Glycoprotein C Gene Based Molecular Subtyping of a Bovine Herpesvirus -1 Isolate from Uttar Pradesh, India

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Abstract Bovine herpesvirus -1 (BHV-1) is the etiological agent of many clinical syndromes in cattle which causes huge economic losses to the animal husbandry sector annually. Since the first report of its presence in India in 1976, the disease is considered to be endemic in the country. In the present study, a case of keratoconjunctivitis in a cow was investigated to find out the underlying cause of the condition. The clinical material (ocular swab) was tested by BHV-1 glycoprotein D gene specific PCR using in house designed primers and found to be positive by the presence of a 212 bp DNA product in agarose gel electrophoresis. The virus was isolated in MDBK cell line in the third passage and the serum from the animal, was positive for antibodies against BHV-1 by ELISA. A 575 bp segment of the glycoprotein C gene of the isolate was amplified by PCR, cloned and sequenced. On phylogenetic analysis, it was seen that the sequence matched with published BHV-1.1 sequences from USA and Uruguay whereas it was divergent from Brazilian BHV-1.1 isolates. This study highlights the isolation, rapid and sensitive detection of BHV-1 virus from clinical cases and its subtyping by nucleotide sequencing and subsequent phylogenetic analysis which gives invaluable information about the molecular epidemiology of BHV-1 subtypes prevalent in the country.

Keywords Bovine herpesvirus 1 · Isolation · Phylogeny · Subtype 1.1 · India

Bovine herpesvirus-1 (BHV-1), is the causative agent of a variety of disease syndromes in cattle, including rhinotracheitis (infectious bovine rhinotracheitis/IBR), vulvovaginitis (infectious pustular vulvovaginitis/IPV), balanoposthitis (infectious pustular balanoposthitis/IPB), conjunctivitis, abortion, enteritis and a generalized disease of newborn calves. BHV-1 belongs to the genus *Varicellovirus* and subfamily *Alphaherpesvirinae* of the *Herpesviridae* family. The 135.3 kbp viral genome is a single, linear double stranded DNA molecule within the protein capsid of approximately 125 nm diameter. Surrounding the capsid is a layer of globular material, known as the tegument, which is enclosed by a typical lipoprotein envelope with numerous small glycoprotein spikes [12]. BHV-1 genome codes for 33 structural proteins, 13 of which are probably associated with the envelope [10] and ten of these have the potential to encode glycoproteins [30]. The virus can be differentiated into subtypes 1.1 responsible for causing IBR and 1.2 further subdivided into 1.2a and 1.2b responsible for causing IPV/IPB [17]. With the exception of few countries free from BHV-1, the virus is distributed worldwide. The virus has been eradicated from some countries of Europe whereas in others like Germany and Italy control programs are implemented [32]. Though an infection with BHV-1 does not cause high mortality among the affected animals, the economic loss due to reduced production, impaired work ability, abortion etc. is enormous.

In India, IBR was first reported in 1976 and the disease is endemic in the country and outbreaks have been reported from almost all the states [2, 7, 9, 15, 27, 29]. In India, there are only few reports on the isolation of BHV-1 from clinical cases [14–16, 18, 24] and semen samples [1, 3]. However, reports on molecular characterization, including subtyping, of the virus from clinical cases of IBR are

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limited [24]. This report deals with the isolation of BHV-1 from a case of keratoconjunctivitis in a cow and its molecular characterization up to subtype level by sequencing of a part of viral glycoprotein C (gC) gene.

The clinical material was an ocular swab collected from a cow with unilateral keratoconjunctivitis and profuse serous lacrimal discharge in a farm in Uttar Pradesh state, India. The swab was transported in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, USA) with 2 % fetal bovine serum (FBS) (Life Technologies, USA) and under chilling conditions to the Virus Laboratory, Centre for Animal Disease Research and Diagnosis (CADRAD), Indian Veterinary Research Institute, Izatnagar, India for virus isolation. Blood samples were also taken in sterile vacutainers (Becton–Dickinson Biosciences, USA), allowed to clot, serum separated by centrifugation and stored at -20°C until tested.

The presence of BHV-1 genomic DNA in the clinical sample was tested by polymerase chain reaction (PCR) targeting a portion of the glycoprotein D (gD) gene of the virus. The DNA was extracted from the clinical material by the sodium dodecyl sulphate (SDS)–proteinase K–phenol chloroform method. Briefly, 200 μl of transport medium which contained the ocular swab was taken and was mixed with 20 μl of 10 % SDS and 10 μl of proteinase K (20 mg/ml) in a microfuge tube and incubated at 60°C for 1 h. After the incubation, DNA was extracted from the mixture employing standard procedure [26].

A set of primers which amplified a 212 bp portion of the gD gene of BHV-1 were in house designed using Primer Select 5.00 software (DNASTAR Inc., USA) and custom synthesized by M/s Eurofins, Bangalore. The sequences of the forward and reverse primers were 5'GACGAGCTGG GACTGATTA3' and 5'CGCAGAACCTTCTTTTGCTC3' (corresponding to nucleotide positions 119361–119379 and 119572–119553 of BHV-1 complete genome accession number AJ004801) respectively. The PCR master mix consisted of 2.5 μl of $10\times$ PCR buffer, 1.5 μl of 25 mM MgCl_2 , 1.0 μl of 10 mM dNTP, 1.0 μl each of 10 mM forward and reverse primers, 0.5 μl of 1.0 U/ μl *Taq* DNA polymerase, 12.5 μl of NFW to which 5 μl of purified template DNA was added. The PCR was carried out in a thermal cycler (Applied Biosystems, USA) and the cycling conditions were initial denaturation at 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s, and final extension at 72°C for 5 min. Total DNA extracted from a Madin Darby bovine kidney (MDBK) cell line adapted BHV-1 isolate (isolate 216) maintained in our laboratory was used as the positive control.

The ocular swab was processed for virus isolation following the standard protocol described by the World Organization for Animal Health [32]. One milliliter of the

processed sample was inoculated on to confluent MDBK cell line in a vented 25 cm^2 tissue culture flask and kept for virus adsorption for 2 h. After the adsorption, the inoculum was poured off and the cell monolayer washed with maintenance medium (DMEM containing 2 % FBS and 50 $\mu\text{g}/\text{ml}$ gentamicin) (Life Technologies, USA). After washing, 5 ml of maintenance medium was added and the flask was incubated at 37°C under 5 % CO_2 tension in a CO_2 incubator (Heal Force, China) and observed daily for 7 days for appearance of cytopathic effect (CPE). MDBK cells without sample inoculation was also incubated as control. If no CPE was observed after 7 days of incubation, the cell culture was freeze thawed three times and clarified by centrifugation. One milliliter of the supernatant obtained after the centrifugation was used as the inoculum for fresh MDBK cells.

The serum sample was tested for antibodies to BHV-1 by using an Indirect ELISA (I-ELISA) kit (Svanovir® IBR-Ab, Svanova Veterinary Diagnostics, Sweden) following the protocol supplied by the manufacturer. After the test, the plate was read at 450 nm in ELISA reader (Thermo Labsystems, USA). The percentage positivity (PP) was calculated using the formula $\text{PP} = (\text{OD of test sample})/(\text{OD of positive control}) \times 100$. Serum samples with a percentage positivity of <25 were considered as negative and those with ≥ 25 were positive.

To ascertain the subtype of the BHV-1 isolate, a 575 bp portion of gC gene was amplified by PCR, cloned and sequenced. The PCR was performed using forward primer (5'CGGCCACGACGCTGACGA3') and reverse primer (5'CGCCGCCGAGTACTACCC3') (corresponding to nucleotide positions 16763–16780 and 17337–17320 of AJ004801 respectively) described by Esteves et al. [6]. The 575 bp PCR product was purified by using GeneJET gel extraction kit (Fermentas, Lithuania) and the purified PCR product was cloned in pJET1.2/blunt vector by using CloneJET PCR cloning kit (Fermentas, Lithuania). The recombinant plasmids containing the 575 bp insert were sequenced by M/s Eurofins Genomics India Private Limited, Bangalore.

BLAST analysis was performed on the nucleotide sequence data obtained within the non-redundant nucleotide database (<http://www.ncbi.nlm.nih.gov/Blast>) to confirm the presence of the gene specific to BHV-1. BHV-1.1, BHV-1.2 and BHV-5 gC sequences from different countries were retrieved from the NCBI nucleotide database. Phylogenetic comparison of a 451 nucleotide portion (corresponding to positions 16825–17275 of AJ004801) of the sequence obtained was performed using MEGA4.0 software [31]. The evolutionary history was inferred using the Neighbor-Joining (N-J) method [25]. The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the isolates analyzed.

The ocular swab sample was tested positive in the first round of PCR as evidenced by a 212 bp DNA band in the

Fig. 1 Madin Darby bovine kidney cells inoculated with bovine herpesvirus-1 suspected sample showing characteristic cytopathic effects such as rounding and clumping of cells to form grape like clusters

agarose gel electrophoresis. The specific DNA band was observed in the positive control also. By 48 h of inoculation in the third passage, CPE was observed in the cell culture inoculated with the processed clinical sample. The CPE was characterized by rounding and clumping of cells to form grape like clusters (Fig. 1). The control cells remained healthy during the period. The infected flask was harvested by freezing at -20°C 24 h after onset of CPE. The serum sample taken from the affected animal had a PP value of 52.08 in indirect ELISA.

On analysis of the extracted DNA by BHV-1 gC gene specific PCR, an expected amplicon of 575 bp was visualized on agarose gel in the suspected sample and in the positive control. The product was cloned into pJET1.2/blunt vector and the nucleotide sequence of the insert obtained by sequencing. BLAST analysis of the nucleotide sequence confirmed that the isolate was of BHV-1 and the sequence has been deposited in GenBank (Accession number

JX127195). On phylogenetic analysis of a 451 nucleotide portion of the sequence, it was seen that the isolate was of BHV-1.1 subtype by virtue of its clustering with other BHV-1.1 sequences taken for analysis. The percentage identity of the isolate with the closely clustered BHV-1.1 isolates (T3, Uy1999 and Cooper) was 100 (451 out of 451) and that with the divergent BHV-1.1 cluster (PG2560 and EVI 123) was 99.7 (450 out of 451). This close clustering of the isolate under study with T3, Uy1999 and Cooper was supported by a relatively high boot strap value of 62. BHV-1.2 sequences formed a separate branch in the phylogenetic tree while BHV-5 formed the outgroup (Fig. 2).

BHV-1 subtypes 1.1 and 1.2 usually infect respiratory and genital tract of cattle, however, it has been suggested that each subtype is better adapted to either respiratory (BHV-1.1) or genital (BHV-1.2) tract [4, 23, 28]. The BHV-1.2 subtype is considered to be less virulent than subtype 1.1 [5]. In the present study it was seen that the case of keratoconjunctivitis was caused by BHV-1.1. The ocular form is more often seen as a sequelae of the respiratory form of the disease. Further, there was seroconversion in the animal indicating that primary infection had occurred sometime back probably as a respiratory infection which had subsequently spread to the eye.

Previously, differentiation of BHV-1 into subtypes was carried out based on restriction enzyme analysis of purified virus DNA [17] or by analyzing epitopes on gC [23]. With the advent of PCR and nucleic acid sequencing, there are many reports from other countries on the use of this technique for determination of subtypes of BHV-1 [6, 11]. In the present study, the BHV-1 isolate was confirmed to be a 1.1 subtype based on PCR amplification of part of gC gene and its nucleotide sequence analysis. The present isolate was having 100 % nucleotide sequence homologies with BHV 1.1 isolates from USA (Cooper) and Uruguay (T3 and Uy1999) but it showed divergence from the Brazilian isolates (PG2560 and EVI 123) as they formed a

Fig. 2 Neighbor joining tree constructed from a 451 nucleotide portion (corresponding to positions 16825–17275 of AJ004801) of glycoprotein C gene of the bovine herpesvirus-1 field isolate and published bovine herpesvirus-1.1, 1.2 and bovine herpesvirus-5 isolates

separate branch with respect to the present isolate. While generating the phylogenetic tree, local isolates could not be incorporated as there are no gC gene sequences of known subtype from India available in public databases. The present work has succeeded in generating invaluable information about the type of BHV-1 isolates circulating in India which will help in studying the molecular epidemiology of BHV-1 in bovines in future. However, to gain a more clear picture about the subtypes of isolates circulating in the country, more number of isolates from different parts of the country, especially BHV-1 strains isolated from abortions, should be sequenced and subtyped.

Since the first report of IBR in India in 1976, there have been various reports of seroprevalence of BHV-1 in cattle and buffaloes [13, 19, 20, 22] from different parts of the country indicating its endemicity. Our findings also support the fact that cases of clinical IBR are still occurring in the field conditions. Besides the many clinical manifestation of the disease caused by BHV-1, two important aspects of BHV-1 infection are latency and, shedding of virus in semen. Stress conditions, and corticosteroid treatment can activate the virus in latently infected animals, resulting in re-excretion of virus [8, 21].

As the virus is known to be spread through semen, breeding bulls used for the natural insemination should be free from BHV-1 infection. Again, semen used for artificial insemination should be negative for presence of BHV-1. The Government of India has recently taken a decision that all the semen samples should be tested for presence of BHV-1 and other viruses and bacteria causing reproductive disorders by PCR before being used to inseminate cows in different states in the field condition. Factors such as unrestricted movement of animals, procurement of animals without proper screening, absence of quarantine before entry to the main herd and lack of prophylactic measures lead to introduction and spread of BHV-1 in a herd. Hence proper managerial procedures and routine screening for the prevalence of the disease in herds should be conducted with provision to separate the diseased animals so that the healthy animals do not pick up the infection. In India, there is no national policy to vaccinate animals against BHV-1 and hence good farm managerial practices and biosecurity measures will definitely help in reducing the incidence of the disease and the associated economic losses due to diminished productivity and reproductive performance.

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