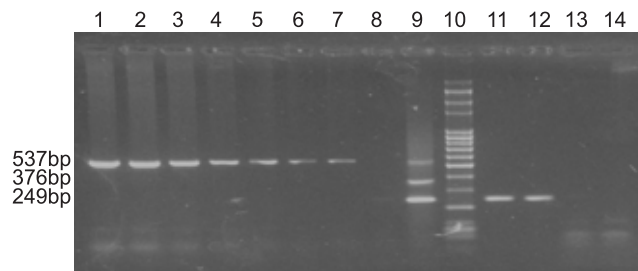


Fig. 1. Multiplex PCR for detection of FMDV in cattle bull semen. Lane No.1 to 7, Positive amplification at size 537bp (1D region) of Type Asia1 spiked semen sample (5×10^5 to 5×10^{-1} TCID₅₀/ml); lane No.8, no amplification in 5×10^{-2} TCID₅₀/ml type Asia1 spiked semen sample; lane No.9, positive control of type O, A, Asia1 viruses; lane No.10, DNA marker of 100bp; lane No. 11 and 12, positive amplification at size 249bp (1D region) of Type O in infected semen samples; lane No. 13, semen sample negative for FMD; lane No. 14, negative control of nuclease free water.

specific PCR inhibitors. QIAamp viral RNA extraction kit and Trizole method were found equally efficient for RNA extraction from pre-treated semen samples for *in vitro* amplification. The advantages of QIAshredder columns for removing non-specific PCR inhibitors in RNA extraction was also noticed by Christopher-Hennings *et al.* (1995). Similarly, siliconized polypropylene tubes and Sephadryl S-400 chromatography can also be used for similar results (Christopher-Hennings *et al.* 1995, Da Silva *et al.* 1995).

Experimental spiking of the semen samples with known titer of virus isolates demonstrated the analytical sensitivity of the assay to be 0.5 TCID₅₀/ml in both whole semen and seminal fraction (Fig. 1). These results were found repeatable and uniform for all the 3 serotype tested. Although, this standard do not mimic a natural case where viruses are cell associated, but can neutralizes the effects of non-specific inhibitors and spermatozoa genomic load on PCR amplification. The detection limit of the mPCR with infected virus cell culture supernatant was calculated to 0.01 TCID₅₀/ml (Giridharan *et al.* 2005). The drop in the lower detection limit may be due to the presence of minor quantity of non specific RT-PCR inhibitors. Another reason could be loss of RNA due to addition of a step in RNA extraction procedure. Da Silva *et al.* (1995) also reported decrease in detection limit in semen samples by 10-fold when compared to tissue culture fluids. Even with this detection limits, the optimized assay would be highly useful for screening of cattle bull semen. The minimum amount of virus to infect cows/heifers by insemination was shown to be $10^{3.4}$ MLD₅₀ (Cottral *et al.* 1968), which is much higher than the detection limit of optimized test.

The farm selected for screening of semen had recent outbreak of FMD with FMDV serotype O, which was confirmed by virus isolation and antigen ELISA (in samples collected from tongue epithelium) in this laboratory. Serology also confirmed the FMD infection as all the animals of the

farm were reactive to r3AB3 and 3ABC FMDV non-structural proteins in indirect DIVA ELISAs.

Semen collected from 20 infected cattle bulls were tested with the optimized mPCR. Fifteen out of these 20 cattle bull semen were found positive for serotype O partial 1D gene amplification (249bp) in the mPCR. The same animals were negative for FMDV in semen stored as frozen straw 15 days before the outbreak (Fig. 1). Nucleotide sequencing of amplified product (partial 1D region of FMDV), further confirmed the specific amplification by mPCR. Processed sequence were aligned with reference 1D sequences of FMDV serotype O, A and Asia 1 retrieved from NCBI database using the Clustal W algorithm accessible in the MEGA4.1 software (Tamura *et al.* 2007). On phylogenetic analysis using neighbour-joining method (Saitou and Nei 1987), it was observed that the sequences obtained from semen samples grouped under serotype O demonstrating the specificity of the mPCR assay (Fig. 2).

The duration of FMDV secretion in semen in some of the infected animals under the study was calculated to minimum of 5 months. Large proportions of the infected animals were found positive for FMDV in semen by mPCR after 1 month post infection (31%). Even after 5 months post infection, 14% of animals were positive in semen. However, all the animals at the farm became negative after 8 months post infection. These findings indicated the duration of FMDV

secretion in semen probably much higher as described by Cottral *et al.* (1968) where FMDV was found after 8 days post infection, but not at 10 days by virus isolation in suckling mice. However, further experimental infection studies are required to ascertain duration of virus secretions in the semen of infected, recovered and subclinical animals in endemic state.

FMD virus being a highly contagious and rapidly transmissible disease demands early diagnosis and rapid measures to check the spread of the virus. Previous studies indicated the shedding of FMDV in semen, which could be source of infection to the susceptible population. Bastos *et al.* (1999) has reported the transmission of FMDV in African buffalo via mating in the infected herd. FMD carrier animals constitute the major threat as animal continues to excrete virus in body secretions without exhibiting clinical signs. The early stage of infection prior to the onset of clinical signs is associated with high titres of virus in secretions, excretions and affected tissues (Burrows *et al.* 1981). It is documented that $10^{1.5}$ to $10^{4.7}$ 50% mouse lethal dose MLD_{50}/ml of FMDV in bull semen is present up to 4 days before clinical signs and $10^{6.2}$ MLD_{50}/ml after development of clinical lesions (Burrows 1972, Cottral *et al.* 1968, Sellers *et al.* 1968). FMDV present in frozen semen have potential to spread in wide area of population due to active secretion of virus before onset of clinical symptoms and survival of virus in frozen straw. Procedures used in processing semen for artificial insemination, such as dilution with extenders and freezing, create conditions conducive to virus survival. Virus may survive as long as 320 days when stored at $-50^{\circ}C$ without loss in virus titer (Cottral *et al.* 1968). Artificial insemination although has reduced the incidence of many infectious diseases especially sexually transmitted disease but on the other hand opened the avenue of virus spreads to vast susceptible population via frozen straw. To ensure FMDV free semen for artificial insemination, regular screening and monitoring of cattle bulls semen in endemic areas are required with a rapid and reliable diagnostic method. Multiplex PCR, requiring routine laboratory facility, has emerged as the best alternative to conventional virus isolation and ELISA. The developed assay has potential to screen the cattle bulls engaged for semen straw production. However, validation of the assay is required by screening more number of farms and testing experimentally infected animals.

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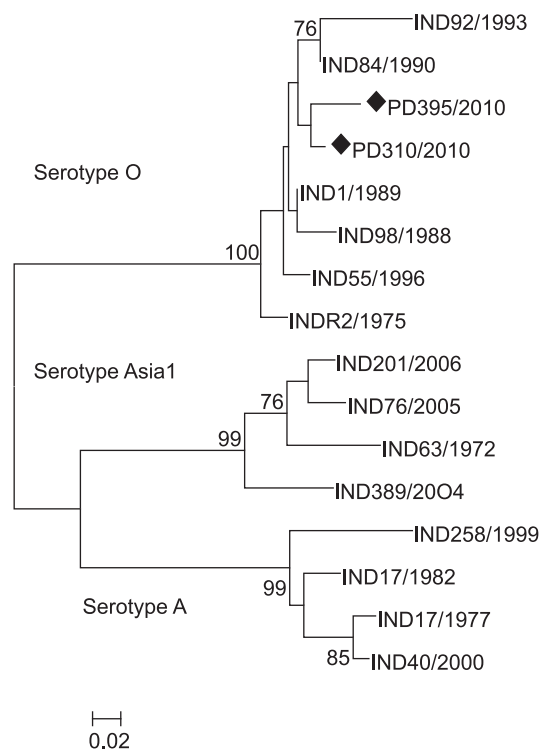


Fig. 2. Phylogenetic tree depicting PD395/2010 and PD310/2010 Type O 1D gene sequence (amplified in cattle bull semen samples) aligned with FMDV serotype A and serotype Asia 1 sequences.

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