



Research Article

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

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ABSTRACT

Bovine herpesvirus 1 (BoHV1) causes infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis and infectious balanoposthitis in cattle and buffalo. It is also one of the main causative agents of bovine respiratory disease complex or shipping fever, which causes colossal economic losses due to productive and reproductive illnesses in infected animals. Glycoprotein B (gB) is one of the most important surface glycoproteins of BoHV1 by inducing strong neutralizing antibody responses and is the target of CD4⁺ T cells. There are only few partial sequences available for gB gene of Indian origin BoHV1. In this study, 2514 bp at carboxyl terminal of gB gene was amplified by using overlapping primers, which were cloned and subsequently sequenced. On *in-silico* analysis, it was found that glycoprotein B gene of Indian isolate of BoHV1 shared more than 99% sequence identity with other BoHV1 strains. The BoHV1-IBR 216 II shown 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. So we can conclude that the gB gene of bovine herpesvirus 1 is highly conserved among isolates, it can be used as a target for designing primer for detection of bovine herpesvirus 1. The deletion found in this study should be further confirmed by sequencing using multiple set of primers.

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INTRODUCTION

Infectious bovine rhinotracheitis (IBR) is an important viral disease of cattle and buffalo. It is caused by bovine herpesvirus 1. The virus belongs to family *Herpesviridae*, sub family *Alphaherpesvirinae* and genus *Varicelovirus*. Based on DNA restriction enzyme analysis, the virus can be divided into BoHV1.1 and BoHV1.2 subtypes. Conventional serological assays cannot distinguish between immune responses induced by subtypes of BoHV1. IBR is caused by BoHV1.1 and the disease is characterized by symptoms such as 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), and 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 assay, 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 and 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% CO₂. 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 number JX898220.1.1). Primers designed for amplification of glycoprotein B gene are presented in the table 1. The PCR was carried out in a 50 µL of reaction mixture. The components of the reaction mixture are presented in Tables 2 and 3.

The resulting mixture was subjected to a following precise thermal profile as initial denaturation of 95 °C for 5 minute denaturation at 95 °C for 1 minute, annealing at 50 °C at 1 minute for gBF1 and gBF3 and annealing temperature of 65 °C for gBF2 and gBF2a, extension at 72 °C for 1.5 minute for gBF1 and gBF3 and 1 minute for gBF2 and gBF2a. Final extension was given at 72 °C for 10 minute. Expected band size was visualized under transilluminator after running 5 µL of PCR product in 1% agarose gel. PCR-amplicons of expected size were gel purified using QIAquick® Gel Extraction Kit (Qiagen, USA) and were used for downstream applications.

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	

Sr. 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
4	Reverse Primer	1 µL
5	Template DNA	1 µL
Total		50 µL

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

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

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

Cloning and Sequencing

The gel purified PCR amplicon was cloned using InsTA cloning Kit (Fermentas, USA). In brief, 1 μ L of vector pTZ57R/T was mixed with 1 μ L 10X Ligation buffer, 4 μ L purified PCR product, 1 μ L T4 DNA Ligase and 3 μ L nuclease free water. The mixture was vortexed, centrifuged for 3–5 sec and incubated for 2.5 h at 22 °C followed by overnight at 4 °C. For transformation, 50 μ L of competent DH5 α from –80 °C was thawed on ice and mixed with 5 μ L of ligated gel purified 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 μ L of PBS and spread over LB agar containing ampicillin (50 μ g/mL), X-gal (30 μ g/mL) and Isopropyl β -D-1-thiogalactopyranoside (IPTG, 40 μ g/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 containing positive inserts were commercially sequenced (SciGenome, Cochin, Kerala).

Sequence trimming and editing of the raw sequence was carried out using Sequencer 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 induces a strong neutralizing antibody response (Babiuk et al., 1987) and is recognised by CD4⁺ helper T lymphocytes (Hutchings et al., 1990). It is also involved in the initial interaction of virus with cell namely attachment (Liang et al., 1991), penetration, cell fusion and cell-cell spread (Kopp et al., 1994).

To amplify some segment of gB gene, glycerol @ 5% 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).

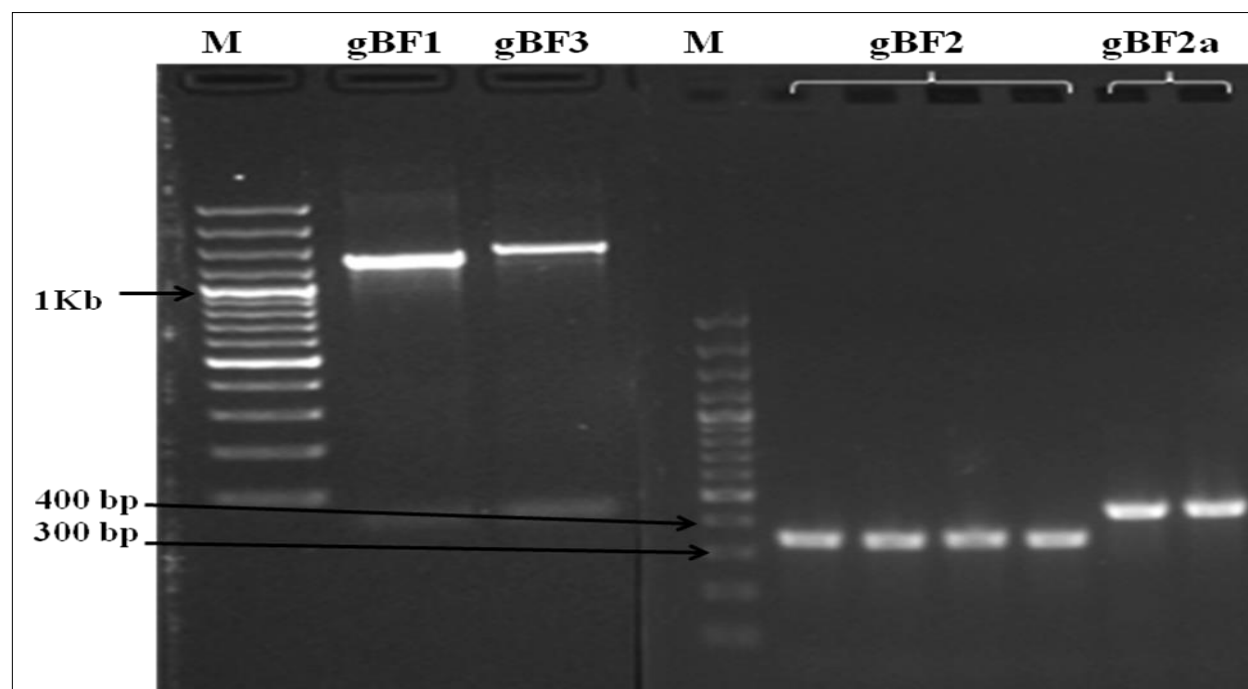


Figure 1: Results of colony PCR for confirmation of presence of amplified glycoprotein B gene insert using universal M13 primers; Lane 1 : 100 bp plus DNA marker; Lane 2: Colony PCR product (using M13 primer) of glycoprotein B fragment 1; Lane 3: Colony PCR product (using M13 primer) of glycoprotein 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

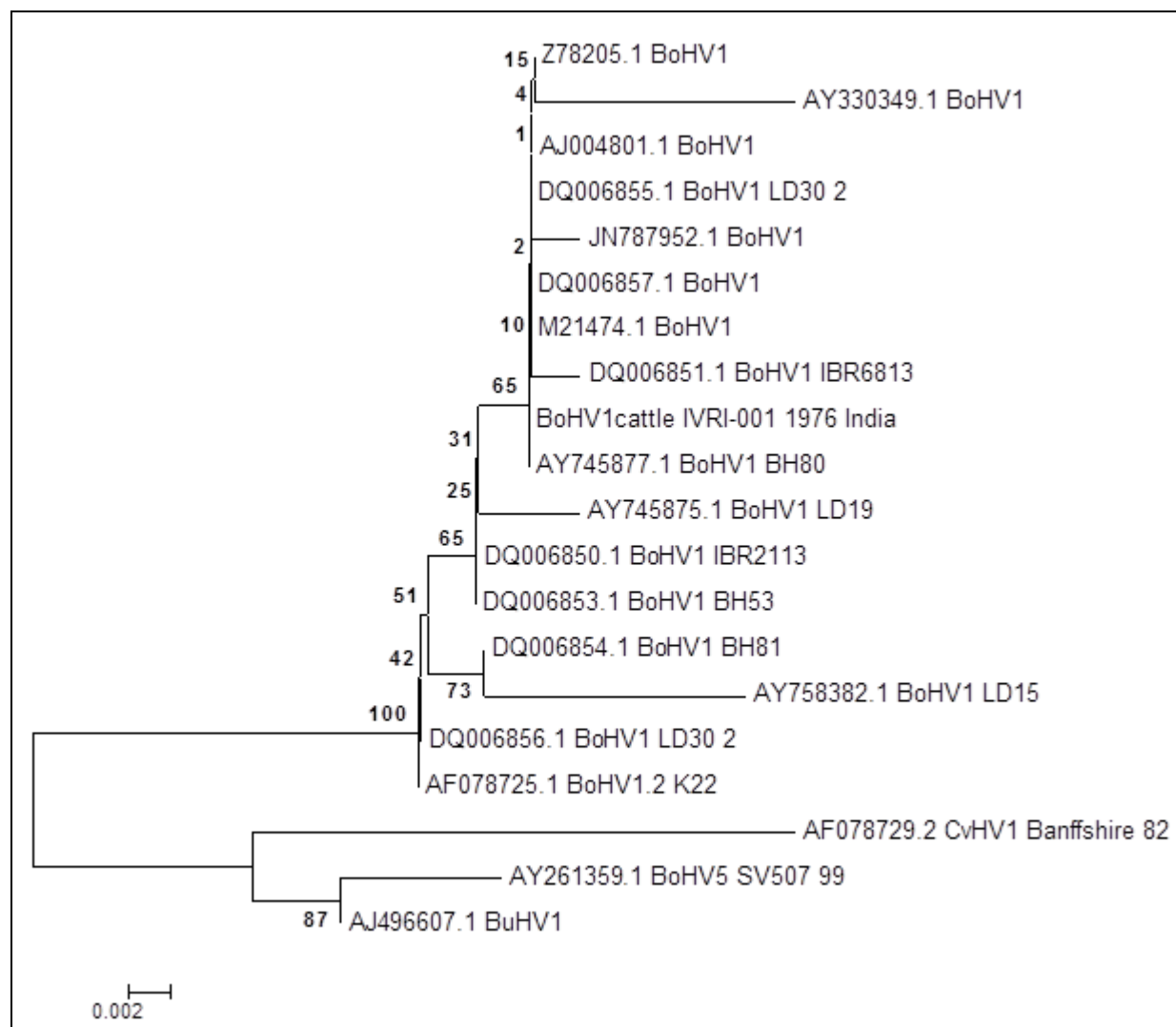


Figure 2: Phylogenetic analysis of glycoprotein B gene of various isolates of BoHV1 with other related members of *Alphaherpesvirinae*.

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/>). 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 (Figure 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.

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, however the cervid herpesviruses forming a separate cluster. Bubaline herpesviruses and bovine herpesvirus 5 were clustered together and formed 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. The deletion was further confirmed with different set of primers for that region (Primers gBF2a-F/gBF2a-R). Detailed molecular and pathogenicity studies are required for understanding the deletion observed in the gB gene.

CONCLUSIONS

This is the first genetic characterization of glycoprotein B gene (UL44) of Indian isolate of BoHV1. Addition of glycerol is essential for amplification of 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 studies are warranted to understand the functional implication of this deletion.

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