Development of a Probe Based Real Time PCR Assay for Detection of Bovine Herpes Virus-1 in Semen and Other Clinical Samples

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Abstract This study describes development of a TaqMan probe based real time PCR assay that can detect BoHV-1 of as low as 0.001 TCID₅₀/0.1 ml in clinical samples, its comparative evaluation with indirect ELISA and virus isolation for detection of Bovine herpes virus-1 (BoHV-1) in semen and swab clinical samples. For this study, we collected samples from 212 animals (cattle and buffaloes) comprising 91 bulls and 121 females. Avidin–biotin ELISA employed on serum samples from 212 animals revealed 74 as seropositive for BoHV-1. On inoculation of semen/swabs on MDBK cell line, nine samples yielded cytopathic changes characteristic of herpes viruses. The isolates were confirmed by VNT and a conventional PCR. A real time PCR assay was standardised by designing a new set of TaqMan probe and primers targeting a 71 bp

region on gB gene of the virus. The assay detected viral antigen in 21 seropositive and 14 seronegative animals, emphasizing the relevance of serology in BoHV-1 diagnosis, particularly in breeding stations. Further, real time PCR assay was 100 % sensitive and 87.19 % specific compared to virus isolation in detection of the BoHV-1 in clinical samples. The assay was validated at reputed national laboratories, with a sensitivity of ≥ 99 %.

Keywords Bovine herpes virus-1 · Indirect ELISA · Virus isolation · Real time PCR

Introduction

Bovine herpes virus type-1 (BoHV-1), a member of the *Alphaherpesvirinae*, infects the respiratory and genital tracts of cattle causing diverse ailments viz., infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), infectious balanoposthitis [11], keratoconjunctivitis [18] and neurological disorders [6]. In BoHV-1 genital tract infections of bulls, the virus replicates in the mucosae of the prepuce, penis and possibly in the distant part of the urethra and semen is likely to be contaminated during ejaculation by virus that sheds from the infected mucosae [2, 7]. Insemination of cows with such contaminated semen reduces conception rate and may cause endometritis, abortion and infertility [9, 13]. During the primary infection, virus is transported along axons and becomes latent in sacral ganglion where it persists for the life of the animal [27], which gets reactivated under stress conditions making the animal carrier for lifetime and potential shedder of the virus. Today, developing countries records the highest outbreaks of BoHV-1 infections in the world. For example, more than 40 % of Indian cattle population are seropositive for

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BoHV-1 antibodies and has been a major hurdle in export of animal products especially the high quality germplasm (mainly the semen) in the international market under the WTO rules. In spite of all these, developing countries like India, do not have a definitive diagnostic test for this major infection. Keeping in view the emerging nature of the disease and its economic impact, present work was designed for comparative evaluation of existing OIE approved diagnostic tests and their clinical relevance in regions which record high seroprevalence inspite of absence of vaccinations against BoHV-1 and development of a probe based real time PCR for BoHV-1 detection.

Materials and Methods

Sample Collection

Fresh semen samples were collected from 91 bulls, stationed at three different semen collection centres located across Karnataka state, India. Semen was collected in viral transport media (VTM) made from Dulbecco's modified eagles media (DMEM) with 2 % foetal calf serum (FCS) and antibiotics.

Vaginal swabs were collected from eighty-six cows in two organised farms in Karnataka state and swab samples (Nasal, conjunctival and vaginal) from thirty-five animals showing BoHV-1 like symptoms, were collected in VTM from Tumukur, Hasan and Mandya districts of Karnataka state. Thus, the present study was conducted on a total of 212 samples comprising 91 semen and 121 swab samples.

Blood samples were collected from all 212 animals under the study for evaluation of serum antibodies against BoHV-1.

Enzyme Linked Immuno Sorbent Assay (ELISA)

Enzyme linked immuno sorbent assay (ELISA) was employed on serum samples collected from all the animals during the study. The Avidin- Biotin ELISA kits supplied by Project Directorate on Animal Disease Monitoring and Surveillance, Bangalore, India, were used for this purpose. 1:30,000 dilution of Biotin anti IgG conjugate and 1:15,000 dilution of Avidin-HRPO conjugate were used in ELISA, as described in the kit procedure.

Virus Isolation

Cell Line

Madin-Darby Bovine Kidney (MDBK) cells obtained from National Centre for Cell Sciences, Pune, India, grown in

DMEM growth media supplemented with 5 % FCS were used in the entire screening work.

Processing and Infection of Cells

Two hundred μ l of semen sample in VTM was diluted with 2 ml of certified FCS (Gibco, Cat log No. 160000-044, Lot No. 1127731, USA), mixed vigorously and incubated for 30 min at room temperature. One ml of semen-serum mixture was inoculated on to confluent monolayer of MDBK cells in tubes. The swabs were well agitated in VTM, filtered in 0.22 μ syringe filters and infected on to MDBK cells by adsorption method. Each sample was given three blind passages. In each passage, the cells were observed for 7 days. Tubes that did not show cytopathic effect (CPE) by third passage were discarded and declared as negative for BoHV-1. Samples that gave CPE by third passage were further passaged for virus propagation [4].

Virus Neutralisation Index (NI)

The virus isolates obtained from the clinical samples during this study were characterised by Neutralisation tests using BoHV-1 specific antiserum available at PD-AD-MAS, Bangalore, India. The virus titration, neutralisation and determination of NI of virus isolates were carried out as per standard protocols [4, 22].

During the entire study, 'PD-ADMAS BoHV-1 virus isolate', obtained from Project Directorate on Animal Disease Monitoring and Surveillance, Bangalore, India, was used as the positive control.

Polymerase Chain Reaction

Isolation of DNA

The method for extraction of DNA from semen was essentially according OIE protocols [4]. In brief, 10 μ l of semen sample was added to 100 μ l of Chelex 100 (Sigma) (10 %, w/v, in sterile, distilled water), 11.5 μ l of 10 mg/ml proteinase K (Sigma), and 7.5 μ l of 1 M DTT (Sigma) and 90 μ l sterile distilled water. The sample mixture was mixed gently and incubated at 56 °C for 30 min. Following brief vigorous vortexing (10 s), the sample tube was placed in a boiling water bath for 8 min. The vortexing was repeated and the samples were centrifuged at 10,000 $\times$ g for 3 min. The supernatant containing DNA was used directly or stored at -20 °C for future use.

The DNA from cell culture supernatants and swabs samples collected from animals was isolated as per the procedures of Lopasev et al. [15] using Quiagen DNA isolation kits.

PCR Assay Protocols

The DNA isolated from clinical samples and cell culture supernatants were subjected to routine PCR in a 20 µl reaction mixture containing a final concentration of 10 mM Tris (pH 9.0), 50 mM KCl, 1.9 mM MgCl₂, 0.2 mM deoxy nucleoside triphosphate, 0.1 µM each of primer P1 Forward 5'-TGGTGGCCTTYGACCGCGAC-3' and Reverse 5'-GC TCCGGCGAGTAGCTGGTGTG-3', 1 U of Taq Polymerase per reaction. All the reagents and primers were obtained from Bangalore Genie Ltd., Bangalore, India. The primer sequence are based on the sequence of BoHV-1 glycoprotein (gB) gene [24]. The PCR mixture was amplified by 35 repeated cycles: 94 °C for 1 min, 50 °C for 30 s and 72 °C for 45 s and final extension at 72 °C for 10 min. Negative control reactions (reaction without template and reaction with BoHV-1 negative semen) were added to each set of PCR. The PCR products were analysed by agarose gel electrophoresis.

TaqMan Real Time PCR

Primers and Probe Sequences

In the present study, we designed a new set of primers and TaqMan 5'-nuclease probe targeting a conserved region on the glycoprotein B gene (gB) of the virus using applied biosystems (ABi) primer design software. The primers and probe were checked for their specificity using the database similarity search program nucleotide–nucleotide BLAST (www.ncbi.nlm.nih.gov/blast) and the sequences were 100 % homologous to those of BoHV-1 strains deposited in the GenBank. The designed TaqMan probe was a sequence-specific oligonucleotide labelled with two different fluorophores, the reporter/donor, 5-carboxyfluorescein (FAM) at the 5' end, and the acceptor/quencher 6-carboxytetramethyl rhodamine (TAMRA) at the 3' end. This real time PCR assay was designed to detect viral DNA of all BoHV-1 strains, including subtype 1 and 2, from bovine semen as well as from clinical samples. The sequences of the designed the primers and probes are as follows:

in the middle gB-F: 5'-TGCTCGACTACAGCGAGAT
ACAG-3',
Primer gB-R: 5'-CACGCGGTCAATGTCGTAG
A3' and
TaqMan probe: 5'-FAM-CCGCAACCAGCTGCA
CGAGC-TAMRA-3'

Standardisation of TaqMan Real Time PCR Components

The real time PCR was carried out using the Platinum Quantitative PCR Super Mix-UDG master mix (Fermentas).

A number of experiments were performed to optimize the PCR protocol, including concentration of reagents and PCR cycling parameters. The optimized PCR assay was established using a total volume of 25 µl. The PCR reaction mixtures were prepared in a clean laboratory room. All the reagents except the test samples were mixed before distribution to each individual reaction tube. Non templates control (NTC, reagents only) and negative controls were included in each test.

Five µl of the DNA isolated from cell culture supernatants/clinical swab/semen sample was added to the PCR reagent mixture to make final volume of 25 µl.

Parameters of TaqMan Real Time Polymerase Chain Reaction

The PCR plates were placed in the real time PCR detection system (7900, Applied bio systems) with following optimised programme parameters: one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 sec, and hold at 60 °C for 45 sec.

The results were analysed using SDS 2.1 version of software provided with 7900 ABI Real time PCR system. Though real time PCR does not require running of gel for PCR products, as a means of authenticity, the real time PCR products were analysed by agarose gel electrophoresis. The standardisation of real time PCR was carried out at Institute of Stem Cells, National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India.

BoHV-1.1 and BoHV-1.2 isolates available at Dept of Veterinary Microbiology, Veterinary College, Bangalore, Karnataka were used for assessing the sensitivity/specificity/cross reactivity of the developed assay.

Results

Sample Collection

During sample collection from filed outbreaks a few of the animals showed typical gross lesions of vulvovaginitis, balanoposthitis, conjunctivitis and respiratory system related lesions. The sample collection in organised farms was made exclusively in the early mornings which recorded sudden change of weather conditions (like sudden rains, adverse cold weather conditions).

ELISA

Avidin–biotin ELISA was employed on serum samples collected from all the 212 animals during this study.

Table 1 gives results obtained during the study. Overall 34.90 % of the animals tested during the study were seropositive for BoHV-1 antibodies.

Virus Isolation and PCR

During virus isolation, inspite of using serum to neutralise seminal enzymes as per the protocol of OIE [4], a few of the semen samples produced extensive degeneration and detachment of cells within 24 h in the first passage and a few of the samples had similar effects even during second passage. Of the 91 semen samples tested, only one sample developed viral specific CPE in MDBK cell line. Out of 121 swab materials collected from organised farms and filed outbreaks, eight samples showed characteristic CPE. The CPE comprised rounding of cells and thread like cellular elongations in 48–72 h post infection with characteristic “bunch of grapes” like aggregation developed by 72 h, finally leading to complete destruction of cell sheet by 96–120 h (Fig. 1).

The titre of the virus isolates varied between $\log 10^{2.24}$ to $10^{7.24}$ TCID₅₀/0.1 ml. All the nine isolates were completely neutralised by BoHV-1 specific serum obtained from PD_ADMAS, Bangalore, India and further all the isolates had more than 1.5 NI (Fig. 2).

The DNA isolated from cell cultures showing CPE when subjected to conventional PCR using primers amplifying gB region of BoHV-1, yielded a specific PCR product of 443 bp as depicted in Fig. 3.

TaqMan Real Time PCR

Optimization of Cut Off Threshold Cycle (Ct)

Standardization of the cut off threshold cycle/end cycle of real time PCR assay was very critical in the present study. The different concentrations/dilutions of known positives and known negatives, and non template controls were

tested using the optimized qRT-PCR reaction mixture and thermal conditions. The set of primers and probe used detected all positives below 40 cycles and no positives were detected after the 40th cycle. Further, there was no nonspecific amplifications of negative samples and NTC even when the real time PCR assay was extended up to 45th cycle, hence the cut off cycle for interpretation of results of positive or negative samples was kept at a Ct of 40 (Fig. 4).

Sensitivity of TaqMan Real Time PCR for BoHV-1 Detection

The analytical sensitivity of the real time PCR assay was evaluated by testing several different types of templates, including virus cultures, spiked negative semen, positive semen diluted with cell culture medium and negative semen. The sensitivity was first determined by testing sequential 10-fold dilutions of virus culture of one BoHV-1 isolate. The detection limit was determined as 0.001 TCID₅₀ per reaction (Fig. 5). The sensitivity of the qRT-PCR assay was also evaluated using serial dilution of positive semen samples with cell culture medium (DMEM). The PCR assay was able to detect a range of positive semen dilutions of up to 1:80 (Fig. 6). Virus isolation was also performed on different dilutions of the semen samples, the virus was isolated only in the dilution of 1:10 and rest of the dilutions did not yield the virus in cell culture system.

When DNA isolated from samples collected from 212 animals under this study were subjected for real time PCR, the assay detected BoHV-1 in all the samples that yielded the virus in cell cultures apart from detecting the virus in 26 animals that were negative for BoHV-1 by virus isolation (Table 1). The sensitivity of qRT-PCR was calibrated in comparison with virus isolation and the real time

Table 1 The comparison of virus isolation and real time PCR in detection of BoHV-1 in seropositive and seronegative animals

Farm	Total number of samples collected	AB-ELISA positives			AB-ELISA negatives		
		Number of Ab positives	Number of virus isolates	Real time PCR positives	Number of Ab negatives	Number of virus isolates	Real time PCR positives
Farm A	49	19	Nil	1	30	Nil	2
Farm B	50	28	2	8	22	2	3
Farm C	38	8	Nil	3	30	Nil	Nil
Farm D	40	7	Nil	Nil	33	Nil	Nil
Field samples	35	12	2	10	23	3	8
Total	212	74	4	22	138	5	13

Fig. 1 Sequential cytopathic changes produced by one of the BoHV-1 isolate

Fig. 2 Virus titration and neutralization (96 well plate stained with crystal violet) of one of the BoHV-1 isolate obtained during the study

Fig. 3 PCR based detection of all nine BoHV-1 isolates obtained during the study by amplification of 443 bp partial gB gene of BoHV-1 *M* marker, *1* to *9* nine BoHV-1 isolates, *P* positive control (PD_ADMAS isolate), *N* known negative

PCR was 100 % sensitive in relation to virus isolation in detection of BoHV-1 on the samples tested.

Specificity of TaqMan Real Time PCR for BoHV-1 Detection

The real time PCR assay detected both the BoHV-1 subtypes 1.1 and 1.2 isolates isolated during this study (Fig. 7). The real time PCR assay was employed on known positive semen samples by spiking negative semen samples with known BoHV-1. The qRT-PCR assay detected all positive semen samples consistently and there was no detection of any negative semen samples tested, without any non specific amplifications even when the assay was run up to 45th cycle (Fig. 8). On statistical analysis, the developed assay was 87.19 % specific in BoHV-1 detection in relation to virus isolation.

Fig. 4 Standardisation of cut off threshold cycle (Ct) for BoHV-1 detection, no amplification curves after 40th cycle of real time PCR run, no non specific amplification when real time PCR was extended up to 45 cycles

Repeatability of TaqMan Real Time PCR for BoHV-1 Detection

The repeatability of real time PCR assay was evaluated from multiple runs with replicated samples in each run. One negative sample and one known positive sample were used. Each sample was tested in triplicate in 10 consecutive PCR assays, to determine the inter- and intra-assay variability. All replicates of negative semen had no Ct. reading in all 10 consecutive test runs. The inter-run coefficient of variation (CV), as determined from the Ct. values obtained from 10 consecutive runs, was 0.84 for positive semen sample. The variability between three replicates in the 10 PCR assays ranged from 0.26 to 1.05 % for positive sample.

Confirmation of Real Time PCR Amplicon by Gel Electrophoresis

Though real time PCR does not require running of gel for PCR confirmation, as a means of authenticity a few of the real time PCR products were analyzed by agarose gel electrophoresis. The real time PCR products under gel electrophoresis yielded a specific amplicon of 71 bp.

Comparison of ELISA, Virus Isolation and Real Time PCR

In the present study, out of 212 animals, 74 animals were found positive for BoHV-1 antibodies by AB-ELISA, nine animals yielded the virus in cell culture system and 35

Fig. 5 BoHV-1 detection limit of real time PCR in tissue culture supernatants (amplification curves from *left to right*, 10 TCID₅₀, 1 TCID₅₀, 0.1 TCID₅₀, 0.01 TCID₅₀, 0.001 TCID₅₀)

Fig. 6 BoHV-1 detection limit of real time PCR in positive semen dilutions (amplification curves from *left to right*, 1:10, 1:20, 1:40, 1:80)

animals indicated the presence of the virus by real time PCR (Table 1). On statistical analysis, the real time PCR was found 100 % sensitive and 87.19 % specific test in detection of BoHV-1 in relation to virus isolation.

The serology, virus isolation and antigen detection by real time PCR were compared for diagnosis of BoHV-1 in animals. During virus isolation out of 74 seropositive animals only four animals yielded the virus but real time PCR

Fig. 7 Detection of both BoHV-1.1 (KVAFSU BNG-1) and BoHV-1.2 (KVAFSU BNG-5) subtypes by real time PCR (*green* base line, *green* amplification curve-BoHV 1.1, *red* amplification curve-BoHV 1.2)

Fig. 8 Sensitivity and specificity of real time PCR assay with known positive samples, known negative samples and non template controls (*green* base line, *blue* line NTC, amplification curves above base line—known positives, *red* and *green* lines below base line—known negatives)

detected BoHV-1 viral antigen in 22 seropositive animals. Out of the samples collected from 138 BoHV-1 seronegative animals, samples from five of these animals yielded cytopathic changes characteristic of BoHV-1 and real time PCR detected the BoHV-1 viral antigen in thirteen of these seronegative animals (Table 1).

Development and Validation of BoHV-1 Detection Kit

Based on the results obtained during this study, a TaqMan real time PCR based BoHV-1 detection kit was developed.

Thus developed real time assay was validated at Bigtec laboratories, Indian institute of Science, Karnataka, India

and Project Directorate on Animal Disease monitoring and surveillance, Indian Council of Agricultural Research (ICAR), Karnataka, India.

The BoHV-1 detection kit comprising of set of primers, probe, reaction mixture and the real time PCR protocols developed during this study have been filed for patent in Government of India (Number: 778/CHE/2012; CBR. No. 2365 dated 01.03.2012).

Discussion

The biggest challenge in herpes viral infections is the carrier status they induce in the animals they prevail in [16], consequent to which presence of antibody in an animal may not indicate an active infection. Considering this major drawback in serum based tests, detection of virus or its antigen becomes mandatory to designate any animal as positive for BoHV-1 [4]. BoHV-1 is of significance to international bovine germplasm trade. Comparative evaluation of available diagnostic tests and their practical relevance on clinical diagnosis is truly the need of the hour in most parts of the world, where there is no vaccination but record high seropositivity. Hence, the present study draws significance with the objectives detailed above.

In organised farms, the clinical samples were collected exclusively in the early mornings which recorded sudden change of weather (like sudden rains, adverse cold weather conditions), so that sudden change of weather could act as stressor in some carrier animals resulting in virus excretion. The selection of season for sample collection was in accordance with the reports of Singh et al. [26], who found that intense winter as the most suitable season for collection of clinical samples from BoHV-1 carrier animals for virus isolation.

The finding of overall 34.90 % seropositivity for BoHV-1 antibodies correlated with previous findings of Ganguly et al. [10], Nandi et al. [20] and the annual reports of India's national laboratory PD_ADMAS who have recorded 40, 39 and 42 % seropositivity respectively, in Indian cattle. 34.90 % of seropositivity seen during this study is truly, a huge percentage considering the fact that India does not practice BoHV-1 vaccinations and possibly, this gives the larger evidence of alarmingly wide spread nature of BoHV-1 in Indian cattle. Due to herpes viral latency, an animal once infected develops antibodies and the animal remains infected for life time and whenever the animal is subjected to stress conditions (disease/change of weather/change of feed/place, etc.) it excretes the virus, probably potentiating further antibody production. So an animal positive for antibody means it contains the virus and will be source of the virus whenever it comes under suitable stressors.

During virus isolation, the semen samples produced extensive destruction of cells in first passage of virus isolation which is attributed to lytic enzymes present in the seminal fluids and this finding was in total agreement with all previous works using semen samples. The finding of CPE of BoHV-1 with "bunch of grapes" like appearance of virus infected cells was in correlation with previous BoHV-1 isolation studies [5, 19]. As per OIE [4] to declare any isolate as BoHV-1, the virus isolate should have a NI of at least 1.5 and all nine isolates obtained in this study were confirmed as BoHV-1 with NI of more than 1.5.

The conventional PCR yielding a specific PCR product of 443 bp on all the nine tissue culture fluids showing CPE further confirmed the isolates as BoHV-1, as the primers used were specific to the conserved gB region of BoHV-1 [21, 24, 28]. Chelex resin based DNA isolation was adopted and was found very sensitive method for DNA isolation from semen samples.

Real time PCR differs from standard PCR in that the amplified PCR products are detected directly during the amplification cycle using a hybridisation probe, which enhances assay specificity. Real time PCR assays have several advantages over the conventional PCR methods. Real time PCR assays using only one pair of primers are able to provide sensitivity close or equal to nested PCR methods with a much lower risk of contamination. The amplification and detection of target is conducted simultaneously. There is no post amplification PCR product handling, which significantly reduces the risk of contamination [4].

We designed a set of primers amplifying conserved sequences of 71 bp on BoHV-1 gB gene. This is significant, since shorter distances between the forward primer, the probe and reverse primer in real time assays increases the specificity of the assay [29]. This is the smallest amplicon described for BoHV-1 diagnosis using real time PCR assay, as on date. Prior to our work Abril and colleagues [1], have reported the first set of primers and probe with an amplicon size of 97 bp for detection of BoHV-1 and these were validated by Wang et al. [29]. The real time PCR developed in this study had a detection limit at 0.001 TCID₅₀/0.1 ml of BoHV-1 in tissue culture supernatants. As on date this is the best detection limit described for BoHV-1 using any diagnostic assay. This ultra low detection limit achieved during the study is attributed to the primers and probe targeted to amplify a very short, conserved region of BoHV-1. Wang et al. [29, 30] have reported a detection limit of 0.36 TCID₅₀/0.1 ml of BoHV-1 in tissue culture supernatants.

The sensitivity of the real time PCR assay in terms of detection limit in dilution of the original sample was also evaluated using serial dilution of positive semen sample with cell culture medium (DMEM). The PCR assay was

able to detect dilutions of positive semen sample up to a dilution of 1:80. Virus isolation was also performed on same dilution of the semen samples, the virus was isolated only in the dilution of 1:10 and rest of the dilutions did not yield the virus in cell culture system, indicating that the assay developed had a much greater sensitivity than virus isolation in detection of BoHV-1 in clinical samples.

Comparison of ELISA, virus isolation, real time PCR and their clinical relevance (Table 1) was the most important objective of the present study. Real Time PCR detected all the samples that yielded virus in cell culture indicator system apart from detecting, twenty-six additional samples as positive for BoHV-1.

During our study all the seropositive animals did not yield the virus or its antigen (Table 1), however, they conclusively indicate that these animals have been infected with BoHV-1 sometime in its life time and they harbour the virus in latent or carrier status and can excrete the virus whenever they are subjected to stress conditions. Thus, a seropositive animal will mandatorily will have to be removed from the breeding herd.

Interestingly, out of 138 seronegative animals, samples from five animals developed CPE characteristic to BoHV-1 and thirteen of these animals were detected positive for BoHV-1 by real time assay (Table 1). This is a very unique situation which states that sero-negative animals need not be negative for the virus. This is probably attributed to the following reasons individually or in combinations. Firstly, an animal can be seronegative and can still yield the virus if the clinical samples are collected in the very early stage of infection before antibody production begins [17, 25], because antibody production requires at least 10–14 days [23]. Substantiating this, Hage et al. [12] have demonstrated that seronegative animals can excrete virus of up to 10^7 TCID₅₀/ml between onset of infection and seroconversion. Secondly, recent study on herpes viruses have described special mechanisms of viral spread between cells such that they never get exposed to the host immune system [3] so virus exists without actually coming in contact with immune effector cells. Thirdly, the carrier status is usually established in neuronal cells/ganglions (sacral ganglion for genital tract infections and trigeminal ganglion for respiratory tract infection) which either express very low levels of MHC or never express MHC on their cell surface [23]. Fourthly, BHV-1 has got unique abilities to destroy the synthesis of MHC molecules in the infected cell [5]. Finally, a recent finding by Laszlo et al. in 2011 [14] have shown that because bovine herpes viral surface proteins are poorly immunogenic, a seronegative animal can still harbour and yield the virus upon isolation.

Therefore, an antibody negative animal can be positive for the virus as noticed during our study hence a seronegative animal (especially in breeding stations) should

always be subjected for antigen detection by a suitable test, as it could act as a source of infection to other animals.

When compared with that of virus isolation, real-time PCR assay was a better test in detection of the virus, with an ability to detect virus at an ultra low concentration (of 0.001 TCID₅₀/0.1 ml) in clinical samples which was further authenticated at validating laboratories. Our finding on real time assay is in agreement with recent finding and was with better sensitivity/detection limits compared to the assays that have been described previously [8, 29, 30]. The assay can be efficiently used for diagnosis of BoHV-1 in clinical samples with special reference to semen samples which contain very low levels of virus and because if unchecked, an infected bull can be source of infection to thousands of female animals.

Considering these results, present study draws following conclusions. Firstly, in breeding stations an animal positive either in antibody detection or by antigen detection should mandatorily be removed from the herd because a seronegative animal can still be positive for virus and a seropositive animal need not yield the virus all the time, as evidenced during this study. Secondly, selection of new breeding animals should be done by both antigen detection as well as antibody detection tests and only those which are negative under both these tests should be selected for breeding purposes. Thirdly, every breeding animal should be subjected for periodical BoHV-1 screening both by antigen as well as antibody detection assays, preferably in winter seasons.

It's a reality that the real time assay cannot be applied at all laboratories in developing countries due to high cost of the real time PCR equipment. However, because of very high sensitivity of the test one recognised laboratory in each state/zone can have the real time facility (equipment and the assay/kit) and samples from across the state can be submitted to this laboratory for routine or periodical testing (especially the semen samples) for BoHV-1 detection.

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