ISOLATION AND MOLECULAR CHARACTERIZATION OF BOVINE HERPES VIRUS-1 BY POLYMERASE CHAIN REACTION


Department of Veterinary Microbiology, Veterinary College, Hebbal, Bangalore– 560 024, India

Received: 28-05-2012 Accepted: 05-02-2013

ABSTRACT

The present study was undertaken to isolate the Bovine Herpes virus-1(BHV-1) from cattle and buffaloes suffering from Infectious Bovine Rhinotrachitis (IBR) and confirmed by polymerase chain reaction (PCR) and compare it with the standard isolate of BHV-1. A total of 40 nasal and conjunctival swabs were collected from cattle and buffalo from different districts of Karnataka. Isolation of BHV-1 was carried out in Madin Darby Bovine Kidney (MDBK) cell lines. Out of 40 samples collected, three virus strains were isolated from three samples with one each from Mandya, Hassan, and Bangalore district. The virus isolates were confirmed and characterized by PCR using set of designed primers targeting Glycoprotein C (gC) gene of BHV-1 followed by restriction endonuclease (RE) analysis and sequencing.

Key words: BHV-1, IBR, Isolation, PCR Characterization.

INTRODUCTION

Bovine herpesvirus 1 (BHV-1), a member of the family Herpesviridae, subfamily Alphaherpesvirinae, genus Varicellovirus, is the causative agent of several infections of the respiratory and genital tracts of domestic cattle and other ruminant species. Apart from respiratory disease, this virus can cause other clinical syndromes such as infectious pustular vulvovaginitis or balanoposthitis, conjunctivitis, encephalitis and generalized systemic infections. In India the first case of IBR was reported by Mehrotra et al. (1976) by isolating the virus from Keratoconjunctivitis form of the disease in an organized farm of Uttar Pradesh. The viral genome consists of double stranded DNA that codes for about 70 proteins based on Western Blot analysis of viral proteins, restriction endonuclease analysis of the viral nucleic acid and differential amplification by PCR. Bovine Herpes Virus -1 strains are classified into two subtypes, BHV-1.1 and BHV-1.2. The glycoprotein C (gC) of BHV-1 is a major viral attachment protein (Okazaki, et al, 1987) that interacts with a cellular heparin like moiety (Okazaki, et al, 1994). It is a 91 kD MW molecule containing both N-linked and O-linked oligosaccharides (Van, et al, 1986) and is a member of the immunoglobulin superfamily (Fitzpatrick, et al, 1989).

Diagnosis of BHV-1 infection can be difficult for a variety of reasons. BHV-1 like other herpes viruses can establish a latent state in ganglionic neurons post infection and the subclinical infections are common. Of the several laboratory methods available for BHV-1 detection, PCR represents an excellent tool for the fast and very sensitive detection of virus in biological and clinical specimens. Various PCR assays for the detection of BHV-1 have been described (Kibenge et al., 1994 and Vilcek et al., 1994).

MATERIALS AND METHODS

Collection and processing of the samples: Collection and processing of the samples and isolation of the virus was done as the method described in OIE, 2008. Nasal and conjunctival swab samples were taken from the cattle and buffaloes which showed symptoms similar to IBR in viral transport medium i.e. Eagle’s Minimum Essential Medium (EMEM).
**Reference Virus:** Standard Reference Virus was procured from Project Directorate, Animal Disease Monitoring and Surveillance (PD-ADMAS), ICAR, Hebbal, Bangalore.

**Isolation of the virus:** In the laboratory, the swabs were agitated in the transport medium to elute virus and left at room temperature for 30 minutes. The swabs were removed and supernatants were filtered through 0.22 μm filters and a 100 μl volume of supernatant from the processed swabs was inoculated into monolayer of MDBK cell culture (tissue culture flask of 25 ml volume) and incubated at 37°C for 1 hour. After 1 hour adsorption, the cells were rinsed, maintenance medium was added and the cultures were subsequently incubated at 37°C in an incubator with 5% CO2.

The inoculated cell cultures were observed daily for CPE under inverted microscope. CPE was characterized by grape like clusters of rounded cells gathered around a hole in the monolayer. In cases, where no CPE was observed within 5 days, the inoculated cell culture was frozen and thawed thrice, the suspension was used for inoculation into fresh monolayer cell culture. In case of no CPE after 5 days, the sample was regarded as negative for BHV-1.

**DNA extraction:** DNA extraction from the samples showing CPE was carried out as per the manufacturer’s protocol. A 200 μl volume of cell culture supernatant was taken and DNA was isolated using QIAamp DNA Mini Kit (Qiagen Pvt. Ltd.) and final elution of DNA was done in 50 μl of elution buffer and stored at -20°C for long term use.

**Polymerase Chain Reaction:** Primers were designed targeting gC gene by semi manual method, using primer designing tool so as to amplify a product of 578 bp. The sequence of primers was selected from conserved region of gC sequence (Fitzpatrick et al., 1989) with ORF sites. The primer sequences were, gC F (560 – GAA GGA GCG CAA GTG GAT GCT CT- 583) and gC R (1138-CGG AGT CGT CGA CCG TAA AGA CGT-1115). The specificity was checked by blasting the primer sequences in NCBI blast and synthesized by Chromous Biotech. The primers were used for detection of BHV-1 directly in field samples as well as in cell cultures.

**Conditions of PCR:** PCR was carried out in a final reaction volume of 25 μl using a 200 μl capacity thin wall PCR tube (Axygen). PCR was performed in a total volume of 25 μl containing 2.5μl of 10x PCR buffer, 2.5 μl of MgCl₂, 1 μl of DNTP, 1 μl of 10 pmol/μl of each primers and Taq DNA polymerase, 5 μl of extracted DNA and 11 μl of DNase free water. The amplification was performed in a thermal cycler (MasterCycler Gradient, Eppendorf, USA). Thermal cycling conditions consisted of an initial denaturation step at 94°C for 15 minutes, followed by 34 cycles of 94°C for 50sec, 65°C for 50 sec, 72°C for 50 sec and a final extension at 72°C for 10 minutes. The negative control consisted of sterile water instead of DNA template while positive control consisted of DNA extracted from reference virus spiked with neat semen. After amplification the reaction mixture was electrophoresed in 2.0 % agarose gel, stained with ethidium bromide. The amplified product was visualized as a single compact band of expected size under UV light and documented by a gel documentation system (Syn Gene, Gene Genius Biolmaging System, UK). A clear, compact band of 578 bp was regarded as a positive for BHV-1.

**Purification of gel cut product for sequencing:** Twenty microlitres of the PCR-amplified products of representative field sample was electrophoresed (60 mV for two hour) along with 100 bp DNA molecular weight marker (GeneRuler, MBI Fermentas) on a 2 % (W/ V) low melting point agarose gel in 1X TAE and stained with ethidium bromide (0.5 μg/ml of 1X TAE buffer). The DNA band of interest (578 bp) was visualized on a UV Trans-illuminator. Using a sharp razor blade, a slice of agarose containing the bands of interest was cut out and transferred into a clean microcentrifuge tube. Later the gel cut was purified by using QIAquick gel extraction kit as per the instructions of manufacturer and the eluted product was stored at -20°C for restriction enzyme analysis and also to send it for sequencing.

**Restriction enzyme analysis:** A 7 μl volume of gel purified product was treated with 1 μl of RE enzyme SAC-1 (GAGGTC ,117/113) single cutter and 2 μl of SAC-1 buffer (Fermentas). This enzyme was chosen from various restriction enzymes for BHV-1 from nebul cutter website. The amplified product was sequenced on a 3130 Genetic Analyzer (ABI) (Mwgagboitech) and the result obtained was compared with the standard BHV-1 strain of PD-
ADMAS, Bangalore to assess any change in the targeted gC gene of BHV-1.

RESULTS AND DISCUSSION

Three BHV-1 strains were isolated from three samples including one each from Mandya, Hassan and Bangalore districts out of 40 nasal and conjunctival swabs screened. The isolated virus strains showed characteristic ballooning or bunch of grapes like CPE in MDBK cell lines.

From the BHV-1 positive samples, DNA was isolated and PCR was done using a designed primer targeting conserved region of gC region. All the three isolates were amplified in the region between 550-600 bp indicating the presence of BHV-1 in the samples (Fig 1). The restriction enzyme Sac-1 cleaved the amplified and purified product at 117 bp and band was seen at 117 and 461 bp respectively (Fig.1.1). The result of sequencing of gel extracted product indicated the matching of the product with the 578bp of standard BHV-1 genome.

Of the three isolates, two were from lactating cows and one from a buffalo calf. The lesser number of isolates from nasal swabs was probably due to the low concentration of virus excreted from the respiratory route. This may also be due to very low adaptability of BHV-1 in the cell culture system. The number of isolates could have been increased if repeated swabs would have been taken from the suspected animals or if swabs would have been taken during stress period of animal. Since maximal virus replication and shedding occurs between the third and sixth days which is early acute phase of the disease, swabs for virus isolation should be taken early in the course of disease when the discharge is serous rather than mucopurulent (OIE, 2008).

Bovine Herpes virus-1 was detected in nasal swabs of cattle earlier by other workers (Mehrotra et al., 1976; Mohankumar and Rajasekar, 1994; Rola et al., 2005). In India, the seroprevalence of IBR was estimated to be as high as 41.23% in 2003-04 (Annual Report, PD-ADMAS, C-2004). Isolation of three isolates of BHV-1 from cattle and buffaloes is further confirmed the prevalence of the disease in Karnataka.

![FIG. 1. Agarose gel electrophoresis pattern of PCR amplified product of BHV-1 gC gene (578 bp).](image)

![FIG. 1.1. Restriction enzyme analysis of DNA from BHV-1 strains with SAC-I.](image)

REFERENCES


