



Cloning of partial glycoprotein B gene and molecular epidemiological studies of bovine herpes virus -1 isolates

B M CHANDRANAIAK¹, D RATHNAMMA², S S PATIL³, S RANGANATHA⁴, RAMESH C KOVI⁵, D S AKHILA⁶, SHRIKRISHNA ISLOOR⁷, C RENUKAPRASAD⁸ and K PRABHUDAS⁹

Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka 585 401 India

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ABSTRACT

The study describes genetic grouping and molecular epidemiology of bovine herpes virus-1 (BoHV-1) through cloning of partial glycoprotein B gene of BoHV-1 isolates, with special reference to isolates recovered from cattle breeding stations. Samples were collected from 212 animals (91 bulls and 121 female cattle). Avidin-biotin ELISA employed on serum samples found 74 animals as seropositive for BoHV-1. On inoculation of 212 semen/swab samples to MDBK cell line for virus isolation, samples of 4 seropositive and 5 seronegative animals yielded cytopathic changes characteristic of BoHV-1. Partial gB gene of these isolates were cloned in pGEM T vector, nucleotide sequences were deduced and phylogenetic tree was constructed. Sequence analysis grouped 5 of these isolates under BoHV-1.1 cluster having highest sequence identities with previously described Indian, European and Brazilian isolates of BoHV-1.1. The other 4 isolates were clustered as BoHV-1.2 subtypes having 100% sequence identity with European strain of BoHV-1.2. We found that, apparently healthy, seronegative animals can be sources of BoHV-1, attributable to the unique pathogenesis/ latency of BoHV-1. This finding necessitates mandatory culling of breeding animals which are positive either in antigen detection or by serology, especially in countries which do not practice vaccination but report high seroprevalence of BoHV-1.

Key words: Bovine herpes virus-1, Clinical relevance, Genogrouping, Molecular epidemiology, Serology, Virus isolation

Bovine herpes virus type-1 (BoHV-1), a member of the subfamily alpha herpes virinae, under the order Herpesvirales, causes diverse ailments in cattle (Patil *et al.* 2012). In genital tract infections of bulls, the virus replicates in prepuce, penis and urethra and the semen gets contaminated during ejaculation. Insemination of cows with such semen causes infertility, endometritis and abortion (Chintu Ravishankar *et al.* 2012). Initially, the virus is transported along axons and becomes latent in sacral ganglion where it persists for the life of the animal (VanEngelenburg *et al.* 1993). The virus gets reactivated under stress conditions

(Chandranaiak *et al.* 2010). India records high seroprevalence of BoHV-1. Though, BoHV-1.1 is the major subtype circulating in cattle worldwide, recent descriptions of other subtypes complexes the understanding of viral pathogenesis. This work describes the molecular epidemiology of the BoHV-1 and the relevance of serology in clinical diagnosis of BoHV-1 in breeding stations.

MATERIALS AND METHODS

Sample collection: Fresh semen samples were collected from 91 bulls in viral transport media (VTM) made from Dulbeccos modified eagles media (DMEM) with 2% foetal calf serum (FCS) and antibiotics. Vaginal swabs were collected from 86 cows in 2 organised farms. The swab samples (nasal, conjunctival and vaginal) were collected from 35 ailing animals during filed outbreaks. All the swab samples were collected in VTM. The present study was conducted on 212 samples comprising 91 semen and 121 swab samples. Serum samples from all the 212 animals were collected for detection of BoHV-1 antibody.

Enzyme linked immunosorbent assay: Enzyme linked immunosorbent assay (ELISA) was employed on 212 serum

Present address: ¹Scientist-2, IAH and VB (dinglychandru@yahoo.com), ²Senior Scientist, PD-ADMAS (sharnaspin123@rediffmail.com), ³Retired Project Director (prabhudask@gmail.com), PD-ADMAS, Hebbal, Bengaluru, Karnataka. ²Professor and Head (doddamanerathnamma@yahoo.co.in), ⁴M.VSc. Scholar (srangavetco@gmail.com), ⁶Research Associate (akhilads87@gmail.com), ⁷Associate Professor, Department of Veterinary Microbiology (kisloor@rediffmail.com), ⁸Vice Chancellor (c.renukaprasad@gmail.com). ⁵Assistant Professor, Department of Veterinary Population Medicine, University of Minnesota, St. Paul, Minnesota, USA (rckovi@gmail.com).

samples collected from all the animals. The Avidin- Biotin ELISA kits were used. 1:30,000 dilution of biotinylated anti IgG and 1: 15,000 dilution of Avidin-HRPO conjugate were used during the test.

Virus isolation

Cell lines: Madin Darby bovine kidney (MDBK) cells obtained from National Centre for Cell Sciences, Pune, India were used. The cell lines grown in DMEM growth media supplemented with 5% FCS were used for the entire screening work.

Processing and Infection to cells: Semen sample (200 micro litres) in VTM was diluted with 2 ml of certified foetal calf serum (USA) mixed vigorously and incubated for 30 min at room temperature. The processed samples were infected onto MDBK cells by adsorption method (OIE 2010). Each sample was given 3 blind passages. In each passage the cells were observed for 7 days. The samples that did not show cytopathic effect (CPE) by third passage were discarded and declared as negative for BoHV-1. The samples that showed CPE by third passage were further passaged for virus propagation.

Virus neutralisation index: The virus isolates thus obtained were confirmed by neutralisation test using BoHV-1 specific antiserum procured from PD_ADMAS, Bengaluru. The virus titration, neutralisation and determination of neutralisation index (NI) of virus isolates were carried out as per standard protocols (Reed and Muench 1938, Chandranaik *et al.* 2010, Anonymous 2010). The BoHV-1 virus isolate (PD_ADMAS BoHV-1), obtained from Project Directorate on Animal Disease Monitoring and Surveillance, Bengaluru, was used as the positive control.

Polymerase chain reaction: All the virus isolates recovered from semen and swab samples were subjected for BoHV-1 glycoprotein B (gB) gene specific conventional polymerase chain reaction (PCR).

DNA isolation: The method for extraction of DNA from semen was essentially according to Wang *et al.* (2008) with some modifications. In brief, 10 µl of semen sample was added to 100 µl of Chelex 100 (10% w/v in sterile distilled water), 11.5 µl of 10 mg/ml proteinase K and 7.5 µl of 1M DTT and 90 µl sterile distilled water. The sample mixture was mixed gently and incubated at 56°C for 30 min. Following a brief vigorous vortexing for 10 sec, the sample was placed in a boiling water-bath for 8 min. The vortexing was repeated and the samples were centrifuged at 10,000 × g for 3 min. The supernatant containing DNA was used directly or stored at -20°C for future use.

The DNA from all virus isolates (in cell culture supernatants) and swab samples was isolated as per the Lopasev *et al.* (1991) using DNA isolation kits.

PCR assay protocols: The DNA isolated from all the samples and virus isolates were subjected to conventional 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 triphosphates, 0.1 µM each of primer P1 Forward 5' TGGTGGCCTTYGACCGCGAC 3' and Reverse 5'GCTCCGGCGAGTAGCTGGTGTG 3', 1U of Taq polymerase per reaction (Ros and Belak 1999). All the reagents and primers were obtained commercially. The PCR mixture was subjected to 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 30 sec and extension at 72°C for 45 sec followed by one cycle of final extension at 72°C for 10 min. Non template control and DNA extracted from BoHV-1 negative semen were used as negative controls. The PCR products were analysed by agarose gel electrophoresis.

Cloning of the partial gB gene of BoHV-1 isolates

Gel extraction and cloning: The PCR amplified products of virus isolates were extracted from agarose gel and eluted in 25 µl of nuclease free water using gel extraction kit as per manufacturer's instructions.

The extracted PCR product of partial gB gene of BoHV-1 was cloned into pGEM T easy vector in 10 µl volume which consisted of 50 ng of vector, 100 ng of PCR product, 3U of T4 DNA ligase, 1X ligation buffer and nuclease free water up to 10 µl and was incubated at 22°C for 1 h. The ligated mixture was transformed into DH 5α strain of *E.coli* cells following standard procedures (Sambrook *et al.* 1989, Patil *et al.* 2006, Ranganatha *et al.* 2013).

Sequencing: The recombinant plasmids were extracted from white colonies using plasmid extraction kit and was checked for the presence of the insert by *Eco*R1 restriction enzyme digestion (Sambrook *et al.* 1989, Patil *et al.* 2006, Ranganatha *et al.* 2013).

The confirmed recombinant clones were subjected to nucleotide sequencing commercially. Nucleotide sequences were aligned with published sequences in GenBank using DNASTAR software by CLUSTAL method. Phylogenetic tree was constructed and compared with reference nucleotide sequences of BoHV-1 available in the GenBank. Sequence analysis was performed with the laser gene 6 package. Phylogenetic analyses of the 443-bp fragment of BoHV-1 gB were conducted and tree was constructed using modules of MEGA version 5, using the maximum parsimony method with 500 bootstrap replicates (Tamura *et al.* 2007).

RESULTS AND DISCUSSION

The biggest challenge in herpes viral infections is the carrier state they induce in the animals (Chandranaik *et al.* 2010, Chintu Ravishankar *et al.* 2012) as a result of which presence of antibody in an animal may not indicate an active infection. Considering this as a major drawback in serum based tests, detection of virus or its antigen becomes mandatory to designate any animal as positive for BoHV-1 (Anonymous 2010). The BoHV-1 is of significance to international bovine germplasm trade. Hence, comparative

Table 1. Comparison of Indirect ELISA and Virus isolation for diagnosis of BoHV-1

Farm	Number of samples collected	Seropositives (Ab positives)		Seronegatives (Ab Negatives)	
		Number of Ab positives	Number of virus isolates	Number of Ab Negatives	Number of virus isolates
Farm A	49	19	nil	30	nil
Farm B	50	28	2	22	2
Farm C	38	8	nil	30	nil
Farm D	40	7	nil	33	nil
Field samples	35	12	2	23	3
Total	212	74	4	138	5

evaluation of available diagnostic tests and their practical significance is, truly the need of the hour and the present study draws significance with the objectives detailed above.

Sample collection: Samples were collected from 212 animals from 4 organised farms and different outbreaks suspected of BoHV-1 infections. During sample collection from filed outbreaks, a few of the animals showed typical gross lesions of vulvovaginitis, balanoposthitis, conjunctivitis and respiratory system related lesions. In organised farms samples were collected exclusively in the early mornings which recorded sudden change of weather conditions such as sudden rains and adverse cold weather. The sudden change of weather could act as stressor in carrier animals resulting in virus excretion. This is in accordance with findings of Singh *et al.* (1986) who have reported the intense winter as the most suitable season for collection of clinical samples from BoHV-1 carrier animals for virus isolation.

Seroprevalence by ELISA: Overall 34.90% of the animals tested were seropositive for BoHV-1 antibodies (Table 1). The finding of overall 34.90% seropositivity for BoHV-1 antibodies correlated with previous findings of Ganguly and Mukhopadhyay (2010) and Nandi *et al.* (2010) with 40% and 39% seropositivity, respectively, in Indian cattle. The annual reports of national laboratory PD_ADMAS also recorded 42% seropositivity in cattle. The high seropositivity of BoHV-1 in this study gave a larger evidence of alarmingly widespread nature of BoHV-1 in cattle. These findings are comparable globally with the seroprevalence of 33.97% in Iran (Kargar *et al.* 2001), 25.9% in Tunisia (Ghram and Minocha 1990), 37.8% in Canada (Durham and Hassard 1990), 37% in Uruguay (Guarino *et al.* 2000), 35.9% in Belgium (Castrucci *et al.* 1997), 34.99% in Italy (Boelaert *et al.* 2000), 40% in Netherland (Castrucci *et al.* 1997) and 38% in Poland (Ackermann and Engels 2006).

Owing to latency of herpes viruses, an animal once infected develops antibodies and the animal remains infected for life time. The latent animal is when subjected to various stress conditions, it excretes the virus. Therefore, an animal positive for antibody means, it contains the virus and will be the source of infection for in contact animals.

Virus isolation and PCR: During virus isolation, in spite of using serum to neutralise seminal enzymes, a few of the

semen samples produced extensive degeneration and detachment of cells within 24 h in the first passage and some samples had similar effects even during second passage. Of the 91 semen samples tested, only 1 sample developed cytopathic effect (CPE) in MDBK cell line. Out of 121 swab materials collected from organised farms and filed outbreaks, 8 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.

In this study, the semen samples produced extensive destruction of cells in first passage which is attributed to lytic enzymes present in the seminal fluids. 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 reports (Benoit *et al.* 2007, Chandranaik *et al.* 2010, James and Edward 2011).

All the 9 isolates were neutralised by BoHV-1 specific serum obtained from PD_ADMAS, Bengaluru, India with neutralisation index of more than 1.5. The titre of the virus isolates varied between log $10^{2.24}$ and $10^{7.24}$ TCID₅₀/0.1ml. As per OIE, to declare isolate as BoHV-1, the isolate should have a neutralisation index of at least 1.5 and all the 9 isolates obtained in this study were confirmed as BoHV-1 with NI of more than 1.5.

All the virus isolates were also confirmed by conventional PCR using primers specific for gB region of BoHV-1, yielding a specific amplicon of 443 bp, which was in accordance with similar previous works (Patil *et al.* 2012 and Patil *et al.* 2006). After PCR confirmation these were serially named as KVAFSU-BNG-1 through 9.

Cloning and sequencing: The PCR products of partial gB gene of BoHV-1 were cloned into pGEM T easy vector, the ligated mixture was transformed into DH 5 α strain of *E.coli* cells (Fig.1). The recombinant plasmids extracted from white colonies were checked for the presence of the desired insert by *EcoR*I restriction enzyme digestion, releasing insert of expected size (Fig.2). The nucleotide sequences obtained from cloned product of 443 bp were initially aligned with the published nucleotide sequences of BoHV-1 isolates in GenBank using DNASTAR software by CLUSTAL method.

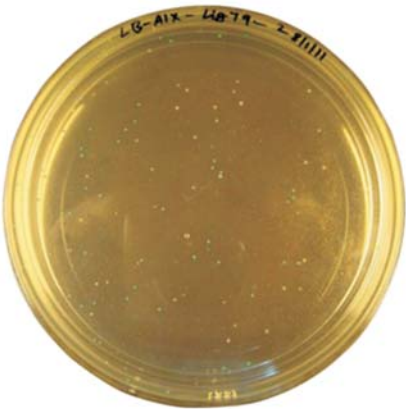


Fig. 1. Cloning of partial gB gene of BoHV-1 in *DH 5α E. coli* cells on LB agar. (Blue colonies without insert, White colonies with insert).

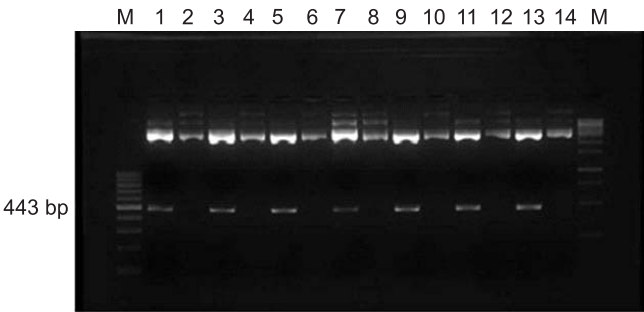


Fig. 2. Confirmation of recombinant clone (With desired 443 bp partial gB gene insert) by *Eco*R1 restriction enzyme digestion. **M**: Marker; **1,3,5,7,9,11,13**: Recombinant plasmids with desired insert of 443 bp; **2,4,6,8,10,12,14**: plasmids without insert.

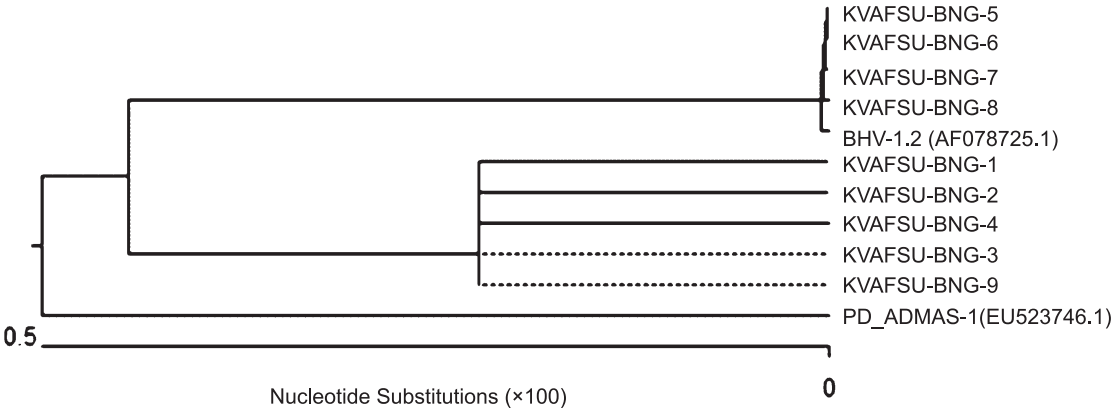


Fig. 3. Phylogenetic tree of BoHV-1 isolates.

Percent Identity												Divergence	
	1	2	3	4	5	6	7	8	9	10	11		
1		99.8	99.8	99.8	100.0	99.3	99.3	99.3	99.3	99.3	99.3	1	KVAFSU-BNG-9
2	0.2		99.5	98.4	99.8	99.1	99.1	99.1	99.1	99.1	99.1	2	KVAFSU-BNG-4
3	0.2	0.5		98.4	99.8	99.1	99.1	99.1	99.1	99.1	99.1	3	KVAFSU-BNG-1
4	0.2	0.5	0.5		98.6	98.2	98.2	98.2	98.2	98.0	98.0	4	KVAFSU-BNG-2
5	0.0	0.2	0.2	0.2		99.3	99.3	99.3	99.3	99.3	99.3	5	KVAFSU-BNG-3
6	0.7	0.9	0.9	0.9	0.7		100.0	100.0	100.0	98.9	100.0	6	KVAFSU-BNG-5
7	0.7	0.9	0.9	0.9	0.7	0.0		100.0	100.0	98.9	100.0	7	KVAFSU-BNG-6
8	0.7	0.9	0.9	0.9	0.7	0.0	0.0		100.0	98.9	100.0	8	KVAFSU-BNG-7
9	0.7	0.9	0.9	0.9	0.7	0.0	0.0	0.0		98.9	100.0	9	KVAFSU-BNG-8
10	0.7	0.9	0.9	0.9	0.7	1.1	1.1	1.1	1.1		98.9	10	PD_ADMAS-1(EU523746.1)
11	0.7	0.9	0.9	0.9	0.7	0.0	0.0	0.0	0.0	1.1		11	BHV-1.2 (AF078725.1)
	1	2	3	4	5	6	7	8	9	10	11		

Fig. 4. Sequence pair distance map of BoHV-1 isolates.

Phylogenetic tree was constructed (Fig. 3) and nucleotide sequence pair identity map was deduced (Fig.4). The virus isolates KVAFSU BNG-1, KVAFSU BNG-2, KVAFSU BNG-3, KVAFSU BNG-4 and KVAFSU BNG-9 shared more than 99% sequence identity with reference strain PD_ADMAS BoHV-1 (GenBank accession number EU 543746.1). The nucleotide sequences of these 5 isolates were submitted to GenBank under accession numbers JF 920418, JF 920419, JF 920420, JF 920974 and JF 920475.

The virus isolates KVAFSU BNG-5, KVAFSU BNG-6, KVAFSU BNG-7 and KVAFSU BNG-8 recovered from 4 cows showing typical symptoms of pustular vulvovaginitis under field conditions were homologous between themselves and shared a sequence identity of 100% with a European strain of BoHV-1.2 (GenBank accession number AF 078725.1). However, these 4 isolates shared a sequence identity of 98.9% and divergence of 1.1 with BoHV-1.1 reference (PD_ADMAS) strain. Upon sequence analysis of these 4 isolates which were clustered under BoHV 1.2, it was found that the nucleotide sequence varied with BoHV-1.1 strains at positions of 45, 84, 27 and 435, wherein T nucleotide at these locations in the PD_ADMAS reference strain sequence was substituted with C, C, G and C nucleotides respectively. Further, at location 432 the nucleotide C in BoHV-1.1 strain was substituted with nucleotide A in all the 4 isolates. These 4 isolates of BoHV-1.2 had consensus sequences and were submitted to GenBank with common accession number JN 022592.

The detailed phylogenetic analysis showed that the isolates which were clustered under BoHV-1.1 were genetically very close to PD_ADMAS strain (Accession number EU 543746.1), the Switzerland strain (AJ 004801) and the Brazilian strains (Accession number AY 58382 and Accession number AY 330349). The isolates which were clustered under BoHV-1.2 were genetically very close to European strain (Accession number AF 078725.1) of BoHV-1.2.

The cloning of partial gB gene of BoHV-1 isolates and their sequencing data were in correlation with the findings of Patil *et al.* (2012) and Surendra *et al.* (2011) who have described BoHV-1.1 strain as the most common type of herpes virus in cattle. However, out of the 9 isolates, the nucleotide sequences of 4 isolates recovered from a single outbreak were 100% homologous with sequences BoHV-1.2 reference virus. The BoHV-1.2 isolates showed more than 98% sequence identity with BoHV-1.1 isolates, indicating that, there is possibility of very minute difference between 1.1 and 1.2 types of BoHV-1. This finding was in accordance with works of Ros and Belak (1999) who have sequenced the gB gene and recorded 98.8% sequence identity between BoHV-1.1 and BoHV-1.2. Further, this was supported by Arce *et al.* (2002) who have shown that monoclonal antibodies (MAbs) against either BoHV-1.1 or BoHV-1.2 will cross react with both the subtypes and MAbs cannot differentiate the subtypes. The non differentiation of subtypes even with

MAbs indicated high degree of similarity between BoHV-1.1 and BoHV-1.2. The present study further confirmed the circulation of BoHV-1.2 in cattle, which could be one of the main reasons for increased herpes viral abortions in cattle in recent times.

The tremendous growth in dairy sector is primarily attributed to the extensive crossbreeding programme undertaken during the periods of white revolution in 1970's and 1980's using exotic breeds or their germplasms. Genetic characterisation of virus isolates obtained during this study could well support the fact that BoHV-1 must have entered during extensive dairy development in India using foreign germplasms. However, as only a small number of BoHV -1 and its subtypes were characterised, sampling errors must be considered, since the isolates examined in this study may not be actual representatives of prevailing viruses found in the country. The PD_ADMAS strain and European strain of BoHV-1.2 which were used as reference strains may not be the actual representatives of most of BoHV-1.1 and BoHV-1.2 isolates circulating in the country. It is likely that variations between strains may be larger than the variations shown in this study. Similar reports on transmissions through germplasm/breeding bulls were noted by Nuotio *et al.* (2007), who observed that one of the seropositive bulls imported from Denmark in 1968 introduced the BoHV-1 to Finland. The large scale spread of bovine herpes virus in several countries (especially Asian countries), may be attributable to similar reasons of dissemination through transport of high yielding bulls or their semen, throughout the world.

In the present study, all the seropositive animals did not yield the virus rather only 4 out of 9 virus isolates were from seropositive animals (Table 1). It was speculated that these animals might have infected with BoHV-1 during their life time, harbouring the virus in latent or carrier state and can excrete the virus under stress conditions. Hence, the present study recommends mandatory culling of seropositive breeding animals from the breeding station.

Out of 138 seronegative animals, samples from 5 animals developed CPE characteristic of BoHV-1 (Table 1). This is a very unique observation which states that seronegative animals need not be negative for the virus. This could be 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 early stage of infection before the antibody production begins (Lowen *et al.* 1985, Rudi Weilben *et al.* 1992) as it requires at least 10–14 days for antibody production (Richard *et al.* 2007). Substantiating this, Hage *et al.* (1996) demonstrated that seronegative animals can excrete BoHV-1 of up to 10^7 TCID₅₀/ml between the onset of infection and sero-conversion. Secondly, Antinone *et al.* (2006) described the special mechanisms of spread of herpes virus between cells in such a way that they never get exposed to the host immune system. The virus exists without actually coming in contact

with immune effector cells. Thirdly, the carrier status is usually established in neuronal cells/ganglions, viz. sacral ganglion for genital tract infections and trigeminal ganglion for respiratory tract infection. These neuronal ganglions either express very low levels of MHC or never express MHC on their cell surface (Richard *et al.* 2007). Fourthly, BoHV-1 has got unique mechanisms to prevent the synthesis of MHC molecules in the infected cell (Benoit *et al.* 2007). Finally, Laszlo *et al.* (2011) showed that, because bovine herpes viral surface proteins are poorly immunogenic, a seronegative animal can still harbour and yield the virus upon isolation.

As a result of unique mechanisms of pathogenesis and immune evasion strategies adopted by BoHV-1, an antibody negative animal can be positive for the virus as observed in the study. Hence, a seronegative animal (especially in breeding/ semen stations) should always be subjected for antigen detection by a suitable test; otherwise an infected bull can be source of infection to thousands of animals.

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