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***Research Article***

# PREVALENCE OF BOVINE HERPES VIRUS - 1 IN ORGANIZED FARMS OF WEST BENGAL, INDIA

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**ABSTRACT: Infectious Bovine Rhinotracheitis, caused by Bovine Herpesvirus-1 (BoHV-1) maintains latency in trigeminal nerve ganglia of bovine. The sero-positive bull infected with BoHV-1 secretes the virus through semen intermittently, when the immune system is compromised. Sera from bulls housed at different bull stations were analyzed using gE protein specific IDEXX Kit, which showed 78.69% positivity. Each batch of semen from sero-positive bull was investigated further for presence of virus in semen by Real Time-PCR technique for validation of presence of virus in the frozen semen doses using gB specific primers and probe, which showed 0.968 % semen batches positive. This study showed that despite high sero prevalence in bull, the semen excretes very negligible amount of the virus indicating the subtypes circulating in farms of West Bengal, India is assumed to be respiratory type.**

**Key words:** IBR, BoHV-1, gB, Prevalence, West Bengal, India.

## INTRODUCTION

Bovine Herpes Virus-1(BoHV-1) is a significant pathogen in cattle, which causes respiratory disease, abortion, and infrequently, encephalitis in calf (Wyler *et al.*, 1989). Like other members of herpesviridae, it maintains latency in the trigeminal ganglion of infected animals. Cattle of all ages and breeds are susceptible to infection from introduction of infected animals in herd (Rock 1993 and 1994). The virus is transmitted through nasal, ocular and semen to healthy animals in farm (Kendrick and McEntree 1987). The virus is also spreaded through semen of infected bulls either through natural insemination, as well as, through artificial insemination (Masri *et al*., 1996).

In West Bengal, BoHV-1 is a major problem of cattle and buffalo as reported to be serologically positive. Therefore it is a prerequisite to validate a suitable laboratory test protocol to screen frozen semen straw so that virus excreted through semen can be stopped. In accordance with the World Organisation of Animal Health (Office International des Épizooties: OIE) Manual (2010), there is no restriction regarding the maintenance of

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BoHV-1 sero-positive bulls in semen stations but it is mandatory for screening of BoHV-1 in each batch of semen from these bulls for distribution of frozen semen in field . The routine method for detection of BoHV-1 in bovine semen is virus isolation in cell culture is one of the prescribed tests of the OIE for international trade. However, this method has limitations, such as sensitivity, time and cost. Hence, in the present study, we performed real time PCR by amplifying gB gene of BoHV-1 to estimate prevalence of BoHV-1 in the semen of IBR-sero positive bulls as per OIE guideline.

## METERIALS AND METHODS

### Collection and processing of serum

Blood samples were collected aseptically in evacuated collection tubes (VAKU-8) from different age group of breeding bulls from different frozen semen bull station farms of West Bengal, India during 2015-2016. We used single container for single animal to prevent cross contamination. After collection blood samples were kept for 3 hours in room temp for clotting and getting maximum volume of serum. After that the blood samples were kept at 4°C over-night for serum separation. Finally sera were separated and poured in serum vials under the laminar hood and were kept at -24°C for further study.

### Competitive ELISA against gE glycoprotein

Competitive ELISA was performed for detection of antibody against gE glycoprotein of BoHV-1, as per manufactures' protocol (IDEEX KIT, Switzerland). First, the antigen coated plate was removed from the pouch and all the reagents and plate are brought to room temperature (18°-25°C). Then sample position were recorded in the X check software using relevant template. Thereafter 50 µl of the sample diluent was added to each well by pipette along with 50 µl negative control and 50 µl positive controls in duplicate in appropriate wells. Fifty micro litre samples in duplicate were added in rest of the wells. Then the plate was incubated for 12-18 hours at 18°C-26°C under tightly sealed condition in a shaking incubator to avoid any evaporation. After that each well was washed with 300 µl of wash solution for five times and the plate is firmly tapped onto absorbent material to remove residual wash fluid from each well. Thereafter 100 µl of conjugate was dispensed into each well. After that the plate was incubated for 30 minutes at room temperature (18-25 °C). Then wells were washed with 300 µl of wash solution five times as mentioned above. Then 100 µl of TMB substrate solution was dispensed into each well. After that the plate was incubated for 10 minutes at room temperature (18-25 °C) in dark. Then 100 µl of stop solution was dispensed into each well to stop the reaction. Finally, the absorbance is measured at 650 nm and the result was analysed using X Check software.

### Real Time PCR for detection of BoHV-1

Extraction of DNA from semen samples: Two frozen semen straws from each batch of sero-positive bulls were processed. Duplicate PCR amplifications were carried out in duplicate for each DNA preparation to ensure detection of DNA in samples containing low levels of virus. The reaction was performed in a single tube by adding the following components in a screw top 1.5 ml tubes: Chelex 100 sodium (Sigma) (10% w/v in distilled deionised water) 100 µl, Proteinase K (10 mg/ml, Sigma) - 11.5 µl, DL-Dithiothreitol (1 M,

**Table 1. Prevalence of BoHV-1 virus, as detected by serology and Real Time PCR in West Bengal.**

| Parameters | Competitive ELISA (gE) | | | Real Time PCR | | |
|---|---|---|---|---|---|---|
| | Total no. of animals | Total no. of positive animals | (%) | Total no. of batches | Total no. of positive batches | (%) |
| Organized Farm-1 | 27 | 24 | 88.89 | 1706 | 14 | 0.82 |
| Organized Farm-2 | 118 | 88 | 74.57 | 673 | 09 | 1.36 |
| Organized Farm-3 | 113 | 91 | 80.53 | 409 | 05 | 1.22 |
| | 258 | 203 | 78.69 | 2788 | 27 | 0.968 |

**Table 2. Primers and Probe used for detection of BoHV-1 by Real Time PCR.**

| Sl. No | Name of the primer/probe for gB gene | Sequence(5'-3') | Binding position on BoHV-1 genome | Product Size (bp) |
|---|---|---|---|---|
| 1. | Forward Primer | TGTGGACCTAAACCTCACGGT | 57499-57519 | 97 |
| 2. | Reverse Primer | GTAGTGTCGTCGAGCAGACCC | 57595-57575 | |
| 3. | Taq Man Probe 5'FAM & 3'TAMRA | AGGACCGCGAGTTCTTGCCGC | 57525-57545 | |

Sigma) - 7.5 µl, Nuclease-free water - 90 µl, Semen sample - 10 µl. Then the components were mixed by pipetting and kept for incubation at 56°C for 30 minutes and then vortexed at high speed for 10 seconds. Subsequently, the tubes are incubated in a boiling water bath for 8 minutes and then vortexed at high speed for 10 seconds. The tubes are centrifuged at 10,000 g for 3 minutes. The supernatant is transferred into a new micro-tube and can be used directly for PCR, or stored at –20°C.

**Real-time PCR of gB gene of BoHV-1**

The PCR reaction mixtures were prepared in duplicates in 25 µl volume using with the following components: 2X Platinum Quantitative PCR Super Mix-UDG - 12.5 µl, gB specific forward & reverse primer (4.5 µM) - 1 µl each, Probe (3 µM) - 1 µl, nuclease free water - 5.5 µl and 5 µl of the DNA extracted as template as per the OIE guideline (Table 2). The cycle was set as one cycle each at 50°C for 2 minutes and at 95°C for 2 minutes followed by two steps 45 cycles at 95°C for 15 seconds and at 60°C for 45 seconds respectively.

The threshold level is usually set according to the manufacturers' instructions for the selected analysis software used. Alternatively, virus isolation of negative semen samples from sero-negative animals was run exhaustively (*e.g.* up to 55 amplification cycles) to determine the background reaction associated with the

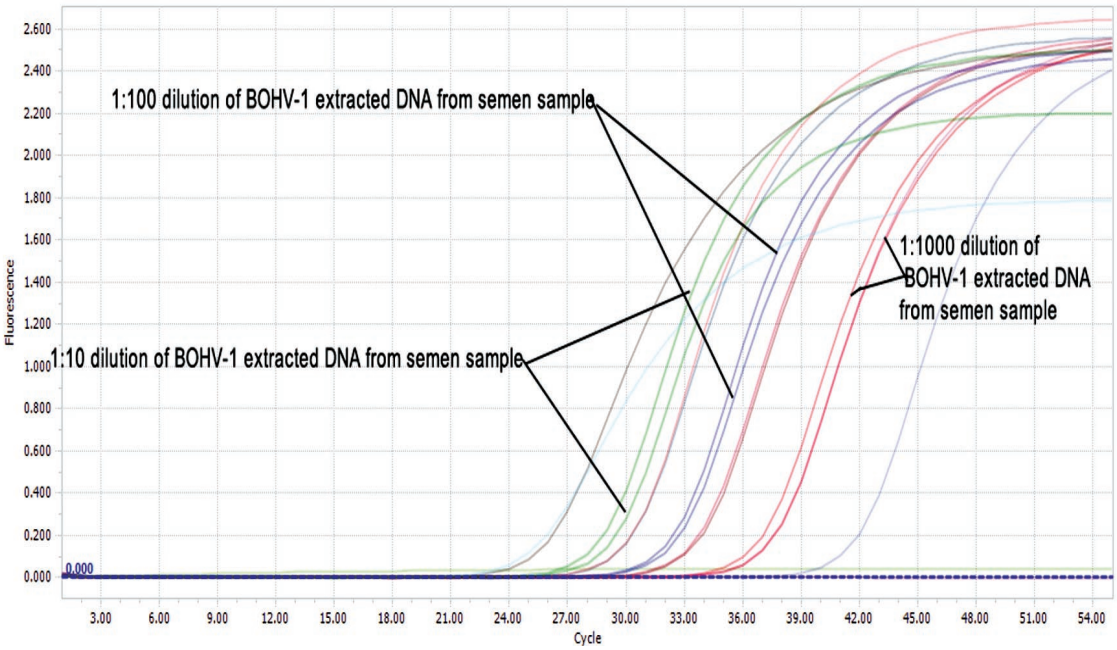


**Fig. 1. Real Time PCR for BoHV-1 detection using gB specific primer probes.**

detection system used. Any sample that has a cycle threshold (Ct) value equal or less than 40 is regarded as positive. Any sample that shows no Ct value is regarded as negative. Negative control and no template control should have no Ct values.

## RESULTS AND DISCUSSION

A total of 258 number sera samples were collected from three semen stations of West Bengal which showed 203 positive animals (78.69%). Each and every batch of frozen semen from all the bulls positive by ELISA was tested by real time PCR. Total 2788 frozen semen straws were tested by gB gene specific primers and probes which showed 27 batches positive (0.968%) (Fig. 1, Table 1).

Peculiarities of BoHV-1 is that it maintains latency in trigeminal nerve ganglia (Pastorel *et al.*, 1984). The virus breaks the latency after exposure to the corticosteroid treatment or hostile environment when the immunity falls. It has been also found that BoHV-1 excretes higher titre of virus during the primary phase of infection than later phase infection when the shedding is intermittent (Bitsch 1973). Several techniques such as virus isolation in MDBK cell lines, immune electron microscopy and PCR targeting various genes of the virus have been tried for the detection of BoHV-1 in bovine semen. Although virus isolation in MDBK cell lines remains the gold standard for detection of the virus, this method has limitations with regard to sensitivity, cytotoxicity of semen. Various PCR methods have been reported to be useful for detection of the virus. However, PCR

inhibitory substances in bovine semen interfere with the amplification of the target DNA. Therefore, attempts have been made to standardise and validate a real-time PCR to detect BoHV-1 in bovine frozen semen from naturally infected cattle and buffalo (Wang *et al.*, 2007). As per Rana *et al.*, (2011) for production of bovine frozen semen, all the batches from semen donating bulls positive in ELISA must be screened through real-time PCR for distribution of IBR virus free semen for Artificial Insemination purpose in field.

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 oligo-probe (TaqMan) for the detection of amplified products. PCR amplification of tenfold serial dilutions of the positive BHV-1 virus spiked with semen negative in BoHV-1 produced mean Ct value of 27.02, 30. 27 and 32.45 up to 1: 1000 dilutions which supports the OIE guideline. The positive samples showed the Ct value with in the mean 38.42. The semen samples showing Ct value up to 40 was included as positive samples and above that it was regarded as negative sample. The results from this study indicate that the real-time PCR assay offers greater sensitivity and will be valuable in detecting low titre of virus in extended semen samples.

## CONCLUSION

The present study clearly indicated that though high sero prevalence of BoHV-1 semen donating bulls, the virus is excreted in very low frequency through semen. Further investigation requires establishing the subtypes of the virus excreted through semen by cloning and sequencing of gC gene of the virus.

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