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Use of real-time polymerase chain reaction to detect bovine herpesvirus 1 in frozen cattle and buffalo semen in India

Samir Kumar Rana[1], Sri Naga Leela Surendra Kota[2], Penchalla Narasimha Rao Samayam[2], Sriraman Rajan[2] & Villuppanoor Alwar Srinivasan[2]

Summary

Bovine herpesvirus 1 (BoHV-1) infection in cattle and buffalo makes these animals life-long carriers of the virus which is intermittently excreted in semen. In the present study, a real-time polymerase chain reaction (PCR) was validated to screen frozen semen from cattle and buffalo for BoHV-1 by amplification of the *gB* gene of the virus. Analysing the intra- and inter-test variability, the assay was found to be highly reproducible. High sensitivity (100%) and specificity (90.04%) of this real-time PCR assay was recorded in comparison to virus isolation. Extended frozen semen samples from 574 cattle and buffalo bulls that were seropositive to infectious bovine rhinotracheitis (IBR) tested by real-time PCR indicated that 1.97% semen batches from cattle and 3.36% batches of buffalo semen were positive for BoHV-1. The real-time PCR protocol will be useful for screening large numbers of semen samples from IBR-seropositive cattle and buffalo bulls as the test is less time consuming and several batches of semen can be tested with ease compared to virus isolation in cell culture.

Keywords

BoHV-1, Bovine herpesvirus, India, Infectious bovine rhinotracheitis, PCR, Polymerase chain reaction, Real-time PCR, Semen, Virus.

Applicazione della RT-PCR per il rilevamento dell'*Herpesvirus* bovino 1 nel seme congelato di bovini e bufali in India

Riassunto

L'infezione da Herpesvirus *bovino 1 (BHV-1) in bovini e bufali rende i soggetti vettori a vita. Il virus viene escreto in modo intermittente nel seme. Il presente studio ha validato l'utilizzo della reazione a catena della polimerasi in tempo reale (RT-PCR) per l'esame del seme congelato di bovini e bufali al fine di eseguire lo screening del BHV-1 tramite amplificazione del gene gB del virus. L'analisi della variabilità intra e intertest ha dimostrato l'elevata riproducibilità dell'esame. La RT-PCR ha evidenziato un'elevata sensibilità (100%) e specificità (90,04%). L'RT-PCR eseguita sui campioni di seme congelato di 574 tori e bufali sieropositivi alla rinotracheite infettiva bovina (IBR) ha indicato come l'1,97% dei lotti di seme di bovino e il 3,36% dei lotti di seme di bufalo fossero positivi per BHV-1. Il protocollo della RT-PCR è risultato utile per lo screening di un ampio numero di campioni di seme di tori e bufali sieropositivi per l'IBR, l'esame è più rapido e permette di testare facilmente più lotti di seme rispetto all'isolamento del virus in coltura cellulare.*

(1) National Dairy Development Board, Indian Immunologicals Ltd, Gachibowli, Hyderabad 500 032, India
vasrini@rediffmail.com, srini@indimmune.com
(2) Research and Development Centre, Indian Immunologicals Ltd, Gachibowli, Hyderabad 500 032, India

Parole chiave

BHV-1, *Herpesvirus* bovino, India, PCR, Reazione a catena della polimerasi, Rinotracheite bovina infettiva, Seme.

Introduction

Bovine herpesvirus 1 (BoHV-1) is a double-stranded DNA (dsDNA) virus and is a member of subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. BoHV-1 infects the respiratory and genital tracts of cattle and buffalo. It causes clinical symptoms including infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) and infectious pustular balanoposthitis (IPB) (8), sometimes leading to abortions and foetal death. After acute infection, the virus can enter neurons and establish a latent infection in sensory ganglia (13). The latent virus can be reactivated either under stressful conditions or after the administration of glucocorticoids (16, 21). Bulls infected with BoHV-1 become carriers throughout life and shed virus intermittently in their semen (6). Transmission of BoHV-1 can occur through contaminated semen during artificial insemination, where a single ejaculate is diluted and may be inseminated into many cows (7). In India, approximately 23% of cattle and buffalo (2) are reported to be serologically positive for BoHV-1 infection and hence it has become essential to validate a suitable laboratory test protocol to screen extended frozen semen so that virus spread through semen can be prevented.

In accordance with the World Organisation of Animal Health (*Office International des Épizooties*: OIE) *Manual* (20), there is no restriction regarding the maintenance of BoHV-1 seropositive bulls in semen stations, but it is mandatory to screen each batch of semen from these bulls for the presence of BoHV-1 and to obtain negative results to prevent virus transmission through semen. The routine method for detection of BoHV-1 in bovine semen is virus isolation in cell culture which is one of the prescribed tests of the OIE for international trade. However, this method has limitations, such as sensitivity, time and cost. Therefore, in the present study, attempts were made to standardise and validate a real-time polymerase chain reaction (PCR) protocol to detect BoHV-1 by amplifying the highly conserved region of the *gB* gene of the virus, in extended frozen semen of cattle and buffalo (18). This is also a prescribed OIE test (20). After validation of the test, a large number of extended frozen semen batches from cattle and buffalo were screened by real-time PCR so as to estimate the presence of BoHV-1 in the semen of IBR-seropositive bulls.

Material and methods

Frozen semen samples

Initially 152 and 32 batches of extended frozen semen from IBR-seropositive cattle and buffalo, respectively, were tested for the presence of BoHV-1 during the standardisation of real-time PCR. Subsequently, 7 967 additional batches of semen were tested using this assay.

Extraction of DNA

Viral DNA was extracted from extended frozen semen following the OIE protocol (20). Briefly, 10 µl of extended semen was mixed with 100 µl of 10% Chelex®-100 (w/v), 11.5 µl of 10 mg/ml proteinase K, 7.5 µl of 1 M dithiothreitol (DTT), and 90 µl of nuclease-free water. The mixture was incubated at 56°C for 30 min, vortexed at high speed for 10 sec, after which the tubes were placed in boiling water for 8 min. Subsequently, vortexing was repeated and the samples were centrifuged at 10 000 × g for 3 min. The supernatant was used for PCR directly, or stored at –20°C.

Development of positive plasmid control

The conserved glycoprotein B (*gB*) gene of BoHV-1 was amplified by sequence specific primers (1) *gB-F*: 5′-TGT-GGA-CCT-AAA-CCT-CAC-GGT-3′ (position 57499–57519 GenBank®, accession AJ004801), *gB-R*: 5′-GTA-GTC-GAG-CAG-ACC-CGT-GTC-3′ (position 57595–57575 GenBank®, accession AJ004801) and cloned into pDrive cloning vector using a PCR cloning kit.

Tenfold serial dilutions of the pDrive harbouring 97 bp fragments of *gB* gene of BoHV-1 (pDrive-BoHV-1) containing variable copy numbers from 1×10^{10}/5 µl molecules up to one molecule in 5 µl were prepared. Real-time PCR reaction (20) was performed, threshold cycle (C_t) values for different dilutions were determined and linearity of the reaction of different dilutions was tested. The C_t values and the corresponding copy numbers were used as positive standards for plotting standard curves in quantitative real-time analysis.

Real-time polymerase chain reaction assay

The real-time PCR described in our study used a pair of sequence-specific primers for amplification of target DNA and a 5′-nuclease oligoprobe (TaqMan) (1) for the detection of amplified products as indicated in the OIE *Manual* (20).

The real-time PCR reaction was conducted in accordance with the OIE *Manual* (20) with minor modifications, in a Bio-Rad iCycler iQ and the C_t of amplification was determined by data analysis software provided with the instrument.

Real-time polymerase chain reaction assay of virus culture supernatant

BoHV-1 was propagated in Madin-Darby bovine kidney (MDBK) monolayer cell lines. Serial tenfold dilutions of the virus culture supernatant were prepared from $10^{5.68}TCID_{50}$/50 µl to $10^{0.068}TCID_{50}$/50 µl. Viral DNA was extracted from each dilution using a QiaBlood mini kit, in accordance with the manufacturer's instructions. The real-time PCR reaction was performed in duplicate, using eluted DNA and the C_t value was calculated.

Intra-assay precision and inter-assay variation

To confirm accuracy and reproducibility of this real-time PCR, intra-assay precision was determined by testing all 184 semen batches, each in duplicate, within one PCR run. Inter-assay variation was investigated by testing 50 randomly selected samples (10 positive and 40 negative samples) out of 184 in two different runs performed using two different premixes of Invitrogen Platinum quantitative PCR supermix. The mean C_t values and standard deviation (SD) for each sample was determined and the coefficient of variation (CV) calculated.

Virus isolation in cell culture and virus confirmation

All 184 batches of extended semen from cattle and buffalo were also tested by virus isolation in cell culture as described in the OIE *Manual* (20). Virus isolation was considered negative if no cytopathogenic effect (CPE) was recorded after three consecutive passages. However, the presence of CPE in any passages in cell culture was considered to indicate the presence of the cytopathogenic agent and, subsequently, these agents were further confirmed as BoHV-1 using the virus neutralisation test (VNT) and PCR assay (15, 17).

Spiked semen doses

To confirm negative semen batches as truly negative, 10 semen batches declared negative by real-time PCR in two consecutive runs and in cell culture were spiked with serially tenfold diluted pre-titrated BoHV-1, viz. ranging from $10^{5.68}TCID_{50}$/50 µl to $10^{0.68}TCID_{50}$/50 µl. From each dilution of pre-titrated BoHV-1 cell culture supernatant, a quantity of 10 µl was mixed thoroughly with 90 µl of the negative frozen semen and 10 µl of this mixture was finally used for extraction of viral DNA for the real-time PCR reaction.

Processing of field semen batches for screening against bovine herpesvirus 1

After standardisation of this real-time PCR assay, an additional 7 967 batches of frozen semen from 439 IBR-seropositive cattle and 135 IBR-seropositive buffalo prepared between 2005 and 2009, were processed for the detection of BoHV-1. As a routine practice, collection of semen from each bull was performed twice daily and twice a week. The cattle bulls were pure Holstein Frisian and Jersey and the pure indigenous breeds, were: Gir, Kankrej, Khillar, Red Sindhi and Sahiwal. Crossbreeds of the above indigenous and

exotic breeds were also included. The buffalo bulls were mainly of the Murrah breed. Presence of BoHV-1 in extended semen batches produced by 574 bulls was analysed in respect to species, season, number of batches produced etc.

Results

Plasmid construct and quantitative polymerase chain reaction

To use a plasmid construct of target DNA in the real-time PCR reaction as a positive control and to make this real-time PCR protocol semi-quantitative, the target 97 bp of BoHV-1 *gB* gene was cloned into a pDrive cloning vector. Positive cloning was confirmed by restriction enzymatic digestion of the plasmid with ECoR1 and also by nucleotide sequencing. Blast analysis of the cloned DNA portion in plasmid matched the published nucleotide sequence of BoHV-1.

The isolated plasmid construct was quantified by UV spectrophotometry and the copy number was determined using the formulae: 1 µg of 100 bp of DNA = 9.1×10^{11} molecules. The quantified plasmid DNA was serially diluted tenfold to obtain a dilution containing plasmid copies ranging from 1×10^{10} per 5 µl to one copy per 5 µl and assayed in a real-time reaction. In real-time PCR, the corresponding C_t values for each dilution ranged from 11.9 for 1×10^{10} copies to 39.4 for 1×10^{1} copies per reaction. However, no amplification was observed in the higher dilution. The increasing pattern of C_t values corresponding to the increased dilution is shown in Figure 1 and Table I. A significant coefficient of correlation ($r = 0.99$) and slope of –3.387 were observed when a standard curve was plotted using log of the starting quantity (copy number) of the dilutions against the C_t (Fig. 2). This standard curve can be used to ascertain the number of copies of target DNA present in the unknown samples in real-time PCR.

Table I
Threshold cycle value obtained in real-time polymerase chain reaction against serial tenfold dilutions of pDrive-bovine herpesvirus 1 plasmid construct

Plasmid construct	Copy number per reaction	C_t value obtained
pDRIVE-BoHV-1	1×10^{10}	11.9
pDRIVE-BoHV-1	1×10^{9}	15.2
pDRIVE-BoHV-1	1×10^{8}	18.9
pDRIVE-BoHV-1	1×10^{7}	21.8
pDRIVE-BoHV-1	1×10^{6}	25.1
pDRIVE-BoHV-1	1×10^{5}	28.2
pDRIVE-BoHV-1	1×10^{4}	31.2
pDRIVE-BoHV-1	1×10^{3}	34.5
pDRIVE-BoHV-1	1×10^{2}	37.2
pDRIVE-BoHV-1	1×10^{1}	39.4

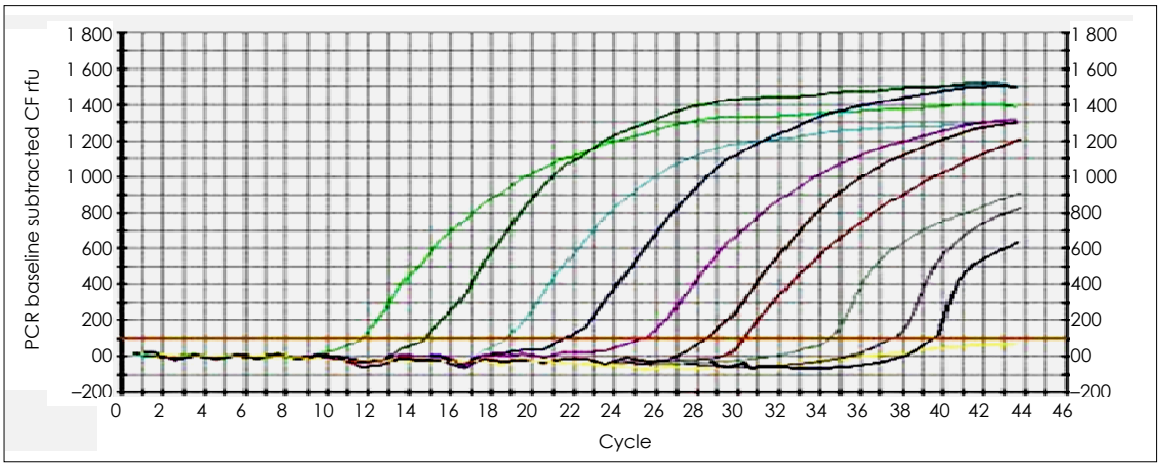

Figure 1
Baseline subtractive curve fit view of serial tenfold diluted positive pDRIVE-bovine herpesvirus 1 constructs with relative fluorescence units (rfu) plotted against cycle number
From left to right, curves represent reactions containing copy number of pDrive-bovine herpesvirus 1 from 1×10^{10} per reaction to one copy per reaction

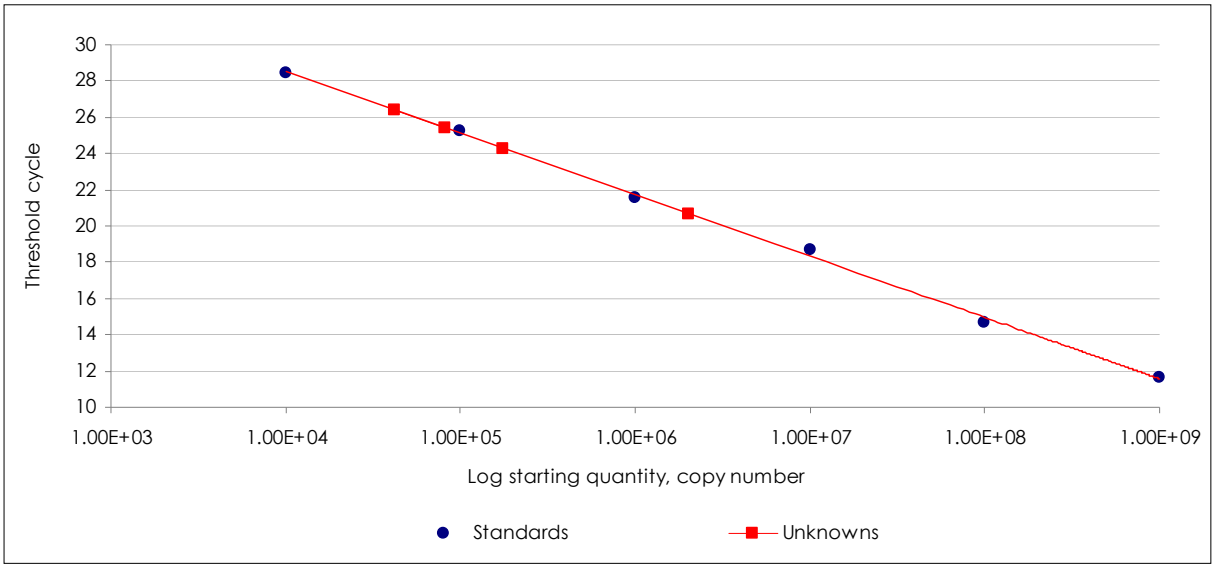

Figure 2
Standard curve analysis of the DNA amplification plot with threshold cycle (C_t) values plotted proportionately against the logarithm of the initial input copy number of pDRIVE-bovine herpesvirus 1
Correlation coefficient: 0.999; Slope: –3.387; Intercept: 42.055; Y: –3.387; X: +42.055; PCR efficiency: 97.4%

No amplification was recorded in PCR reaction with the known negative control, showing the C_t value above 40. Hence, the samples showing a C_t value of <40 was considered positive and >40 was considered negative.

Real-time polymerase chain reaction

Viral DNA extracted from cell culture supernatant of varying virus titres was subjected to real-time PCR and amplification was recorded up to the highest dilution of the culture supernatant with a virus titre $10^{0.68}TCID_{50}$ of BoHV-1/50 µl. The C_t value ranged from 12 to 39.5 from the lower to higher dilution, respectively.

Out of 184 batches of frozen semen tested initially by real-time PCR, 32 (17.39%) were positive and the remaining 152 batches (82.61%) were negative. A total of 29 positive samples (15.76%) were from cattle and the remaining 3 (1.63%) were from buffalo. Amplification efficiency of real-time PCR reactions of these semen batches varied between 95% and 99%.

Analytical sensitivity and variability of real-time polymerase chain reaction

To determine the intra-run precision, each of the above samples was tested in duplicate reactions and two C_t values of each sample were compared. The average SD and CV for all C_t values was recorded as 0.10 and 0.27, respectively. Intra-run assay precision of real-time PCR in terms of C_t values obtained from a total of 32 positive semen batches are indicated in Figure 3.

The inter-run variability of the assay was determined to ascertain reproducibility. A total of 10 positive and 40 negative randomly selected batches of frozen semen out of 184 were again amplified in duplicate reactions in two separate runs, using different stock of supermix from the same manufacturer and the results were compared in terms of C_t values. The positive and negative results of all these samples (#50) remained unchanged in two different runs and, also matched the previous results. The mean C_t values for the positive samples in two different runs were 36.16 and 36.57. The SD between mean C_t values of each sample performed in two different runs ranged from ±0.21 to ±1.79, with an average of ±0.29. The CV of all these samples ranged from 0.54 to 5.49. The overall CV of 0.80 was calculated from the average mean C_t value (36.36) and SD (0.29). Hence, the test was found to be highly reproducible (Fig. 4).

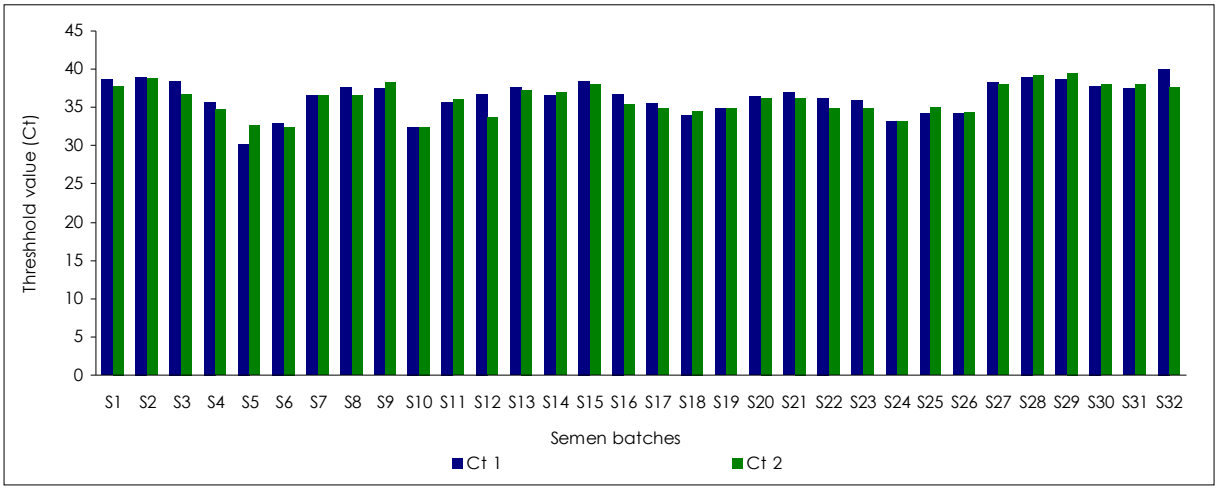

Figure 3
Intra-run assay precision of real-time polymerase chain reaction in terms of threshold cycle (C_t) values obtained from bovine herpesvirus 1-positive semen batches

Virus isolation in cell culture and virus confirmation

All 184 semen batches screened by the real-time PCR were also processed for virus isolation in MDBK monolayer cells and 16 samples gave positive results when tested for the presence of cytopathogenic agents. These agents were confirmed as BoHV-1 by the results obtained using the VNT (with known positive sera against BoHV-1), as well as by PCR amplification using *gB* and *US1.67* gene-specific BoHV-1 primers.

Spiking of negative semen

Ten batches of semen which were declared negative by cell culture as well as by real-time PCR were spiked with pre-titrated virus and again processed in real-time PCR. All 10 semen batches were subsequently declared positive. The positive amplification recorded in the negative semen after spiking with known virus in turn provides confirmation that they were true negatives before spiking and also revealed the absence of PCR inhibition and inhibitors in the earlier reactions.

Sensitivity and specificity

The sensitivity and specificity of the test was calculated taking virus isolation as the gold standard. A total of 184 frozen semen batches collected from IBR-seropositive bulls were tested, of which 16 positive virus isolates were obtained in cell culture, whereas 32 samples were declared positive by real-time PCR. All 16 samples which were positive by virus isolation were also found to be positive by real-time PCR. In comparison to virus isolation, the sensitivity and specificity of real-time PCR were found to be 100% and 90.04%, respectively.

Processing of field semen batches for screening against bovine herpesvirus 1

Overall, 2.22% (177 batches) of semen batches examined were found positive for BoHV-1 out of the total of 7 967 batches tested. Although semen batches from 574 IBR-seropositive bulls were tested, only semen from 86 cattle (19.5%) and 31 buffalo (22.96%) were positive for BoHV-1 by real-time PCR. Semen batches from buffalo showed higher levels of positivity than those of cattle (Table II). In cattle, 2.44% from first ejaculation and 2.93% from the second ejaculation of semen batches were found positive for BoHV-1. However, in buffalo, 7.03% and 3.86% of semen batches were recorded as positive in the first and second ejaculates, respectively.

Discussion

BoHV-1 is one of the important infectious agents which is transmitted through the semen of cattle and buffalo through natural service as well as through artificial insemination (10, 14).

Figure 4
Inter-run assay variability in terms of threshold cycle (C_t) values obtained from representative batches of bovine herpesvirus 1-positive semen

Over a period of time, several methods, namely: virus isolation in cell culture, immune electron microscopy, dot-blot hybridisation and PCR targeting various genes of the virus have been attempted for the detection of BoHV-1 in bovine semen (4, 12). Although virus isolation in cell culture remains the gold standard for detection of the virus, this method has limitations with regard to sensitivity, cytotoxicity of semen, time and cost. Various PCR methods have been reported to be useful for detection of the virus (15, 17). However, PCR inhibitory substances in bovine semen interfere with the amplification of the target DNA. PCR, in combination with southern blot hybridisation, was reported to be more sensitive than virus isolation and dot-blot hybridisation (20) but screening a large number of extended semen by these methods is cumbersome. Therefore, attempts have been made to standardise and validate a real-time PCR protocol (18, 19, 20) to detect BoHV-1 in extended frozen semen from naturally infected cattle and buffalo, and to utilise a method that is sensitive, specific and reproducible. Such an assay will facilitate handling large numbers of semen batches at a time.

In the present study, a pair of *gB* gene-specific published primers designed to detect viral DNA of all BoHV-1 strains, including subtypes 1.1 and 1.2, were used for amplification of the highly conserved 97 bp fragment of *gB* gene augmented with the use of a 5′-nuclease-oligoprobe (TaqMan) for the detection of amplified products. This real-time PCR assay was also reported as having been applied in a ring trial with satisfactory reproducibility (19) and subsequently has been accredited by the OIE (20).

In the present study, PCR amplification of tenfold serial dilutions of the plasmid (pDrive-BoHV-1) was obtained with >97% efficiency through linear amplification and the sensitivity

Table II
Species-wise results of semen batches tested by real-time polymerase chain reaction for bovine herpesvirus 1

Species	No. of bulls	No. of semen batches processed	No. of bulls positive for BoHV-1 in semen	Percentage of semen batches positive for BoHV-1
Cattle	439	6 538	86 (19.5%)	129 (1.97%)
Buffalo	135	1 429	31 (22.96%)	48 (3.36%)
Total	574	7 967	117 (20.38%)	177 (2.22%)

BoHV-1 bovine herpesvirus 1

of the assay was performed with the detection limit of 10 copies of target genome, which concurred with other published reports (11). The standard curve generated from the positive BoHV-1 control can be used to determine the copy numbers of the target DNA in the unknown samples. To the best of our knowledge, the construction of pDrive-BoHV-1 harbouring target 97 bp of *gB* gene and its use in real-time PCR as a positive control appears to be novel for this assay.

To confirm the specificity of the assay and to determine the limit of detection of BoHV-1, cell culture supernatant with a known virus titre was subjected to real-time PCR. Amplification was recorded as low as $10^{0.68}$TCID$_{50}$/50 µl of the virus and a linear correlation was observed between C_t values and log dilutions of the virus, which confirms the specificity of the assay with high sensitivity (22).

A total of 32 (17.39%) out of 184 batches of semen gave positive results using this real-time PCR; 16 of these PCR-positive batches were also positive by cell culture isolation, indicating the higher sensitivity of this PCR protocol in comparison to virus isolation. Fewer samples turning positive in virus isolation in cell culture may be due to lower sensitivity of virus isolation or due to the cytotoxicity of semen to the cells (5, 22). The semen batches that were positive by PCR and declared as negative for virus isolation could also be due to the presence of lower quantities of virus or due to the presence of inactivated virus. BoHV-1 excretes much high titres during the primary phase of infection than in the later phases of infection when shedding is also often intermittent (3, 9). Therefore, the application of a sensitive test is of critical importance to detect low titres of virus during the phases of intermittent virus excretion. Less SD with an acceptable CV in intra- and inter-run assays indicated the accuracy and reproducibility of the test. Unlike virus isolation, BoHV-1 detection in real-time PCR not only depends on the amount of infectious virus particles (i.e. TCID$_{50}$) in the sample but also detects inactivated virus (22). The amount of virus shedding in the semen may vary from sample to sample. From the results obtained, the mean C_t value of samples which were positive by both real-time PCR as well as virus isolation and only by real time PCR were comparable. To test the absence of PCR inhibitory subtances in semen, BoHV-1-negative batches of frozen semen were spiked with live virus and tested with real-time PCR. These samples turned positive in the PCR assay, thereby indicating the absence of PCR inhibitory factors (5, 18).

The sensitivity and specificity of the real-time PCR in comparison to virus isolation in cell were found to be 100% and 90.04%, respectively. The results from this study indicate that the real-time PCR assay offers greater sensitivity and will be valuable in detecting low titres of virus in extended semen samples. As far as the specificity is concerned, the PCR assay appeared to be more specific in comparison to virus isolation (5).

With this understanding, the use of this assay was exploited for screening of larger numbers of extended frozen semen batches against BoHV-1. In the present study, frozen semen produced by 439 cattle and 135 buffalo were tested by real-time PCR and only 86 (19.5%) cattle and 31 (22.96%) buffalo were found positive. Semen from the remaining 457 sero-positive bulls was never declared positive during the course of study; even after testing more than 20 batches of semen from each bull. By analysis of batch-wise positivity, semen samples produced by buffalo were found to exceed those of cattle.

As described earlier, semen batches were collected from bulls twice a day (first and second ejaculation) and twice a week. In buffalo, a higher percentage of semen batches later revealed positive results between the first ejaculation (3.63%) and the second ejaculation (2.40%) collected on the same day. However, in cattle, not much difference in positivity was recorded between the two ejaculations.

As described earlier, semen batches were collected from bulls twice a day (first and second ejaculation) and twice a week. In buffalo, a higher percentage of semen batches that had been collected in first ejaculation (3.63%) turned positive in comparison to the

second ejaculation (2.40%). However, in cattle, not much difference in positivity was recorded between the first and second ejaculations.

The present study indicated that the semen of a majority of bulls turned positive from either the first or second ejaculation, whereas a low percentage (3.26%) of semen batches from a bull was positive in both ejaculations collected on the same day. The presence of BoHV-1 in both semen batches collected on the same day was found to be higher in buffalo (7.69%) than in cattle (1.52%).

Ranges in C_t values for positive semen were between 31.5 and 38.8, with a mean of 36.9, which indicates that the quantity of virus excreted by different bulls in their semen varies from animal to animal and from day to day. However, no statistically significant difference in mean C_t values was recorded in positive semen of cattle and buffalo.

The rate of excretion of BoHV-1 in semen in seropositive bulls during various seasons of the year (winter, summer and the rainy season) was analysed (data not presented). In buffalo, a significantly higher percentage of semen batches were positive for BoHV-1 when collected during the summer, followed by the monsoon and winter seasons. However, in cattle no significant difference in positivity percentages was recorded during the various seasons.

Conclusion

In conclusion, the real-time PCR assay validated in the present study can be used to screen large numbers of frozen semen samples. In addition, the authors concluded that a very small proportion of semen produced by seropositive bulls is positive for BoHV-1. This study appears to be the first report on the detection of BoHV-1 using real-time PCR in extended frozen semen from naturally infected buffalo.

References

1. Abril C., Engels M., Liman A., Hilbe M., Albini S., Franchini M., Suter M. & Ackermann M. 2004. Both viral and host factors contribute to neurovirulence of bovine herpesviruses 1 and 5 in interferon receptor-deficient mice. *J Virol*, **78**, 3644-3653.
2. Anon. 2002. Report on animal disease monitoring and surveillance (ADMAS) 2002. ADMAS, Bangalore, Indian Council of Agricultural Research, New Delhi, 7-10.
3. Bitsch V. 1973. Infectious bovine rhinotracheitis virus infection in bulls, with special reference to preputial infection. *Appl Microbiol*, **26**, 337-343.
4. Brunner D., Engels M., Schwyzer M. & Wyler R. 1988. A comparison of three techniques for detecting bovine herpesvirus type 1 (BHV-1) in naturally and experimentally contaminated bovine semen. *Reprod Domest Anim*, **23** (1), 1-9.
5. Deka D., Ramneek M.N.K. & Oberio M.S. 2005. Detection of bovine herpesvirus-1 infection in breeding bull semen by virus isolation and polymerase chain reaction. *Rev Sci Tech*, **24**, 1085-1094.
6. Dennet D.P., Barsa J.Q. & Johnson J.H. 1976. Infectious bovine rhinotracheitis virus: studies on the venereal carrier status range of cattle. *Res Vet Sci*, **20**, 77-83.
7. Drew T.W., Hewitt-Taylor C., Watson L. & Edwards S. 1987. Effect of storage conditions and culture technique on the isolation of infectious bovine rhinotracheitis virus from bovine semen. *Vet Rec*, **121**, 547-548.
8. Gibbs E.P.J. & Rweyemamu M.M. 1977. Bovine herpesvirus, Part I: Bovine herpesvirus 1. *Vet Bull*, **47**, 317-343.
9. Goffaux M., Harlay T. & Allietta M. 1976. Untersuchungen ueberdas IBR-IPV-virus im samen von besamungsbullen. *Dtsch Tieraerztl Wschr*, **83**, 544-547.
10. Kahrs R.F., Gibbs E.P.J. & Larsen R.E. 1980. The search for viruses in bovine semen, a review, *Theriogneol*, **14**, 151-165.
11. Lu X., Chittaganpitch M., Olsen S.J., Mackay I.M., Sloots T.P., Fry A.M. & Erdman D.D. 2006. Real-time PCR assays for detection of bocavirus in human specimens. *J Clin Microbiol*, **44** (9), 3231-3235.

12. Pacciarini M., Agresti A., Desimone F., Poli G., Torretta E., Siccardi A.G., Meneveri R. & Ginelli E. 1988. Detection of bovine herpesvirus 1 (BHV-1) semen infections by a dot-blot hybridization assay. *Br Vet J*, **144**, 55-63.
13. Pastoret P.-P., Thiry E., Brochier B., Derboven G. & Vandevogle H. 1984. The role of latency in the epizootiology of infectious bovine rhinotracheitis. *In* Latent herpesvirus infection in veterinary medicine (G. Wittmann, R. Gaskell & H.J. Rzhia, eds.). Martinus Nijhoff, The Hague, 191-209.
14. Philpott M. 1993. Dangers of disease transmission of artificial insemination and embryo transfer. *Br Vet J*, **149**, 339-343.
15. Rola J., Polak M.P. & Źmudziňński J.F. 2003. Amplification of DNA of BHV1 isolated from semen of naturally infected bulls. *Bull Vet Inst Pulawy*, **47**, 71-75.
16. Thiry E., Saliki J., Pastoret P.-P., Lambert A.F. & Ligot J. 1984. Failure to demonstrate infectious bovine rhinotracheitis virus reactivation in parturient cows. *Vet Res*, **8**, 248-249.
17. Vilcek S. 1993. Detection of bovine herpesvirus-1 (BHV-1) genome by PCR. *J Virol Methods*, **41**, 245-247.
18. Wang J., O'Keefe J., Orr D., Loth L., Banks M., Wakeley P., West D., Card R., Ibata G., Van Maanen K., Thoren P., Isaksson M. & Kerkhofs P. 2007. Validation of a real-time PCR assay for the detection of bovine herpesvirus 1 in bovine semen. *J Virol Methods*, **144**, 103-108.
19. Wang J., O'Keefe J., Orr D., Loth L., Banks M., Wakeley P., West D., Card R., Ibata G., Van Maanen K., Thoren P., Isaksson M. & Kerkhofs P. 2008. An international inter-laboratory ring trial to evaluate the real-time PCR assay for the detection of bovine herpesvirus 1 in extended bovine semen. *Vet Microbiol*, **126**, 11-19.
20. World Organisation for Animal Health (Office International des Épizooties: OIE) 2008. Manual of diagnostic tests and vaccines for terrestrial animals, 6th Ed. OIE, Paris, 752-767.
21. Wyler R., Engles M. & Schwyzer M. 1989. Infectious bovine rhinotracheitis/vulvovaginitis (BoHV-1). *In* Herpesvirus diseases of cattle, horse and pigs (G. Wittmann, ed.). Kluwer Academic, Boston, 1-72.
22. Xia J.Q., Yason C.V. & Kibenge F.S. 1995. Comparison of dot blot hybridization, polymerase chain reaction, and virus isolation for detection of bovine herpesvirus-1 (BHV-1) in artificially infected semen. *Can J Vet Res*, **59** (2), 102-109.

