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Evaluation of a specialized filter-paper matrix for transportation of extended bovine semen to screen for bovine herpesvirus-1 by real-time PCR

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ABSTRACT

The extended frozen semen (EFS) batches produced from infectious bovine rhinotracheitis (IBR) sero-positive cattle and buffalo bulls housed in various semen stations in India are transported to the testing laboratory in liquid nitrogen (LN_2) for screening bovine herpesvirus-1 (BoHV-1). This procedure is laborious and poses LN_2 related hazards. An alternative logistics for transportation of samples was investigated. Use of Flinders Technology Associates (FTA®) elute card was evaluated for transportation of extended bovine semen to screen BoHV-1 DNA by real-time PCR targeting gB gene and the method was compared with the OIE approved Chelex resin based method. A protocol for extraction of BoHV-1 DNA from FTA® card spotted with extended semen was optimized. The viral DNA was found to be stable on FTA® card for at least 28 days when the cards are stored at 4°–37 °C. The analytical sensitivity for the assay was determined using variable dilutions of BoHV-1 spiked semen and positive plasmid harbouring gB gene (97bp) spotted onto FTA® card and it was found to be $10^{0.8}$ $TCID_{50}$/ml or 100 copies respectively in real-time PCR. The test could detect as low as $10^{0.008}$ $TCID_{50}$/ml or 1 copy of positive plasmid when more number of replicates (n = 6) of the same sample were tested. This sensitivity was found to be comparable to Chelex method and both the methods demonstrated a very strong correlation (r = 0.9774; 95% CI: 0.9620–0.9860) in terms of Ct value ($p < 0.0001$). The diagnostic sensitivity and specificity of the FTA method in comparison to the Chelex method was 83.08% (95% CI: 71.73%–91.24%) and 93.23% (95% CI: 89.38%–96.01%) respectively when 316 samples were screened by both the methods. The degree of agreement between these two tests was good (Kappa value: 0.738; 95% CI: 0.646–0.829). The method was found to be robust and highly repeatable in inter-assay and intra-assay precision testing. The result suggests that the FTA® card holds promise as an alternative system for transportation of EFS for downstream screening of BoHV-1 DNA.

1. Introduction

Bovine herpesvirus 1 (BoHV-1) infection in cattle and buffaloes manifests into economically important disease syndromes – infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV). The disease is characterized by rhinotracheitis, pustular vulvovaginitis, balanoposthitis, conjunctivitis, enteritis, general respiratory disease, encephalitis, decreased milk production, weight loss and abortion (Muylkens et al., 2007).

The BoHV-1 virus belongs to genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, family *Herpesviridae* and order *Herpesvirale* (Murphy et al., 1999; Muylkens et al., 2007). Following primary infection, the virus becomes latent in the sensory neurons of trigeminal/sacral ganglia. During stress or immune suppression, reactivation of the virus occurs and the virion is shed in various body secretions *viz.* nasal, lachrymal, seminal and genital secretions (Jones et al., 2006; Kutish et al., 1990). The BoHV-1 virus is intermittently excreted in the semen of IBR sero-positive bulls and thus the virus can be transmitted from infected animal to susceptible animal thorough such contaminated semen during natural or artificial insemination (AI) (Mars et al., 2000). Although culling of sero-positive bulls are recommended, it is difficult to follow in many settings. Hence, in such cases it is advocated that each ejaculate of the bull be tested for the absence of BoHV-1 either by real-time PCR or by cell culture. (DADF, 2014; OIE, 2017a).

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Currently all frozen semen batches produced from IBR sero-positive bulls are transferred from semen stations to the testing laboratory in liquid nitrogen (LN_2) which is cumbersome, expensive and is associated with problems in transportation. The requirement of LN_2 for sample transport could be obviated by use of alternative method like chemically treated filter papers spotted with clinical materials.

Use of the filter paper-based system in specimen collection and transport for the diagnosis of infectious agents has gained momentum in recent years as it obviates the requirement of cold chain for preserving the fitness of the specimen during transportation and storage (Smit et al., 2014). FTA® (Flinders Technology Associates) cards are specially treated filter papers that inactivate the pathogenic bacteria/virus upon contact, thereby allowing safe and easy transport of materials to the laboratory (Muthukrishnan et al., 2008). A number of studies have evaluated use of FTA® cards for screening of infectious diseases of veterinary importance namely, rabies virus, foot-and-mouth disease virus, newcastle virus, swine influenza virus, porcine reproductive and respiratory syndrome (PRRS), avian metapneumovirus, bovine respiratory disease complex (BVDV, BRSV, coronavirus, BoHV-1) (Inoue et al., 2007; Maldonado et al., 2009; Muthukrishnan et al., 2008; Perozo et al., 2006, Picard-Meyer et al., 2007; Prickett et al., 2008; Wacharapluesadee et al., 2003). Most of these studies have shown comparable sensitivity and specificity of detection with samples transported through traditional cold chain. The findings of these studies suggest, FTA® card can be used as an alternative system of sample transport and storage for nucleic acid based diagnosis. OIE also suggests that FTA® cards can be used as an alternative system for transport of specimens for detection of rabies virus using molecular techniques (OIE, 2013). However, detection of pathogen from samples spotted onto FTA cards is dependent on several factors *viz.* nucleic acid concentration in the sample, the inherent stability of nucleic acid and the extraction procedure adopted.

In the present work attempts have been made to develop a protocol for retrieval of nucleic acid from extended bovine semen through FTA® card for detection of BoHV-1 DNA by real-time PCR.

2. Materials and methods

2.1. Virus, semen and positive plasmid DNA construct

In this study, virus strain Guk 1/1983 (BoHV-1.1 genotype), available in the repository of National Dairy Development Board Research and Development (NDDB R&D) laboratory, Hyderabad, India as infected MDBK cell culture supernatant was used as reference virus. The infectivity titer of the virus was $10^{6.8}$ $TCID_{50}$/ml (Reed and Muench, 1938). For the standardization, two sets of serial ten-fold dilution of the BoHV-1 virus were prepared; one in minimum essential media (MEM) and another in known BoHV-1 negative extended semen. The diluted virus was aliquoted and stored at −80 °C for future use.

Extended frozen semen (EFS) batches collected from IBR sero-negative animals and tested negative in real-time PCR were used as known BoHV-1 negative semen samples. EFS batches collected from sero-positive bulls and tested positive by real-time PCR were used as known BoHV-1 positive semen samples. The Tris-egg yolk-glycerol extender (Tris Buffer – 24.2 gm, Citric Acid – 13.6 gm, Fructose – 10 gm, Glycerol – 70 ml, Egg Yolk – 200 ml, Benzyl Penicillin – 10 lakh IU, Streptomycin – 1 gm and distilled water to make up volume 1000 ml) was used as diluent in the frozen semen doses.

A positive plasmid construct containing the target 97bp of BoHV-1 glycoprotein B (*gB*) gene cloned into pDRIVE cloning vector (Rana et al., 2011) was used as positive control in the real-time PCR reaction. The concentration of the plasmid construct was 1.893×10^{10} copies/µL. For the standardization, a working stock of 1×10^{10} copies/5 µL was prepared. Further, the standards with variable copy number starting from 1×10^{10} copies/5 µL to $1 \times 10^{\circ}$ copies/5 µL was prepared by serial ten-fold dilution of the working stock, aliquoted and stored at −20 °C for future use.

2.2. Extraction of DNA from semen samples by Chelex resin based method

The extraction of DNA from EFS was performed as per the protocol laid down in OIE terrestrial manual (OIE, 2017b). Briefly; 10 µL of extended semen sample was added to 0.6 ml tube containing lysis solution {11.5 µL of 10 mg/ml proteinase K (Sigma), 7.5 µL of 1 M DL-Dithiothreitol (DTT) (Sigma) in 90 µL nuclease free water (NFW)} and 100 µL of 10%, w/v Chelex 100 (Sigma). The sample mixture was vortexed for 10 seconds (s) and incubated at 56 °C for 30 minutes (min). The tube was vortexed again for 10 s and incubated at 98 °C for 8 min. The vortexing was then repeated and the samples were centrifuged at 10,000 × *g* for 3 min. The supernatant containing DNA was transferred to 96 well PCR plate and stored at −20 °C for future use.

2.3. Retrieval of DNA from FTA® elute cards spotted with extended semen

The retrieval of DNA from the FTA® elute cards (GE Healthcare Life Sciences) was carried out as per manufacturer's protocol with slight modification (GE Healthcare Life Sciences, 2011). For extended semen samples spotted onto FTA® card, overnight DTT treatment step was included before proceeding for the manufacturer's protocol. Briefly, 30 µL of extended bovine semen was added to the FTA® card and allowed to dry at room temperature. After complete drying, 10 µL of 0.1 M DTT was added to each spot and the card was left overnight for drying at room temperature. The next day one disc was punched out from the centre of the area spotted with semen using single hole paper punch (4.5 mm size) and the disc was transferred to a sterile 1.5 ml micro centrifuge tube. The sample disc was then washed by addition of 500 µL NFW, pulse vortexed for 3 times of 5 s each followed by removal of NFW. This washing step was repeated thrice and the disc was transferred to a fresh 0.6 ml tube. Then 30 µL of NFW was added to the disc and the tube was incubated in the PCR machine (with heated lid) for 30 min at 98 °C for elution of DNA. The tube was then pulse vortexed for approximately 60 times of 1 s each followed by brief centrifugation for 15 s. The FTA® matrix disc was discarded using a sterile pipette tip. The eluate containing DNA was stored at −20 °C.

2.4. Prevention of carry-over contamination

To prevent carry over contamination through punching device different decontamination procedures involving dipping with disinfectant and/or isopropyl alcohol (IPA) for different time interval followed by punching sterile blank filter paper were evaluated. The optimized procedure for cleaning of paper punch consisted of dipping in IPA for one minute, wiping it dry with tissue paper followed by five serial punches on Whatman filter paper. To further confirm if the method prevented carry-over contamination, the filter paper disc from the fifth punch was also subjected for DNA extraction. The elution from this disc was included in the downstream real-time PCR assay as a carry-over contamination control. One of this control was maintained for every four samples processed during standardization and for every seven samples processed during clinical sample screening.

2.5. Real-time PCR for detection of BoHV-1 DNA

The DNA extracted by Chelex resin based method or eluate from FTA® cards were processed by real-time PCR for detection of BoHV-1 viral DNA. The primers and probe targeting *gB* gene of BoHV-1 were used (Wang et al. 2007) and the real-time PCR was performed as per the protocol (Rana et al., 2011). Briefly, for a single reaction 12.5 µL TaqMan® Universal PCR Master Mix (ABI), 0.5 µL of each primer (gB-F and gB-R, 4.5 µM), 1 µL of probe (3 µM) and 5.5 µL of NFW were added. Finally, 5 µL DNA template was added. The real-time PCR was carried out in a Quant Studio 7 flex (ABI) or Step one Plus (ABI). The PCR

cycling parameters were, 2 min at 50 °C, 10 min at 95 °C, followed by 60 cycles of 15 s at 95 °C and 45 s of 60 °C. The threshold level was usually set according to the manufacturer's instructions for the selected analysis software used. Alternatively, the threshold was set manually at a level significantly higher than that of background. Samples were considered to be positive when the Ct (cycle threshold) value was less than 40.

2.6. Repeatability of the assay

The repeatability of the assay was evaluated by inter-assay and intra-assay variation. For the evaluation, semen samples consisting of (i) highly positive (known negative extended semen spiked with $10^{4.8}$ $TCID_{50}$/ml BoHV-1 virus) (ii) moderately positive (known negative extended semen spiked with $10^{1.8}$ $TCID_{50}$/ml BoHV-1 virus) (iii) known naturally infected EFS and (iv) known BoHV-1 negative EFS batches were spotted onto FTA® card. The DNA was extracted in quadruplets for three consecutive days followed by real-time PCR.

2.7. Stability of DNA spotted onto FTA card

Highly positive, moderately positive, known naturally positive EFS and known BoHV-1 negative EFS batches were spotted onto FTA® cards and the cards were stored at different temperature *viz.* 4 °C, room temperature (approximately 25 °C) and 37 °C for 28 days. To check the effect of humidity, semen spotted FTA® cards were kept at 4 °C in zip-lock pouch; (i) with silica gel and (ii) without silica gel. DNA was extracted from those cards in triplicate on days 1, 7, 14, 21, 28 and real-time PCR was carried out to check the amplification efficiency over a period of time.

2.8. Analytical sensitivity (limit of detection) of the assay

The analytical sensitivity for the assay was determined using serially ten-fold dilutions of (i) BoHV-1 ($10^{6.8}$/ml $TCID_{50}$) spiked extended semen and (ii) positive plasmid harbouring gB gene (97bp) in extended semen. DNA was extracted from each dilution series in triplicate by both Chelex based method as well as FTA method and real-time PCR was carried out.

2.9. Determination of diagnostic sensitivity and specificity

The diagnostic sensitivity and specificity were determined by testing EFS batches as per OIE guidelines. For each batch of EFS, 6 straws (250 µL each) were taken and were tested in triplicate (3 pools of 2 straws). The contents from two straws were transferred onto a 1.5 ml tube and 30 µL of semen was spotted onto FTA® card whereas 10 µL of the semen was used for processing by Chelex method. The DNA extracted by both the methods were tested in duplicate by real-time PCR. So in total there were 6 replicates of each sample. Any of the six replicates having Ct value less than 40 was declared positive. In each run, extraction control ($10^{1.8}$ $TCID_{50}$/ml BoHV-1 virus spiked semen; IBR negative semen) and reagent control (positive plasmid and non-template control) were included. A total of 316 EFS batches were tested by both the methods and the diagnostic sensitivity and specificity was calculated considering Chelex resin based method as gold standard.

2.10. Quality by design (QBD) experiment

The optimized protocol was evaluated for the robustness by deliberate modifications in the conditions (changing elution temperature and time, number of washing steps) and reagent concentrations (DTT concentration, different master mix). The effect of elution temperature and time was evaluated at three different temperature (80 °C, 90 °C and 98 °C) and at three different time interval (15 min, 30 min and 45 min). The effect of DTT concentration was evaluated by treating the semen spotted cards with different volumes of 0.1 M DTT (0, 0.1 µL, 1 µL, 2.5 µL, 5 µL). Further, four commercially available master mix {PREMIX EX TAQ™ (Probe qPCR), (TAKARA); Kappa Probe Fast Universal qpcr kit (KAPPA); VETMAX™PLUS qpcr master mix (thermo fisher) and TAQMAN® Universal PCR master mix (ABI)} were evaluated for their effectiveness in the real-time PCR reaction.

3. Results

3.1. Optimization of the protocol

The manufacturer's protocol was adequate for retrieval of the DNA from the FTA® card spotted with BoHV-1 virus diluted in MEM but not for the BoHV-1 spiked semen. A number of experiments were performed with the BoHV-1 virus diluted in MEM to optimize the general procedures for spotting and retrieval of DNA from the FTA® card *viz.* quantity of samples to be spotted, the size of the disc to be punched out, the number of discs to be used for extraction, elution volume, temperature and time. The above parameters were decided with reference to the Ct value obtained in the real-time PCR assay for the corresponding samples extracted using Chelex-resin based method. The manufacturer's protocol for DNA extraction did not result in any amplification in the real-time PCR assay for the BoHV-1 spiked extended semen spotted FTA® cards. Therefore, additional processing steps in the DNA extraction procedure were attempted to ensure optimum extraction of viral DNA. Four different types of modifications were attempted *viz.* (i) treatment of the extended semen sample with trypsin prior to spotting, (ii) treatment of punched out disc with lysis buffer (proteinase K and DTT), (iii) pre-treatment of the FTA® card either with PK + DTT, PK alone or DTT alone before spotting of extended semen; and (iv) treatment with either PK + DTT, PK alone or DTT alone of dried semen spotted FTA® card. The real-time PCR result suggests all above modifications were successful in ensuring extraction of viral DNA (data not shown). However, the following observations were made. The treatment of extended semen with trypsin resulted in higher Ct value relative to the corresponding sample where DNA was extracted using Chelex resin. The incorporation of lysis solution (PK + DTT) for FTA® card treatment compromised the integrity of the paper leaving fine paper fibres into the elution. The pre-treatment of cards either with PK + DTT or only DTT before spotting of samples resulted in positive amplification. However, pre-treatment of cards before sample spotting may not be practicable in field settings. The treatment of PK + DTT and DTT alone on the dried semen spot, followed by overnight incubation before proceeding for DNA extraction using manufacturer's instruction had the desired results. The real-time PCR results of these samples matched that of the results from the corresponding Chelex based DNA extracted samples. Since the treatment with PK + DTT and DTT alone did not have any appreciable difference in the results, the final protocol involved the addition of only DTT (data not shown).

3.2. Quality by design (QbD) protocol

The study on effect of elution temperature and time suggest 45 min is required for proper elution of DNA at 80 °C. Statistically there was no difference when elution was carried out at 90 °C or 98 °C. The result also revealed that, by 15 min the DNAs are eluted and keeping up to 45 min for elution do not have any adverse effect. The treatment of the semen spotted cards with DTT was found necessary as samples processed without DTT addition did not result in DNA amplification. Further, no difference in Ct value was observed for semen spotted onto FTA® cards treated with different volumes of 0.1 M DTT. All the master mixes showed similar results both for Chelex as well as FTA extracted DNA samples. These results suggests that the protocol can be readily adopted at other laboratories.

3.3. Analytical sensitivity

The serial ten-fold dilutions of BoHV-1 spiked semen and positive plasmid harbouring gB gene (97bp) were tested by FTA method as well as the Chelex method in triplicate. The conservative analytical sensitivity (all six replicates showing positive amplification in real-time PCR) was found to be $10^{0.8}$ $TCID_{50}$/ml or 100 copies of positive plasmid respectively. However, the test could detect as low as $10^{0.008}$ $TCID_{50}$/ml or 1 copy of positive plasmid (at least one of the six replicate showing positive amplification in real-time PCR). This sensitivity was found to be comparable to Chelex method (data not shown).

3.4. Repeatability

The repeatability of the assay was checked by inter-assay and intra-assay variation. All the replicates of the negative semen were found negative in all the test runs. The inter-day (inter-run) co-efficient of variation (CV) was calculated from the Ct values obtained in octuplicate. The CV was found to be 7.14, 1.20 and 0.79 for highly positive (spiked semen sample), moderately positive (spiked semen sample) and known naturally positive semen batch respectively. Similarly, the intra-day/between-run variability between octuplicate was found to be 5.22 for highly positive (spiked semen sample), 0.51 for moderately positive (spiked semen sample) and 2.10 for known naturally positive EFS respectively. This suggests that the test is highly repeatable.

3.5. Comparability between FTA and Chelex method

The two DNA extraction methods were compared based on the Ct value obtained from limit of dilution experiment as well as repeated testing of extraction control ($10^{1.8}$ $TCID_{50}$/ml BoHV-1 spiked semen). The data were analysed by scatter diagram and Bland-Altman plot. The correlation coefficient (r) was found to be 0.9774 (95% CI: 0.9620–0.9866) which indicated a very strong correlation between the two methods and $p < 0.0001$ indicated the low probability of this association to chance. Bland-Altman plot was used to identify relationship between the differences and the averages, to evaluate any systematic biases and to identify possible outliers. The results suggest on an average, the Ct value obtained in FTA method is 0.29 (95% CI: −0.58 to −0.006) less than that of Chelex method. The positive extraction control (known negative extended semen spiked with $10^{1.8}$ $TCID_{50}$/ml BoHV-1 virus) was used in every batch of DNA extraction in both Chelex and FTA method. The Ct values obtained for the positive control sample (19 times on different days) was checked for normal distribution. The mean Ct value and standard deviation of Chelex method was 38.2779 and 1.14; and for the FTA method was 37.8563 and 1.33 respectively. While, all the Ct values were found to be within two standard deviations for the Chelex method; only one replicate showed Ct values higher than two standard deviation in the FTA method.

3.6. Stability of the DNA on FTA® card

The weekly screening of FTA® cards stored at different temperature showed comparable Ct values over time period and at different temperature (4 °C, 25 °C and 37 °C) (Data not shown). The inter-class correlation between different days and temperature was found to be 0.99. IBR negative sample was negative throughout the period. This suggests, DNA in the semen samples after spotting onto FTA® card is stable at least up to 28 days when store at +4 °C to 37 °C and could be detected by real-time PCR. Similarly, there was no significant difference in Ct value obtained for cards stored at 4 °C with or without silica gel. This suggest slight changes in humidity do not significantly affect the quality of the DNA (data not shown).

Table 1
2 × 2 Contingency table showing the result of extended semen batches in real-time PCR. The comparison was carried out between DNA extracted by Chelex method and FTA method from the same samples.

2 × 2 contingency table	Chelex method of DNA extraction		Total
FTA method of DNA extraction	negative	positive	
Negative	234	11	245 (77.5%)
positive	17	54	71 (22.5%)
Total	251 (79.4%)	65 (20.6%)	316

Frequency table	Percentage	95% CI
Sensitivity	83.08%	71.73%–91.24%
Specificity	93.23%	89.38%–96.01%
Positive Likelihood Ratio	12.27	7.65–19.66
Negative Likelihood Ratio	0.18	0.11–0.31
Positive Predictive Value	76.06%	64.46%– 85.39%
Negative Predictive Value	95.51%	92.11%–97.74%

3.7. Diagnostic sensitivity and specificity

Diagnostic sensitivity and specificity was determined by testing EFS by both Chelex method and FTA method of DNA extraction followed by real-time PCR. A total of 316 EFS batches were tested and the diagnostic sensitivity and specificity of the FTA method with respect to Chelex method was found to be 83.08% (95% CI: 71.73%–91.24%) and 93.23% (95% CI: 89.38%–96.01%) respectively (Table 1). The Positive Predictive Value was 76.06% (95% CI: 64.46%–85.39%) and Negative Predictive Value 95.51% (95% CI: 92.11%–97.74%). The degree of agreement between the two tests was found to be good (kappa value: 0.738; 95% CI: 0.646–0.829). The range of Ct values obtained for the positive clinical samples in Chelex and FTA methods are presented in Table 2.

4. Discussion

In the present study, a method for retrieving of BoHV-1 viral DNA from FTA® cards spotted with EFS was standardized. The test method was validated with Chelex based method following the OIE guideline “comparability of assays after changes in a validated method" (OIE, 2016).

The manufacturer’s protocol for elution of DNA from the FTA® cards spotted with spiked extended semen did not result in positive amplification in real-time PCR. This could be either due to the presence of proteins and polyamines (spermine and spermidine) in the semen acting as PCR inhibitor or due to sliminess of the semen which prevent elution of DNA from the card (Ahokas and Erkkilä, 1993; St-Laurent et al., 1994; Wiedmann et al., 1993). The DNA extraction protocol from the FTA® card has been standardized mostly for blood samples where the major PCR inhibitors are haem proteins.

The addition of DTT to the dried semen spot, followed by overnight

Table 2
Comparison of Ct values obtained in real-time PCR for positive samples detected by Chelex method and FTA method of DNA extraction. The number of samples positive by both the methods are provided for different range of Ct values. (*The lowest Ct value obtained among the six replicate was considered for classifying the sample into the Ct values range).

Ct value range	Chelex method*	FTA method*
Below 34	0	0
34–36	4	3
36–38	6	9
38–40	55	59
Total	65	71

incubation and then proceeding for nucleic acid extraction as per manufacturer's instruction was found to provide the desired result. de Gannes (2014) has also observed that PK + DTT to the sperms do not yield more DNA than DTT treatment alone. DTT is a reducing agent that cleaves disulphide bonds and allows proteins to unfold. In semen, it dissociates protamines from DNA and also helps to remove the sliminess of the semen by breaking the proteins. The other advantages of DTT are its effectiveness, low toxicity, mild odour and its ability to work efficiently compared to β-mercaptoethanol (Griffin, 2013).

Inhibitors, if present, in the samples have a significant impact on the sensitivity of detection in PCR based tests. The DNA eluted from FTA® cards spotted with BoHV-1 spiked semen batches as well as known naturally infected extended semen batches could be successfully amplified in real-time PCR and the Ct values matched with the result of Chelex resin based DNA extraction method. This indirectly suggest that PCR inhibitory substances bound to the FTA® card were not released during elution. These experiments, however, do not ascertain either the presence or effect of PCR inhibitors on the sensitivity of the assay. Therefore, as an important quality control step, inclusion of necessary reagents (primers and probe) for the simultaneous detection of housekeeping gene and the target DNA could be considered in further development of the assay (duplex real-time PCR). Wang et al (2007) has evaluated the performance of duplex real-time PCR targeting the *gB* gene of BoHV-1 and bovine growth hormone (bGH) as internal control and have shown equivalent performance with the individual PCR assays. Alternatively, in place of housekeeping gene, an internal control template (a cryptic DNA) can be spiked to the extended semen spotted FTA® card during the DTT solution addition step of DNA extraction and the same can be amplified during the real-time PCR.

One of the major limitations of nucleic acid based assay is occurrence of false positive results due to contamination. To prevent carry over contamination, different procedures for decontamination of the punching device were evaluated. Dipping the punch for one minute in IPA, wiping it dry by tissue paper followed by punching five times on blank sterile filter paper was found effective. This procedure was able to prevent carry-over contamination even at handling $10^{4.8}$ $TCID_{50}$/ml BoHV-1 virus spiked in extended bovine semen. This concentration of the virus is not normally expected in clinical samples. Therefore, it can be presumed that serial punching of blank filter paper between samples is adequate for the desired purpose. Further, the filter paper disc from the fifth punch was subjected for DNA extraction, as carry-over contamination control, for every seven sample processed during clinical sample screening. The elution from this disc showed no amplification in the real-time PCR assays.

The conservative analytical sensitivity of the real-time PCR assay in the extended semen matrix was $10^{0.8}$ $TCID_{50}$/ml for BoHV-1 virus and 100 copies of positive plasmid respectively. Further, the assay could detect, as low as $10^{0.008}$ $TCID_{50}$/ml BoHV-1 virus or 1 copy of positive plasmid when the number of replicates (n = 6) of the same sample was tested. Wang et al. (2007) have evaluated the sensitivity of the real-time PCR for detection of BoHV-1 by testing serial 10-fold dilution of the virus culture and determined the LOD as 0.076 $TCID_{50}$ per reaction. Rana et al. (2011) observation indicated that test could detect up to 10 copies of the target genome and they could amplify as low as $10^{0.68}$ $TCID_{50}$/50 μL of the virus. In this study, the analytical sensitivity of the assay was same for DNA extracted by both Chelex and FTA method.

Determination of analytical specificity was not necessary as this assay only describes an alternate method of DNA extraction. It uses the real-time PCR developed by Wang et al. (2007) and the assay could successfully amplify BoHV- 1.1, 1.2 and BoHV-5. They evaluated the analytical specificity and observed it to be adequate for the intended purpose. In this study, semen from 16 IBR sero-negative animals were tested by FTA method of DNA extraction and all turned negative suggesting the method to be specific.

The manufacturer's claim that DNA spotted onto FTA® card is stable for at least 5 years. However, it varies based on type of nucleic acid with DNA being comparatively more stable than RNA. In the present study, the DNA spotted onto FTA® card was found to be stable for at least 28 days when the cards were stored at +4 °C to 37 °C and slight change in humidity did not have any adverse effect on the quality of DNA. Liang et al. (2014) studied the stabilization of nucleic acid (RNA from BVDV, BRSV, Corona virus and DNA from BoHV-1) spotted onto FTA® card for 14 days over a temperature range of −27 °C to +46 °C and observed no significant difference in the Ct values were noticed in real-time PCR.

Process (quality) control was an integral part both during optimization and screening process. In every batch, one positive semen control ($10^{1.8}$ $TCID_{50}$/ml BoHV-1 spiked semen) and one negative semen control (Semen from IBR sero negative animal) spotted onto FTA® card was included for DNA extraction. Similarly, for quality control and to check the cross contamination during real-time PCR, BoHV-1 positive plasmid and NTC were included in each plate of real-time PCR. If any deviation in the result of the controls was observed, the test was repeated from sample spotting onto FTA® card. For direct comparison of the Ct value obtained from respective FTA® and Chelex method of DNA extraction, real-time PCR was carried out in single plate.

The diagnostic sensitivity and specificity of the FTA method was 83.08% (95% CI: 71.73%–91.24%) and 93.23% (95% CI: 89.38%–96.01%) respectively, when Chelex method was chosen as the gold standard test. The analytical sensitivity of the FTA method matched (100%) with the Chelex method in both virus spiked semen as well as in plasmid spiked semen samples. However, the number of samples found positive varied for the two methods. While the FTA method detected 71 positive samples, the Chelex method detected 65 samples as positive. Both Chelex and FTA method detected 54 samples. The difference in detection between the two methods was noticed when the virus concentration in the clinical sample is low (reflected by higher Ct values of 38–40). This can also be corroborated from the observation that when the virus concentration in the extended semen is high (Ct values below 38 or 4–6 replicates showing positive amplification in real-time PCR) there is 100% agreement between the two methods. The disparity in the samples declared positive by either of the methods mostly occurred when positive amplification was recorded in only one or two of the six replicates (Ct values 38–40). Linhares et al. (2012) observed 100% sensitivity and specificity of FTA method for detection of PRRSV in serum, blood, tissue samples of experimentally infected animals. However, for field samples the sensitivity was 89% (95% CI: 77.35–95.03) for serum, 100% (95% CI: 80–100) for lung sample and 45% (95% CI: 19.97–73.01) for oral fluids respectively. Wang et al. (2014) observed higher specificity (79%) in FTA® card as compared to liquid sample carrier (71.6%) collected from patients with human papilloma virus infection.

In conclusion, a protocol for extraction of DNA from FTA® elute card spotted with extended bovine semen for screening BoHV-1 by real-time PCR was optimized. The method was found to be robust and reproducible. The DNA spotted onto the FTA® cards was found to be stable for at least for 28 days when the cards are stored between +4 °C to 37 °C. This bench validation (stage I and II) suggests, FTA® card is a promising alternative for the transhipment of EFS from semen stations to LN_2 to the laboratory for screening against BoHV-1. Further, inter-laboratory assessment (stage IV) and screening of more number of semen batches (field validation) has to be carried out before it can be recommended for routine use.

Conflict of interest

The authors declare no conflict of interest.

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