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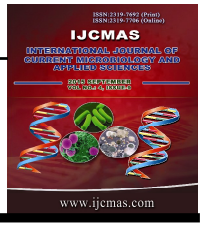

Original Research Article

Sequence and Phylogenetic Analysis of Glycoprotein B Gene of Indian Isolate of Bovine Herpesvirus 1

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K e y w o r d s

Bovine herpesvirus 1, BoHV1, Glycoprotein B, UL27, IBR

A B S T R A C T

Bovine herpesvirus 1 (BoHV1) infects mainly cattle and buffalo causing infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis and infectious balanoposthitis. It is also one of the causative agents of bovine respiratory disease complex or shipping fever. It causes colossal economic losses due to productive and reproductive losses in infected animals. Glycoprotein B is one of the most important surface glycoproteins of BoHV1. It induces strong neutralizing antibody response and is the target of $CD4^+$ T cells. It is the major protein involved in attachment, penetration and cell fusion. There are only few partial sequences available for gB gene of Indian isolate BoHV1. In this study carboxyl terminal 2514 bp of gB gene was amplified by using overlapping primers, cloned and sequenced. On in-silico analysis, it was found that glycoprotein B gene of Indian isolate of BoHV1 is shared over99% sequence identity with other BoHV1 strains. BoHV1-IBR 216 II had divergence of 4.3%, 5.6% and 3.6% with BoHV5, BuHV1 and CvHV1, respectively. Phylogenetic analysis revealed that the Indian isolate clustered with other strains of BoHV1 around the world.

Introduction

Infectious bovine rhinotracheitis (IBR) is an important viral disease of cattle and buffalo. It is caused by BoHV1. The virus belongs to Family-*Herpesviridae*, Sub family-*Alphaherpes virinae* and Genus-*Vericelovirus*. Based on DNA restriction enzyme analysis it can be divided into BoHV1.1 and BoHV1.2. Conventional serological assays cannot distinguish between Immune responses induced by subtypes of BoHV1. BoHV1IBR is caused by BoHV1.1 and the disease is characterized by symptoms like fever, coughing, anorexia, depression, decreased milk production, weight loss, increase respiratory rate, nasal and ocular discharge which is serous at beginning and become mucopurulent later and increased salivation may also accompany these respiratory tract problems. BoHV1.2 may be less virulent than BoHV1.1. BoHV1.2 can be further subdivided into BoHV1.2a and BoHV1.2b. BoHV1.2a causes infectious pustular vulvovaginitis (in cows) characterized by pustular lesions of the genital tract in females or males and may lead to abortion and infectious pustular balanoposthitis (in bulls) characterized by extensive adhesion,

annular constriction and penile distortions. BoHV1.2b is less virulent compared to the earlier one (Metzler *et al.,* 1985). Sequence homology between BoHV1.1 and 1.2 is more than 95% (Engels *et al.,* 1986).

BoHV1 glycoproteins are homologous in structure and function to HSV1 glycoproteins and they are involved in several steps of the viral cycle such as attachment, penetration, maturation and release of the virus. At least 10 genes code for glycoproteins, among them 6 are present in UL region gK (UL53), gC (UL44), gB (UL27), gH (UL22), gM (UL10), gI (UL1) and 4 are in Us region gG (US4), gD (US6), gI (US7), gE (US8). Glycoproteins gP, gI and gE possess the Fc receptor and binds with the IgG molecule (Schwyzer and Ackermann, 1996). Glycoprotein B is one of the major glycoprotein found in the virus envelop and plasma membrane of virus infected cell (Marshall *et al.,* 1986). Immunity to gB appears extremely early post infection and immunity to it following infection is long lived. Its gene maps in the UL region of the genome and codes for a poly peptide of 932 amino acid with an unusually long signal sequence and transmembrane sequence and 5 potential sites for addition of N–linked oligosaccharide (Misra *et al.,* 1988). Glycoprotein B induces a strong neutralizing antibody response (Babiuk *et al.,* 1987) and is recognised by $CD4^+$ helper T lymphocytes (Hutchings *et al.,* 1990). In the initial interaction of virus with cell namely attachment (Liang *et al.,* 1991), penetration, cell fusion (Liang *et al.,* 1991) and cell-cell spread (Kopp *et al.,* 1994). In order to develop a diagnostic assays genetic characterization of major immunogenic protein such as gB is important. At present very limited information is available on characterization of Indian isolates of BoHV1. The present study describes the genetic and phylogenetic analysis of Indian isolate of BoHV1.

Materials and Methods

Cells and virus

Madin-Darby bovine kidney (MDBK) cells were grown in Dulbecco's Modified Eagle Medium + GlutaMax-I (Invitrogen, USA) with supplementation of 10% Newborn Calf Serum (Invitrogen, USA). The cells were incubated at 37 °C in the presence of 5% CO_2. An Indian isolate of BoHV1 viz., (BoHV1/IBR 216 II/ 1976/ India) maintained at Division of Virology, IVRI, Mukteswar was used for the study.

Extraction of viral DNA

The DNA was extracted using commercial kit as per the manufacture's instruction (Promega, USA). In brief, MDBK cells infected with BoHV1 when showing 80% CPE were harvested and supernatant discarded. The infected cell layer was added with 200 µL of phosphate buffer saline and freeze-thawed thrice (30 min each at -80 °C and 37 °C). Then, 600 µL of nuclei lysis solution was added to the above lysate and mixed by pipetting. To remove RNA, 3 µL of RNase solution was added, mixed and incubated at 37 °C for 30 min. About 200 µL of protein precipitation solution was added, vortexed and chilled on ice for 5 min. The suspension was centrifuged at 13000 ×g for 4 min and the supernatant was transferred to a new tube. To precipitate the DNA, about 600 µL of isopropanol was added to the supernatant and centrifuged at 13000 ×g for 1min. After decanting the supernatant, about 600 µL of 70% ethanol was added and centrifuging at 13000 ×g for 1 min. After removing ethanol by aspiration and air drying, the DNA pellet was rehydrated with adding 100 µL of

rehydration solution and incubating for 1 h at 65 °C.

Polymerase chain reaction (PCR)

Overlapping oligonucleotides primers were designed based on sequence of the reference strain of BoHV1 (Genbank accession ID JX898220.1.1). Primers designed for amplification of glycoprotein B gene are presented in the table.

The PCR was carried out in a 50 µL of reaction mixture. The component of the reaction mixture is presented in tables 2 and 3.

The resulting mixture was subjected to a precise thermal profile as followed by table 4 and 5.

Expected band size was visualized under transilluminator after running 5 µL of PCR product in 1% agarose gel. PCR amplicons of expected size was gel purified using QIAquick® Gel Extraction Kit (Qiagen, USA) and used for downstream applications.

Cloning and sequencing

The gel purified PCR amplicon was cloned using InsTA cloning Kit (Fermentas, USA). In brief, 1 µL of vector pTZ57R/T was mixed with 1 µL 10X Ligation buffer, 4 µL purified PCR product, 1 µL T4 DNA Ligase and 3 µL nuclease free water. The mixture was vortexed, centrifuged for 3-5 sec and incubated for 2.5 h at 22 °C followed by for overnight at 4 °C. For transformation, 50 µL of competent DH5α from -80 °C was thawed on ice and Mixed with 5 µL of ligated PCR product and kept on ice for 30 min. The mixture was subjected to heat shock at 42 °C for 1 min, followed by 2 min on ice. 1 mL of LB broth was added to the tube and mixed by inversion, and incubated at 37 °C in a shaker incubator for 2 h. The cells were centrifuged at 5000 xg for 2 min; the pellet was resuspended with 50 µL of PBS and spreaded over LB agar containing ampicillin (50 µg/mL), X-gal (30 µg/mL) and Isopropyl β-D-1-thiogalactopyranoside (IPTG, 40 µg/mL). The plate was incubated at 37 °C for overnight at upright position. At least ten white colonies were selected and confirmed for having the desired construct by colony PCR using M13 primers. The confirmed bacterial containining positive inserts were commercially sequenced (SciGenome, Cochin, Kerala).

Sequence trimming and editing of the raw sequence was carried out using Sequencher 4.7 (Gene Codes Corporation, MI, USA). Reference sequences include BoHV1 and other members of *Alphaherpesvirinae* were downloaded from GenBank. The Indian isolate in the current study and references sequences were aligned using the MEGA 5 software (Tamura *et al.,* 2011). The phylogenetic tree was constructed using neighbor-joining algorithm with Kimura-2 parameters correction and 1000 bootstrap replications.

Results and Discussion

The gB is one of the major glycoproteins found in the virus envelope and plasma membrane of virus infected cell (Marshall *et al.,* 1986). It helps in attachment (Liang *et al.,* 1991), penetration, cell fusion and cell-cell spread (Kopp *et al.,* 1994) of the virus and also involved in inducing strong neutralizing antibody response (Babiuk *et al.,* 1987). It is also recognised by CD4+ helper T lymphocytes (Hutchings *et al.,* 1990).

To amplify some segment of gB gene, glycerol @ 10% has been used in PCR reaction. Without adding glycerol, no

amplification could be obtained with enzymes from different manufactures' and/or PCR conditions. It is difficult to amplify sequences having high G+C contents (guanine-cytosine content) because of the low efficiency of template dissociation as there is alteration in the melting point of the DNA template (Ros and Belák, 1999).

Several researchers used glycerol in PCR mixtures to enhance the specificity and/or the yield of the PCR from high GC templates (Gupta *et al.,* 2006), (Nandi and Kumar, 2011). The G+C content of BoHV1 ranges from 71-72% (Nandi and Kumar, 2011). Glycerol helps in strand-separation and primer-annealing temperatures and also reduces secondary structure that could inhibit the progress of the polymerase. It lowers the temperature of strand separation by lowering the concentration of water, thus the dielectric constant, thus the force needed for breaking Hydrogen-bridges(http://worldwide.promega.com/resources/product-guides-and-selectors/protocols-and-applications-guide/pcr-amplification/).

Table.1 Primers designed for amplification of glycoprotein B gene

Primer	Sequence	Primer length (bp)	Primer Location (genome)	Product Length (bp)
gBF1-F	5´-GACGGCGACGACGCCGCCAG-3´	20	55679-55698	1151
gBF1-R	5´-AGCTCCTGCAGGTACAGCTT-3´	20	56829-56810	
gBF2-F	5´-CACGTGCTGTCGGGCAGCTT-3´	20	56723-56742	346
gBF2-R	5´-CCAGGCGGCTGAACATGGTGT-3	21	57068-57048	
gBF3-F	5´-ACATCCAGGACCACGTGAAC-3´	20	57030-57049	1265
gBF3-R	5´-CCGCCTCCAAGAAAAACA-3´	18	58294-58277	
gBF2a-F	5´-CCGCTGAGCGACTGCGTGAT-3´	20	56648-56667	423
gBF2a-R	5´-GGCCAGGCGGCTGAACATGG-3´	20	57070-57051	

Table.2 Composition of PCR reaction mixture (for gBF1-F/gBF1-R, gBF3-F/gBF3-R, gDF1-F/gDF1-R, gDF2-F/gDF2-R)

Sl. No.	Components	Quantity
1.	Maxima® Hot start Green PCR Master Mix (Fermentas)	25 µL
2.	Nuclease free water	22 µL
3.	Forward Primer	1 µL
5	Reverse Primer	1 µL
6	Template DNA	1 µL
Total		**50 µL**

Table.3 Composition of PCR reaction mixture (gBF2-F/gBF2-R, gBF2a-F/gBF2a-R)

Sl. No.	Components	Quantity
1.	Maxima® Hot start Green PCR Master Mix (Fermentas)	25 µL
2.	Nuclease free water	17 µL
3.	Glycerol	5
3.	Primer 1	1 µL
5	Primer 2	1 µL
6	Template DNA	1 µL
Total		**50 µL**

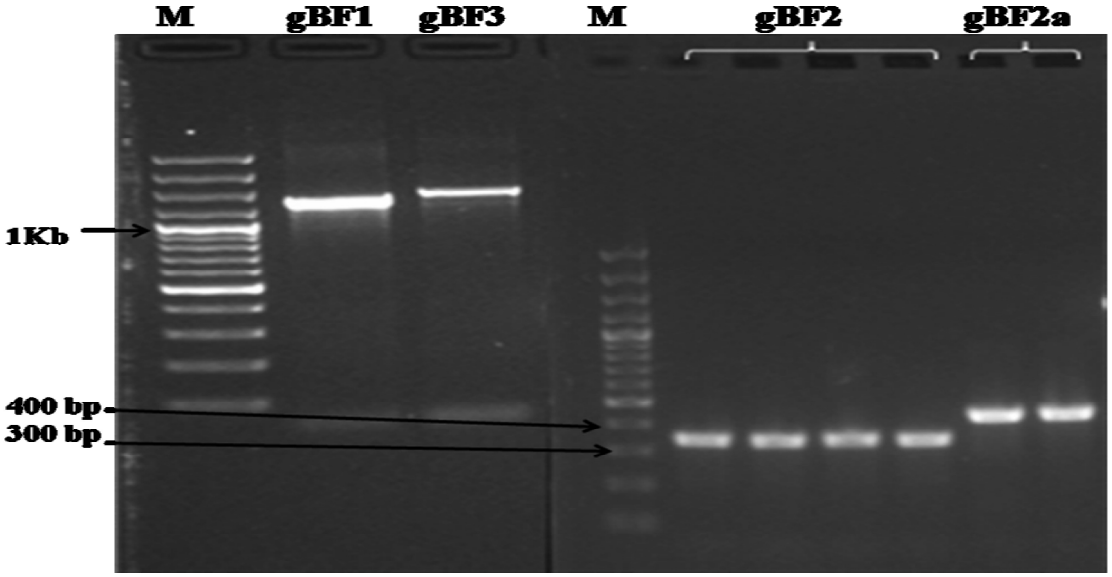
Table.4 Cyclic conditions for gBF1-F/gBF1-R, gBF3-F/gBF3-R

	Initial Denaturation	Denaturation	Annealing	Extension	Final extension
Temperature	95 °C	95°C	50°C	72°C	72°C
Time (min.)	5	1	1	1.5	10
No. Of cycle (s)	1		35		1

Table.5 Cyclic conditions for gBF2-F/gBF2-R, gBF2a-F/gBF2a-R

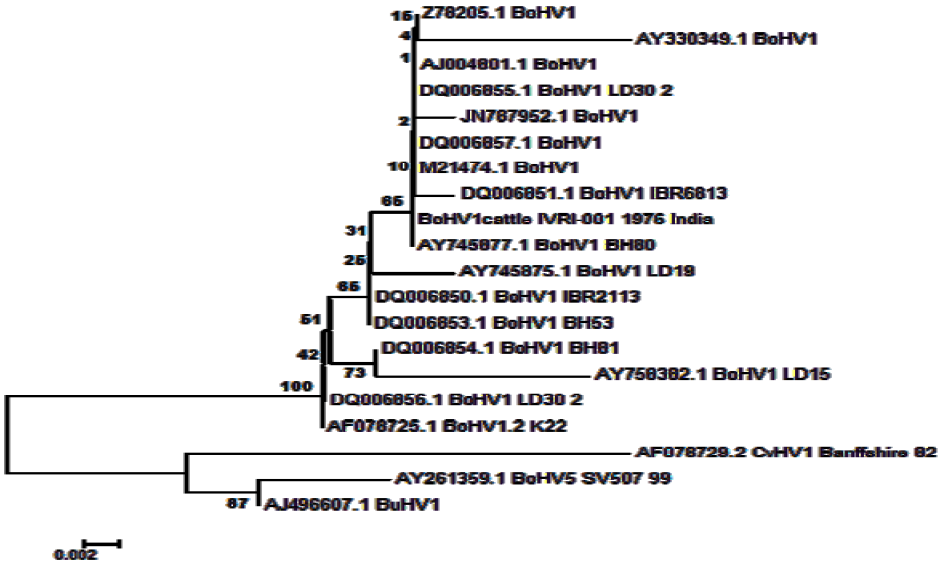
	Initial Denaturation	Denaturation	Annealing	Extension	Final extension
Temperature	95 °C	95°C	65°C	72°C	72°C
Time (min.)	5	1	1	1	10
No. Of cycle (s)	1		35		1

Figure.1 Result of colony PCR for confirmation of presence of amplified glycoprotein B gene insert using universal M13 primers



Lane 1 : 100 bp plus DNA marker; **Lane 2** : Colony PCR product (using M13 primer) of glycoprotein B fragment 1; **Lane 3** : Colony PCR product (using M13 primer) of glycoprotein B fragment 1. **Lane 4** 100 bp plus DNA marker; **Lane 5-8** : Colony PCR product (using M13 primer) of glycoprotein B fragment 2; **Lane 9-10** : Colony PCR product (using M13 primer) of glycoprotein B fragment 2a

Figure.2 Phylogenetic analysis of glycoprotein B gene of various isolates of BoHV1 with other related members of *Alphaherpesvirinae*



On agarose gel electrophoresis of PCR product, fragments gBF1, gBF2, gBF2a, gBF3 yielded expected product size of 1151 bp, 346 bp, 423 bp and 1265 bp respectively (Fig. 1). A total of 2514 bp out of 2799 bp of glycoprotein B has been amplified. Sequencing of the cloned product also confirms that the amplified fragments are for gB gene of BoHV1 (Sequencing was outsourced to Scigenome limited, Kochi). Genetic distance of gB gene of bovine herpesvirus 1/ IBR 216 II/ 1976/ India with other BoHV1 and other related groups of viruses was analysed using MegAlign 5.0 of DNASTAR and phylogenetic analysis by using MEGA 5 (Tamura *et al.*, 2011). Analysis of gB gene showed >99% identity with other BoHV1 isolates. Genetic divergence with other related groups *viz*. BoHV5, BuHV1 and CvHV1 are found to be 4.3%, 5.6% and 3.6%, respectively. Phylogenetic analysis of gB gene of bovine herpesvirus 1/ IBR 216 II/ 1976/ India with other BoHV1 and related groups of viruses revealed that all BoHV1 viruses are clustered within single clade. The cervid herpesviruses forming a separate cluster. Bubaline herpesviruses and bovine herpesvirus 5 are cluster together and forming a separate group.

Interestingly, deletion of 61 nucleotides from position 1490 to 1550 was observed in the BoHV1 used in the current study. Analysis of the missing nucleotides from reference sequences revealed that this portion contains high GC content (93.4%). The deletion of 61 nucleotides leads to a new amino acid sequence after this portion. Therefore, deduced amino acids analysis was not possible.The possible reason for missing nucleotides could be of polymerase slippage or frame-shift mutation. However, this requires further confirmation by repeating the sequence study of the gene with different passage of the virus. In the DNA virus, the fidelity of individual DNA replication complexes, whether of cellular or viral origin, is sufficiently high that records of polymerase slippages and frameshift alterations are rare. To expand or alter their limited coding potential, DNA viruses mainly appear to rely on the use of multiple, alternative splicing sites for processing

transcripts that then allow different proteins to be expressed from a single gene, as observed, for example, with papovaviruses and adenoviruses. A classical case of infidelity, however, in the DNA viruses involves the lysozyme gene of bacteriophage T4. This has been attributed to stuttering of a replication complex at short runs of A residues at two sites in the gene (Streisinger and Owen, 1985). Polymerase slippages that alter mini- and microsatellite sequences in DNA are also well documented (Debrauwere *et al.,* 1997) and, as seen with the RAD50 gene in colorectal cancer and gastric carcinomas, may be related to tumorigenesis (Klein *et al.,* 1967). Elsewhere, sequence slippages have been reported in clinical isolates involving herpes simplex virus type 2 (Jan-Åke *et al.,* 1999). Xue *et al.,* 2003 reported, multiple frameshifts in repetitive sequences within an Epstein-Barr virus unspliced early gene, LF3, which is associated with the viral replicative cycle and also transcriptionally expressed in many virally associated tumours. Detailed molecular and pathogenicity studies are required for understanding the deletion observed in the gB gene. The deletion was further confirmed with different set of primers for that region (Primers gBF2a-F/gBF2a-R).

Addition of glycerol is essential for amplifying GC rich portion of BoHV1 genome. Glycoprotein B gene of BoHV1 is highly conserved in all the isolates and it can be used as a target for designing of primers for diagnosis of IBR using PCR. As it is an immunodominant protein it can also serve as an ideal candidate of subunit vaccine production against BoHV1 infection. Deletion of 61 nucleotides at GC rich region of the gene was observed in this study after repeated amplification and sequencing of that portion. It may be due to polymerase slippage or frame shift mutation. Further study is warrant to confirm this deletion.

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