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RESEARCH ARTICLE
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Genetic characterization of complete open reading frame of glycoprotein C gene of bovine herpesvirus 1

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Abstract

Aim: To characterize one of the major glycoprotein genes viz., glycoprotein C (gC; UL44, unique long region 44) of bovine herpesvirus 1(BoHV1) of Indian origin at genetic and phylogenetic level.

Materials and Methods: A bovine herpesvirus 1 isolate viz., (BoHV1/IBR 216 II/ 1976/ India) maintained at Division of Virology, IVRI, Mukteswar was used for the current study. The DNA was extracted using commercial kit and the complete ORF of gC gene was amplified, cloned, and sequenced by conventional Sanger sequencing method. The sequence was genetically and phylogenetically analysed using various bioinformatic tools. The sequence was submitted in the Genbank with accession number Kc756965.

Results: The complete ORF of gC gene was amplified and sequenced. It showed 100% sequence homology with reference cooper strain of BoHV1 and divergence varied from 0% to 2.7% with other isolates of BoHV1. The isolate under study had divergence of 9.2%, 13%, 26.6%, and 9.2% with BoHV5 (Bovine herpesvirus 5), CvHV1 (Cervid herpesvirus 1), CpHV1 (Caprine herpesvirus 1), and BuHV1 (Bubaline herpesvirus 1), respectively.

Conclusion: This is the first genetic characterization of complete open reading frame (ORF) of glycoprotein C gene (UL44) of Indian isolate of BoHV1. The gC gene of BoHV1 is highly conserved among all BoHV1 isolates and it can be used as a target for designing diagnostic primers for the specific detection of BoHV1.

Key words: BoHV1, glycoprotein C, PCR, UL44.

Introduction

Bovine herpesvirus 1 (BoHV1), the causative agent of infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), infects mainly cattle (both domestic as well as wild) and buffaloes, but sheep and goats, swine, yaks, mithuns, mink and ferrets are also affected [1-3]. BoHV1 causes mainly three clinical syndromes in affected cattle and buffalos *viz*. infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), and infectious pustular balanopostitis (IPB) [4]. The virus may also cause mastitis, infertility, abortion and is an important causative agent of shipping fever [4].

The BoHV1 genome encodes 73 recognized open reading frames (ORFs) within a 135301 bp double-stranded DNA genome. Three subtype variants of BoHV1, designated BoHV1.1a, BoHV1.2a and BoHV1.2b, are recognized based on genomic DNA restriction endonuclease profiles and clinical observations [5]. 10 Ten genes code for glycoproteins and among them 6 are present in unique long (UL) region i.e. gK (UL53), gC (UL44), gB (UL27), gH (UL22),gM (UL10) and gI (UL1) and 4 are in unique short (US) region i.e. gG (US4), gD (US6),gI (Us7), gE (US8). Glycoprotein C (gC) is one of the major glycoproteins present in the envelope of virion and plasma membrane of virus infected cell [6]. It encodes a protein of 521 amino acids with 4 potential sites for addition of N linked oligosaccharide and a serine /threonine rich region (amino acid 32-92) for addition of O linked oligosaccharide. It induces neutralizing antibody response [6] and , is recognized by CD4+ and CD8+ T lymphocytes [6], and it is the major protein involved in attachment to heparin like receptor on tissue culture cells [7]. It was observed that residue 76 of BHV-1.1 gC is valine and residue 76 of BHV1.2 gC is glycine [8].

The disease was first reported by Mehrotra et al., in 1976 [9] . Since then the disease has been reported in several states. The disease was found to be more prevalent in exotic and crossbred cattle than in indigenous breeds. However, very few studies have been focussed on molecular characterization of Indian isolates of BoHV1. In fact, no complete sequence of any ORF/gene is available for Indian isolates.

The present study describes the complete sequences of gC genes of BoHV1 which was isolated in 1976 from calf suffering from keratoconjunctivitis [9].

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 10% Newborn Calf Serum (Invitrogen, USA). The cells were incubated at 37 °C in the presence of 5% CO2. A BoHV1 isolate *viz*., (BoHV1/IBR 216 II/ 1976/ India) maintained at Division of Virology, IVRI, Mukteswar was used for the present 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 (PBS) and thrice freeze-thawed (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 x 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 x g for 1min. After decanting the supernatant, about 600 µL of 70% ethanol was added and centrifuging at 13000 x 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): Oligonucleotides primers were designed based on sequence of the reference strain of BoHV1 (Genbank accession ID JX898220.1.1). Set of primers was designed for full length amplification of glycoprotein C gene (gC-F-5'-ATGGGCCCGCTGGGGCGAGC-3'; gC-R- 5'-CTACAGGCGCGCCCGGGCCTTG-3)'. The PCR was carried out in a 50 µL of total volume of reaction mixture containing 25 µL of 2X Maxima® Hot start Green PCR Master Mix (Fermentas, USA), 5 µL of glycerol (Amresco, USA), 1 µL of forward primer (0.2 µM final conc.), 1µL of reverse primer (0.2 µM final conc.), 1 µL of template DNA and nuclease free water to 50 µL. The resulting mixture was subjected to a precise thermal profile as follows: 95 °C for 5 minutes; 35 cycle at 95 °C for 1 minute, 55 °C for 1 minute, 72 °C for 1.5 minutes followed by one cycle at 72 °C for 10 minutes. Expected band size was visualized using ultraviolet transilluminator after running 5 µL of PCR product in 1% agarose gel. PCR amplicons of expected size was gel purified (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 nuclease free water was added to 10 µL. 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, 50µL 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 rpm for 2 min; the pellet was reconstituted in 50 µL of PBS and spreaded over Luria Bertani (LB) agar plate 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 and gene specific 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 4.9 (Gene Codes Corporation, MI, USA). References sequences includes BoHV1 isolates and other members of Alphaherpesvirinae were downloaded from GenBank. The gC gene sequence of the Indian isolate and references sequences were aligned using the MEGA 5 software [10]. The phylogenetic tree was constructed using neighbor-joining algorithm with Kimura-2 parameters correction and 1000 bootstrap replications.

Results and Discussion

To amplify complete ORF of gC gene, glycerol @ 5% has been added in PCR reaction. Without adding glycerol, no amplification could be obtained with different manufactures' enzymes 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 due to alteration in the melting point of the DNA template [11]. Several researchers used glycerol in PCR mixtures to enhance the speci? city and/or the yield of the PCR from high GC templates [12]. The G+C content of BoHV1 ranges from 71-72% [13]. 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/). The results are in accordance with the previous reports of use of glycerol for amplification of BoHV1 genes [12, 13].

On agarose gel electrophoresis of PCR product, an expected band size of ~ 1527 bp could be visualized (Figure-1). Sequencing of the cloned product also confirms that the length of the gC gene of the isolate studied is 1527 bases (Accession No. KC756965) as that reference Cooper strain. Sequence identity of the product was reconfirmed by blasting in NCBI blast. It

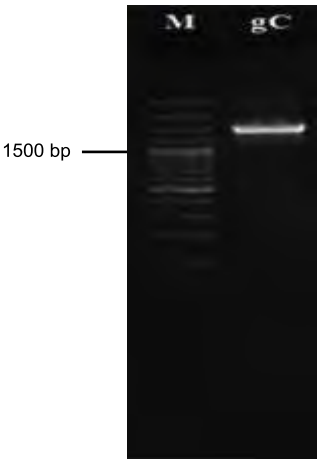

Figure-1. Result of colony PCR for confirmation of presence of amplified glycoprotein C gene insert using universal M13 primers.
Lane 1: 100 bp plus DNA marker
Lane 2: Colony PCR product

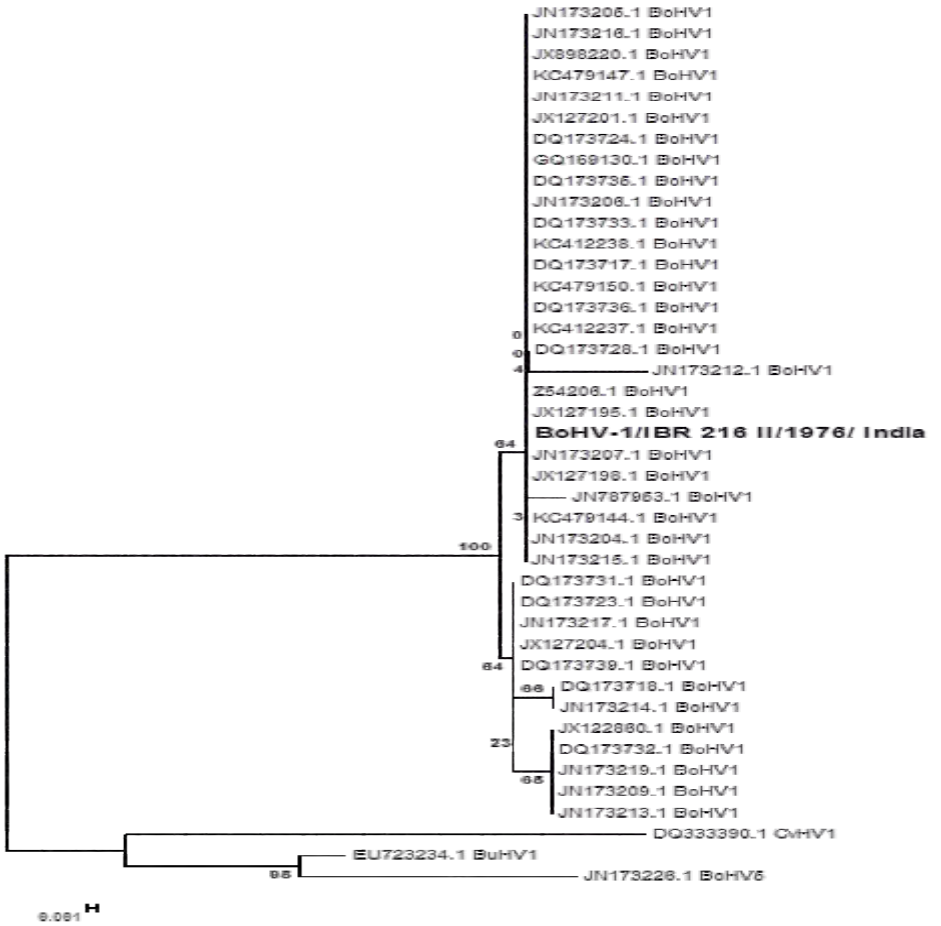

Figure-2. Phylogenetic analysis of glycoprotein C gene of various isolates of BoHV1 with other related members of *Alphaherpesvirinae*

showed 100% sequence homology with Cooper strain (Genbank accession ID JX898220.1.1) and divergence varied from 0% to 2.7% with other BoHV1 isolates. The Indian isolate had divergence of 9.2%, 13%, 26.6%, and 9.2% with BoHV5, CvHV1, CpHV-1, and BuHV1, respectively.

In the deduced amino acid analysis, Indian isolate of the current study has valine at position 76 of gC gene. It was reported that the amino acid position 76 of gC is an marker for differentiating BoHV1.1 (valine) and BoHV1.2 (glycine) [8]. In the present study, available gC gene sequences of all BoHV1 isolates were downloaded and multiple alignment was carried out. At nucleotide position 1104, all the BoHV1 subtype 1.1 isolates had T whereas BoHV subtype 1.2 isolates had C at this position. Based on the above observations, the Indian isolate under study was classified under subtype BoHV1.1. In this isolate four N- glycosylation sites were present at amino acid position 93, 111, 164 and 208 which is in accordance with previous reports [14]. The N-glycosylation of viral glycoprotein plays an important role in stability, antigenicity and host cell invasion of the virus [15].

Phylogenetic analysis of gC gene revealed that all BoHV1 isolates including under study are clustered within single clade. The CvHV1, CpHV1, and BoHV5 viruses formed individual clusters with respective viruses. In conclusion, this is the first genetic characterization of complete ORF of glycoprotein C gene (UL44) of Indian isolate of BoHV1. This study will lay foundation stone for further studies on BoHV1 in respect of development of vaccine or new diagnostics.

Conclusion

This is the first genetic characterization of complete ORF of glycoprotein C gene (UL44) of Indian isolate of BoHV1. Addition of glycerol is essential for amplifying GC rich portion of BoHV1 genome. Glycoprotein C 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.

Authors' contributions

SM, ABP and MAR implemented the study design. SM and MAR carried out the work and analyses part. All authors drafted and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests

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