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Isolation and Molecular Characterization of Bovine Herpes Virus Type 1 from Upper Respiratory Tract of Bovines

Rakhi Gangil, Gurpreet Kaur, P.N. Dwivedi

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ABSTRACT

Background: Bovine herpes virus type 1 (BoHV-1) the causative agent of Infectious bovine rhinotracheitis (IBR) is of great concern to dairy farmers and veterinarians due to great economic impact caused by the virus in terms of loss of production and abortion. Therefore, the study was planned to detect the virus circulating in the bovine population of the region under study. This virus is an important pathogen of bovine respiratory diseases. The aim of the present study was to isolate the BoHV-1 virus from the upper respiratory tract of bovines.

Methods: A total of 13 nasal swab samples were subjected to virus isolation in Madin Darby Bovine Kidney (MDBK) cells lines. A PCR assay was applied to confirm the BoHV-1 DNA by targeting *gI* glycoprotein gene in isolates.

Result: Total two IBR virus isolates were recovered from 13 nasal swab samples of bovines. Both isolates exhibited cytopathic effects *i.e.* clumping and rounding of cells. A 468 base pair of amplified product from both isolates confirmed the IBR virus in *gI* gene specific PCR for BoHV-1. This study concludes that IBR virus exists among cattle population of Punjab and it is present in the upper respiratory tract of infected animal and shed through respiratory route. The PCR detection assay for detection of BoHV-1 from nasal swab samples is considerably more sensitive than virus isolation.

Key words: Bovine, BoHV-1, Isolation, Upper respiratory tract, PCR.

INTRODUCTION

Infectious Bovine Rhinotracheitis (IBR) is caused by Bovine Herpesvirus -1 (BoHV-1) a member of the genus *Varicellovirus* in the sub family *Alphaherpesvirinae* which belongs to the family *Herpesviridae* (Murphy *et al.,* 1999) and newly assigned order *Herpesvirales* (MacLachlan and Dubovi 2011). BoHV-1 is one of the most important pathogens of respiratory disease complex called shipping fever. IBR is an economically important viral disease of domestic and wild cattle. It has been described as an acute, contagious, febrile infection of cattle, characterized by an intense inflammation of the upper respiratory passages and trachea. It is accompanied by dyspnea, depression, nasal discharge and loss of condition. Besides respiratory infection, BoHV-1 is associated with ocular, reproduction, central nervous system, enteric, neonatal and dermal infections of cattle and other species (Ata *et al*., 2008). The disease has been reported not to be highly fatal but it causes considerable economic loss due to abortion, loss of body condition, reduction of milk yield, temporary failure of conceptions, secondary bacterial pneumonia and cost of treatments.

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. Subsequently, isolation of virus from the cases of abortion in cows was also reported (Mehrotra, 1977). Serological investigations, thereafter, have indicated the prevalence of IBR infection in alarming proportion in most of the states of India (Patil *et al.,* 2017). The disease was found to be more prevalent in exotic and crossbred cattle than in indigenous breeds. The disease has been recorded from Kerala (Sulochana *et al.,* 1982), Gujarat (Singh *et al.,* 1983), Tamil Nadu (Manickam and Mohan, 1987), Orissa (Mishra and Mishra, 1987) Andhra Pradesh (Satyanarayana and Suri Babu, 1987), Karnataka (Mohankumar *et al.,* 1994), Uttar Pradesh (Pandey *et al.,* 2000) and West Bengal (Ganguly *et al.,* 2008).

BoHV-1 infection generally occurs above 6 month of age when maternal immunity has waned (Bennet and Ijpelaar, 2003). The virus is excreted through various routes *viz*., nasal and ocular secretions, placenta of aborted animals and most importantly through semen (Jithin *et al.* 2019). Further the BoHV-1 virus remains latent in the neurons of trigeminal ganglia in case of respiratory infection and sacral ganglion after genital infection for the entire life in the host (OIE, 2008). Any stressful condition such as parturition, transportation, high ambient temperature (in case of pure and crossbreeds), high milk yield and artificial stress cause reactivation of the latent virus and consequently intermittent

Department of Veterinary Microbiology, Guru Angad Dev Veterinary and Animal Science University, Ludhiana-141004, Punjab, India.

Corresponding Author: Rakhi Gangil, Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Mhow-453 446, Madhya pardesh, India. Email: rgangil@gmail.com

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shedding of virus into the environment which is a potent source of infection to other healthy animal (Radostits *et al.* 2000 and OIE, 2008).

Current methods of BoHV-1 detection used in diagnostic virology laboratories include virus isolation, ELISA and PCR. Diagnosis of BoHV-1 virus can be difficult due to latency of virus in ganglion. Various PCR assays for the detection of BHV-1 have been described (Kibenge *et al.,* 1994 and Vilcek *et al.,* 1994). Many researchers have been made the isolation of IBR virus from different areas (Rangnatha *et al.,* 2013, Patil *et al.,* 2016, Ravishankar *et al.,* 2012 and Surendra *et al.,* 2015).

In India no routine vaccination against Infectious Bovine Rhinotracheitis (Bovine Herpes virus type-1) is undertaken in cattle till now. Present work has been aimed to isolate the BoHV-1 virus from the upper respiratory tract of bovines thereby any BoHV-1 isolate from cattle would be the field strain virus.

MATERIALS AND METHODS

Collection of samples

A total of 100 nasal swab samples were collected from all ages of cattle and buffaloes exhibiting clinical signs of respiratory infections *viz*., coughing, depression, fever, nasal and ocular discharge and anorexia during the period from November 2016 to March 2018 from different Gaushalas in and around Ludhiana and Teaching Veterinary Clinical Complex, College of veterinary Science, Guru Angad Dev Veterinary Science University Ludhiana, Punjab. Samples were collected with sterile swab in phosphate buffer saline (PBS) in a 15 ml sterile centrifuge tube. All the samples were screened by PCR for BoHV-1. Among them only three nasal swab samples were found positive. In this study, all three BoHV-1 PCR positive samples including 10 other randomly selected samples were used for virus isolation in MDBK cell lines.

Preparation of virus inoculum/ sample processing

Two ml of nasal swab samples were centrifuged at 825x g for 15 minutes to collect the supernatant. Then 15ìl of antibiotic antimycotic solution (100X, Himedia) was added to the supernatant and incubated at 37°C for 30 minutes. The processed samples were stored at -20°C to be used further for virus isolation.

Cell lines

MDBK cell line was procured from the School of Animal Biotechnology, GADVASU, Ludhiana and stored in liquid nitrogen (LN_2) till further use.

Reference virus

Standard reference virus was procured from the School of Animal Biotechnology, GADVASU, Ludhiana.

Isolation of BoHV-1 in cell culture

MDBK cells were distributed in 12 well cell culture plate to grow for 70% confluence. The growth media was discarded and washed three times with maintenance media. Processed samples (virus inoculum) were filtered through 0.22 µm filters and cells were infected with 100µl filtrate of inoculum. For each plate, cell control and virus control were included. The plates were kept at 37°C in CO_2 incubator for one and half hour for viral adsorption. After adsorption, the cells were washed with DMEM to remove unbound virus particles and replaced with 500 µl of maintenance media (containing 2% FBS). The infected cells were incubated at 37°C in an incubator with 5% CO_2. Daily observation was carried out at 24 hr, 48 hr, 72 hr, 96 hr and 120 hr post inoculation for development of cytopathic effect (CPE). CPE was characterized by grape like cluster of rounded cells in the monolayer. After 5 days of incubation, whether CPE has been observed or not, plates were subjected to alternative freezing and thawing for three times and then the cell culture supernatant was harvested aseptically. The harvested cell culture supernatants were stored at -80°C for virus identification. The samples that did not exhibit CPE in first passage were further subjected to 2nd, 3rd upto 5th passage. In case of no CPE observed after fifth passage, the sample was regarded as negative for BoHV-1.

DNA extraction

The DNA was extracted from the cell culture supernatant and reference virus using phenol chloroform DNA extraction method (Sambrook and Russell, 2001). The DNA was eluted in 30 µl of NFW and stored at -20°C till further use.

Polymerase Chain Reaction

For identification of virus, PCR amplification was carried out for conserved sequence of glycoprotein I (*gI*) gene using specific published primers (Vilcek *et al.,* 1994). The primers sequence used were *gI* F (624-CACGGACCTGGT GGACAAGAAG-645) and *gI* R (1070-CTACCGTCACGTGA GTGGTACG-1091) for amplification of 468 bp product. The PCR reaction mixture contained 2.5 µl of 10X PCR buffer (with 15mM $MgCl_2$), 1.0 µl of forward and reverse primer (20 pm/µl) each, 1.0 ìl of dNTPs mix (10mM each), 0.2 µl Taq DNA polymerase (5 units/ µl), 10 µl of extracted DNA (150 - 400ng) and the reaction was made up to 25 µl using nuclease free water. The thermocycler (BioRad, Thailand) conditions were initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 51.8°C for 1 min, extension at 75°C for 1 min and final extension at 75°C for 10 min. The negative control consisted of sterile water instead of DNA template while positive control consisted of DNA extracted from reference virus. After amplification the reaction mixture, it was electrophoresed in 1.5% agarose gel and stained with ethidium bromide. The amplified product was visualized as a single compact band of 468 bp under in gel documentation system (Syngene, USA).

RESULTS AND DISCUSSION

BoHV-1 is the causative agent of IBR which is important emerging disease of bovines distributed worldwide including

India (Gibbs and Rweyemamu, 1977; Kilari *et al.,* 2000 and Dhand *et al.,* 2002). This virus has been associated with a variety of clinical disease manifestations including rhinotracheitis, vulvovagititis, balanoposthitis, abortion, conjunctivitis and generalized systemic infections in newborn calves (Gibbs and Rweyemamu, 1977; Rola *et al.,* 2005). Naturally occurring BoHV-1 in respiratory form usually go unnoticed and does not cause high mortality amongst affected animals, but persistence of the virus causes the economic losses due to reduced production, impaired work ability, abortion *etc.* (Jones and Choudhary, 2007; Ravishankar *et al.,* 2012). As virus remains in carrier stage, serum based test are not suitable for accurate diagnosis of BoHV-1 infection. Diagnosis of IBR virus is usually based on ELISA, PCR and virus isolation test. Virus isolation is known as a gold standard for diagnosis of IBR infection. Present study was aimed to isolate the virus from nasal swab samples. However, the report of isolation of virus in pneumonic cases of bovine is scare in India except few solitary reports of isolation in nasal secretion (Saha *et al.* 2010 and Patil *et al.,* 2016).

In present study, two virus isolates were recovered from 13 nasal swab samples. Cytopathic effect in the form of rounding and clumping of cells has been described as a characteristic phenomenon of BoHV-1 by Mehrotra (1977) and Suresh *et al.* (1993). It was observed that virus isolates did not produce CPE till second passage in any sample. Both the samples showed characteristic CPE in the third passage. Although, one sample (N-1) exhibited extensive CPE after 48 hrs in the form of clumping of cells like bunches of grapes. In another sample (N-2), clumping of cells was seen after 72 hrs of third passage (Figure 1). Remaining samples did not exhibit any CPE even after fifth passage.

Growth of virus in cell culture confirmed by bunch of grape-like clustering and rounding of cell, which was in correlation with previous BoHV-1 isolation studies (Das *et al.,* 2014). Similarly, Saha *et al.* (2010) and Ranganatha *et al.* (2013) attempted IBR virus isolation from nasal swab samples in MDBK cell lines. In their study, only one and three isolates were obtained from 65 and 40 nasal swabs respectively. In this study, lesser number of isolates was recovered from nasal swab samples; it might be due to low concentration of virus excreted through respiratory route. In this study CPE was observed within 48-72 hrs of third passage. Mohankumar *et al.* (1994) isolated the virus from nasal and conjunctival swabs and a virus isolate was recovered after five serial passages in MDBK cells. Dhami (2001) also isolated the virus in MDBK cells and CPE was visible by fifth passage level.

DNA was isolated from suspected BoHV-1 isolates having CPE and PCR was performed using a gI gene specific primer. In PCR 468 bp product was amplified from both virus isolates which confirmed the presence of IBR virus (BoHV-1) in nasal swab samples (Fig 2). Both isolates were obtained from adult female crossbred cattle. Out of three PCR positive samples only two samples could be detected as positive virus for isolation. In PCR we detect the nucleic acid and can be detected in sample even if the virus in the sample is not fine. But in isolation of virus from sample, live virus is needed. The cell culture was not done simultaneously along with the conducting PCR on the samples and the samplers were stored for further isolation of virus. There is quite possibility that the virus was not live in one of the PCR positive sample which could not be isolated. The results in our study show that the PCR detection in nasal swab samples is considerably more sensitive than virus isolation. The reason behind could be the fact that PCR detects and amplify the dead virus particles where as virus isolation cannot. Engelenburg *et al.* (1995) compared PCR with routine virus isolation methods and reported that PCR

Fig 1: Clumping of MDBK cells like “bunches of grapes” 72 hrs of post infection at 10X.

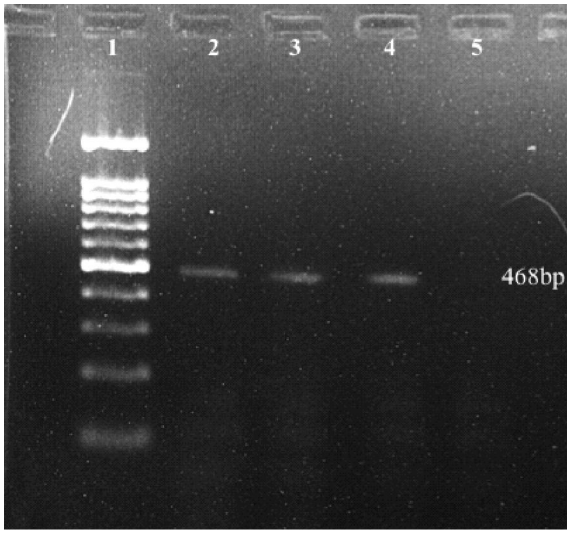

Fig 2: PCR amplification of gI gene of isolates of IBR virus.
Lane 1 - DNA ladder.
Lane 2 - Positive Control.
Lane 3 - Cell culture isolate of nasal swab sample (N-1).
Lane 4 - Cell culture isolate of nasal swab sample (N-2).
Lane 5 - Negative control.

was 2 to 100 fold more sensitive, moreover results of PCR was available within a day in comparison to the virus isolation which takes many more days and many more passages and at least 7 days to complete. PCR is an invaluable tool for fast and sensitive detection of BoHV-1 in biological and clinical specimens (Mahajan *et al.,* 2013). Deka *et al.* (2005) also suggested that PCR is more sensitive method for BoHV-1 screening in Bulls. Both the isolates were confirmed by 468 bp amplified product in gI gene specific PCR. Many researchers used amplification of gI gene of BoHV-1 for diagnosis and understanding the molecular epidemiology and disease distribution of disease in India and worldwide (Nisavic *et al.,* 2018; Hashemzehi *et al.,* 2017, Deka *et al.,* 2005 and Singh *et al.,* 2013).

Both isolates were from adult female crossbred cattle. More susceptibility of these cattle might be due to less adaptation of crossbred cattle in Indian climate and high production stress in female. Moreover it is known that semen is the main source of infection. Above findings are according to Rajesh *et al.* (2003) who reported higher prevalence of IBR in above three years of age cattle population of Kerala. Further they revealed that risk of infection is more in animals that had a parity of two of more and crossbred of Holstein Friesian. The increase in the incidence of IBR infection with age could be due to the fact that as animals grow older, they are more likely to be exposed to the virus since they are more likely to come into contact with other animals which have recovered from the disease but remains carrier. In present scenario, animal husbandry development is directed towards the rearing of crossbred cattle to get more production in tropical and subtropical countries and crossbred animals are more susceptible to heat stress. The virus remain latent for lifelong in BoHV-1 infected animal. The virus excreted through secretions (nasal ocular and vaginal) in any stress condition and imposes the threat of virus dissemination (Pistl *et al.,* 2003).

The low recovery of IBR virus from nasal secretion was due to the fact that BoHV-1 might not be the incriminate cause of respiratory problems of sampled animals. Sampling for virus isolation should be done in early course of the disease because virus shedding occurs during the early acute phase of the disease (between third to sixth days), when the discharge is serous rather than mucoprulent (OIE 2008).

CONCLUSION

This study concludes that IBR virus exists among cattle population of Punjab. The virus is present in the upper respiratory tract of infected animal and shedding the virus through respiratory route. Several seroprevalence reports demonstrate that IBR is now established and endemic in India. Further studies with isolation and molecular characterization of viruses collected from different geographical areas will contribute to understand the epidemiology and development of diagnostic and effective vaccine against IBR.

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