

Isolation and Characterization of BoHV-1 from Cattle in West Bengal, India

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Received: December 13, 2009

Accepted: August 4, 2010

Abstract

Isolation of BoHV-1 was attempted from nasal swabs as well as aborted fetuses and vaginal swabs in Madin Darby Bovine Kidney (MDBK) cell line to find out the prevalent strain in the state of West Bengal, India. The virus was isolated from only one case out of 65 nasal swabs whereas no virus was isolated from aborted fetuses and vaginal swabs. The isolate was typed as BoHV-1.2 (Strain India 3).

Keywords: BoHV-1, isolation, cattle

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Introduction

Infectious Bovine Rhinotracheitis (IBR) and Infectious Pustular Vulvo-vaginitis, caused by Bovine Herpes virus-1 (BoHV-1), is the most important emerging disease worldwide such as in India (Gibbs and Rweyemamu, 1977; Kilari *et al.*, 2000 and Dhand *et al.*, 2002). Besides the respiratory form of IBR, reproductive form is also highly prevalent in different states of India including West Bengal (Mehrotra and Rajya, 1981). Mehrotra and Rajya (1981), Suri Babu *et al.*, (1984), Gill *et al.*, (1987), Satyanarayana and Suri Babu (1987), Mohan *et al.*, (1989), Vaid *et al.*, (1991), Suresh *et al.*, (1999), Chinchkar *et al.*, (2002), Dhand *et al.*, (2002) and Rajesh *et al.*, (2003) reported IBR seropositive cows in different states of India in the past. The BoHV-1 virus becomes latent following a primary infection with field virus or vaccination with an attenuated strain (Radostits *et al.*, 2000). The viral DNA remains in the neurons of trigeminal ganglia in case of respiratory infection and sacral ganglion after genital infection for the entire of life in the host (OIE, 2009). Stressful situation, such as transport, parturition, high ambient temperature (in case of pure and cross-breeds), high milk yield and artificial stress induced by steroid injection cause reactivation of the latent virus from the ganglia and consequently intermittent shedding of virus into the environment thereby act as a potent source of infection to other healthy cattle (Radostits *et al.*, 2000 and OIE, 2009). Sero-evidence indicates the presence of the disease in the bovine population of the country, India in most of the organized farms (Suri Babu *et al.*, 1984; Mohan *et al.*, 1989). Infectious Bovine Rhinotracheitis virus was first identified in calves by Mehrotra *et al.*, (1976) in India.

The present work has been undertaken to isolate the prevalent Bovine herpes virus strains from cattle and further characterization of the isolate which can provide valuable clues to epidemiological investigations on BoHV-1 infections in tropical and sub-tropical

countries like India. In India particularly in the state like West Bengal no routine vaccination against Infectious Bovine Rhinotracheitis (Bovine Herpes virus type-1) was undertaken in cattle till now, thereby any BoHV-1 isolate from cattle would be the field strain virus.

Materials and methods

During the stress, cattle liberate the latent virus from all secretions and excretions; thereby isolation from seropositive cattle was attempted from nasal secretion during respiratory illness and during any abortion from aborted fetuses and subsequent vaginal secretions during January, 2005 to June, 2007.

Seroprevalence analysis

Seroprevalence analysis was conducted by virus neutralization test (VNT) as the method recommended by OIE, 2009 to detect the BoHV-1 seropositive cattle in the organized cattle farms of West Bengal, India. Virus neutralization test was performed in Madin Darby Bovine Kidney (MDBK) cell line in Eagle's Minimum Essential Medium (EMEM) with antibiotics (Penicillin 100,000 I.U., and Streptomycin 100 mg per liter of medium) and foetal calf serum (10%). The Standard Strain of Bovine herpesvirus type-1 (BoHV-1 strain 2204) virus procured from Central Animal Disease Research and Diagnosis (CADRAD) centre, Indian Veterinary Research Institute, Uttar Pradesh at the Institute of Animal Health & Veterinary Biologicals, Kolkata, was used as standard virus where the entire work was conducted. The virus was propagated in MDBK cell line and infectivity titer in cell culture i.e. TCID₅₀ per ml was recorded at regular interval.

Isolation and identification of BoHV-1

Collection and processing of the samples and isolation of the virus was attempted as the method described by OIE, 2009.

Samples of nasal swab, vaginal swab or in case of abortion either the aborted foetus or the foetal cotyledons were taken in viral transport

medium i.e. Eagle's Minimum Essential Medium (EMEM) with antibiotics (Penicillin 100,000 I.U. and Streptomycin, 100 mg per liter of medium) and foetal calf serum (5%).

Samples were collected from seropositive cattle when they showed respiratory syndrome. Nasal swabs were collected with commercially available sterile swabs when the secretion was serous rather than mucopurulent in nature. Isolation from conjunctival swabs was also attempted in this study. Nasal swabs were collected by vigorously rubbing cotton swabs against mucosal surfaces. When the seropositive cows were affected with vulvovaginitis the vaginal swabs were also collected aseptically by opening the vulva with vaginal speculum, vigorously rubbed against the vaginal wall, suspended in transport medium which was kept in ice packing and quickly carried to the laboratory.

In case of abortion, the aborted foetus if available or the foetal cotyledons were packed in ice and quickly brought to the laboratory. In case of aborted foetus, foetal liver, lungs, spleen, kidney and placental cotyledons were homogenized to about 20% suspension in cell culture medium (EMEM) and centrifuged at 1500 g for 10 minutes. The supernatants of the samples were then filtered through 0.45µm filters and used for isolation (OIE, 2009).

After submerging into viral transport medium, the nasal and vaginal swabs were transported to the laboratory in ice packs. In the laboratory, swabs were agitated in the transport medium to elute virus and left at room temperature for 30 minutes. The swabs were removed and the transport medium was clarified by centrifugation at 1500g for 10 minutes. The supernatants were filtered through 0.45µm filters and a 100µl volume of supernatant from the processed swabs was inoculated into monolayer MDBK cell culture (tissue culture flask of 25 ml volume) and incubated at 37°C for 2 hours. After 2 hours 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 7 days, the inoculated cell culture was frozen and thawed thrice, clarified by centrifugation and the supernatant was used for inoculation into fresh monolayer cell culture. In case of no CPE after 7 days, the sample was regarded as negative to BoHV-1.

The isolated virus was then freeze dried in glass ampoule for transportation. The freeze dried virus samples were sent to the OIE and National Reference Laboratory for BoHV-1 (Friedrich-Loeffler-Institut, Institute of Diagnostic Virology, Federal Research Institute for Animal Health, Insel Riems, Germany) for confirmation and subsequent typing in dry ice. Cell culture, immunofluorescence study, real time PCR, restriction fragment length polymorphism (RFLP) analysis and sequencing of the virus sample were performed.

Results

One BoHV-1 was isolated out of 65 nasal swabs screened but no virus could recover from any of the 19 aborted foetuses and genital swabs.

The isolated virus showed characteristic CPE and was neutralized by respective convalescent serum. The BoHV-1 reference virus (BoHV-1 strain 2204) was neutralized to a comparable extent by the same convalescent serum (Mohan Kumar *et al.*, 1994) and there were four fold rises in antibody titers in paired sera sample after 21 days. Electron microscopy study showed icosahedrons shaped virus of 100nm in diameter (Fig. 1).

In immunofluorescence analysis the isolate stained clearly and homogeneously positive for BoHV-1 gB, gD, gG, gC and gE. These tests were performed for infected monolayer as well as for single viral plaques grown under semisolid medium to exclude the possibility of mixtures of different strains within the

samples.

Quantitative real time PCR tests showed distribution of equal amounts of gE and gB in the sample. These findings exclude the concomitance of BoHV-5 and verified the classification as Bovine herpesvirus type 1.

To clarify whether the isolate exhibited different capabilities of cell to cell spread, serial dilutions were grown in cell culture under semisolid medium and stained for BoHV-1 gB. Diameters of at least 30 plaques from each isolate were determined using a graduated ocular and correlated to the plaque size of BoHV-1 Schönböken which was set as 100%. Plaque sizes were analyzed and no satisfactory significant difference could be demonstrated among the isolates.

Restriction fragment length polymorphism (RFLP) analysis identified the isolate as BoHV-1. The isolate was assigned to genotype BoHV-1.2 (Strain India 3).

Nucleotide sequencing: Parts of the regions encoding gH, gB and gC were amplified and sequenced on a 3130 Genetic Analyzer (ABI).

In gH sequence analysis, strain India 3 showed identical sequences with the other isolates from India (Strains India 4, India 5 and India 6 are isolated in West Bengal by inducing artificial stress and typed in OIE laboratory, Germany). In a phylogenetic analysis using maximum likelihood (MEGA version 4.0/Tamura, Dudley, Nei, and Kumar 2007), strain India 3 formed a unique cluster related to previously reported strains like BoHV-1 "Cooper" and "Jura" (sequence from GenBank).

In gB sequence analysis, the isolate showed an identical sequence with other isolates from India (strains India 4, India 5 and India 6) in the analyzed 450 base pair of the gB gene. The sequence was named "India gBcons". India gBcons was 100% matching with sequences of BoHV-1 strains from GenBank. A clear difference to non-BoHV-1 ruminant alphaherpesviruses (e.g. CpHV-1, CvHV-1, BoHV-5) with only 87-95% homology could

be observed.

In gC sequence analysis the isolate showed an identical sequence with other isolates from India (Strains India 4, India 5 and India 6) in the analyzed 158 base pair spanning overlapping region of gC gene. The sequence was named "India gCcons". The BoHV-1 gC sequences from India are also related to other gC sequences of other BoHV-1 strains, but could be clearly differentiated from ruminant alphaherpesviruses like CpHV-1 or BoHV-5.

Discussion

Only one BoHV-1.2 (Strain India 3) was isolated out of 65 screened nasal swabs from seropositive cattle. The seroprevalence of BoHV-1 in West Bengal was 33.97% in 2004-05, 39.1% in 2005-06 and 47.92% in 2006-07 which has an increasing trend (Annual report, 2004-05, RDDL.; Annual report, 2005-06 and Annual report, 2006-07, RDDL). There are no reports regarding isolation of the BoHV-1 from cattle suffering from respiratory form of the disease (Infectious Bovine Rhinotracheitis) in this region, in spite of high percentage of seropositive animals. This is the first reported isolate of BoHV-1 in West Bengal and eastern region of India. The low recovery of BoHV-1 from nasal swab was due to the fact that the respiratory problems might have been due to other etiologies rather than viral causes. Since maximal virus replication and shedding occurs between the third and sixth days (during the 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, 2009). Moreover, as virus shedding is inconsistent, it appears that more number of sampling on the specific days could have yielded more number of isolates. Mehrotra *et al.*, (1976), Pritchard *et al.*, (1997) and Rola *et al.*, (2005) had also detected BoHV-1 from nasal swabs of cattle. Mehrotra *et al.*, (1988) had also isolated IBR-IPV virus strain from cases of conjunctivitis in India. Penny *et al.*, (2002) reported bovine herpes virus type 1 (BHV-1) being associated with upper respiratory tract infection in calves

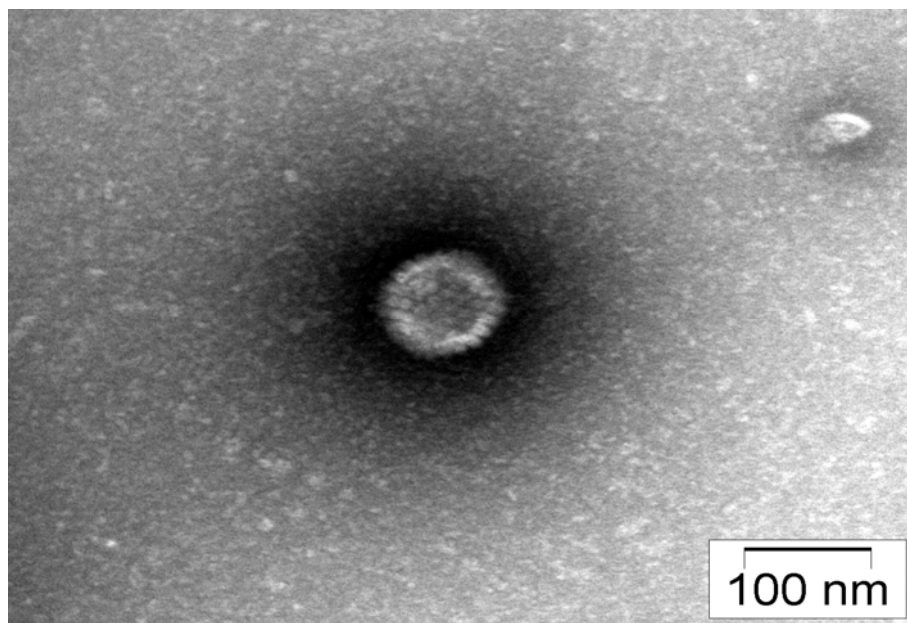


Figure: BoHV-1 under electron microscope (100nm)

in U.K.

A total 19 early aborted foetuses and swabs from genital tract were screened for BoHV-1 but no virus could recover from any of the sample. This may be due to the fact that in genital form of IBR, the foetus was expelled long after its death in the uterus resulting in autolysis of the foetus that may reduce the chance of isolation. Moreover, the sample size taken in this study was also meager. In India, Misra and Mishra (1987) isolated BoHV-1 from two aborted materials and one uterine mucus sample from cattle.

Molecular study of the isolate found to be BoHV-1 genotype i.e. BoHV-1.2. Sequence analysis confirmed the clustering with known BoHV-1 strains and showed a clear difference to other ruminant herpesviruses. A regional clustering of the BoHV-1 isolates for the analyzed gC sequence showed a marginal difference to the known isolates in Europe or the United States of America. Investigation of further Indian BoHV-1 isolates will be necessary to confirm the significance of this observation. The analysis of highly conserved genome regions gB did not reveal significant differences to previously reported BoHV-1.

In tropical and sub-tropical countries

animal husbandry development is directed towards the rearing of cross-bred cattle and they are more susceptible to environmental stress like heat stress. In BoHV-1 infected cattle, the virus remains latent for life-long and is excreted through secretions (nasal, ocular and vaginal) during any type of stress and thereby imposes a threat of disseminating the virus to other cattle (Pistl *et al.*, 2003). In India, the seroprevalence of IBR was 41.23% in 2003-04 (Annual Report, 2003-2004, PD-ADMAS) which is alarming and no prevention and control measures have been adopted by the Government of India yet. Moreover, prevention and control of the disease caused by BoHV-1 needs thorough hygiene, vaccination, mainly by marker vaccine and isolation of the virus (Pistl *et al.*, 2003). Thus, isolation and characterization of more viruses in countries like India will contribute to the control and prevention of IBR.

Acknowledgements

The authors are grateful to the Joint Director, Institute of Animal Health & Veterinary Biologicals, West Bengal for necessary permission to carry out this work in their institute. The authors convey their special

thanks to the Director, Indian Institute of Chemical Biology, Jadavpur and the Director, National Institute of Cholera & Enteric Diseases (NICED), Kolkata for giving necessary permission for ultra-centrifugation and transmission electron microscopy of the isolated virus. The authors are indebted to PD Dr. Martin Beer and Dr. Patricia Koenig of OIE and National Reference Laboratory for Bovine herpesvirus type 1, Friedrich-Loeffler-Institut, Germany for typing of the virus.

References

- Annual Report (2003-2004) Animal Disease Monitoring And Surveillance (PD-ADMAD), Hebbal, Bangalore.
- Annual Report (2004-2005) Regional Disease Diagnosis Laboratory (Eastern Region), Institute of Animal Health & Veterinary Biologicals, Kolkata, West Bengal.
- Annual Report (2005-2006) Regional Disease Diagnosis Laboratory (Eastern Region), Institute of Animal Health & Veterinary Biologicals, Kolkata, West Bengal.
- Annual Report (2006-2007) Regional Disease Diagnosis Laboratory (Eastern Region), Institute of Animal Health & Veterinary Biologicals, Kolkata, West Bengal.
- Chinchkar, S.R., Deshmukh, V.V., Abdulaziz, and Gujar, M.B. (2002) Seroprevalence of infectious bovine rhinotracheitis in Maharashtra state. *Indian Veterinary Journal* **179**, 78-79.
- Dhand, N.K., Singh, G., Sharma, D.R. and Sandhu, K.S. (2002) Seroprevalence of infectious bovine rhinotracheitis in Punjab. *Indian Veterinary Journal* **72**, 850-852.
- Epizooties, O.I.d. (2009) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. pp 752-767.
- Gibbs, E.P.J. and Rweyemamu, M.M. (1977) Bovine herpesvirus. Part I. Bovine herpesvirus 1. *Veterinary Bulletin* **47**, 317-343.
- Gill, B.S., Sharma, D.R., Kwatra, M.S., Kumar, A. and Gill, J.S. (1987) Seroprevalence of infectious bovine rhinotracheitis in the Punjab state. *Journal of Research, Punjab Agricultural University* **24**, 317-318.
- Kilari, S., Singh, S.N., Buche, A.V., Sawarkar, S. and Ghosh, S. (2000) Evaluation of immunogenicity of infectious bovine rhinotracheitis vaccine in Indian tropical climate. *Indian Veterinary Journal* **77**, 185-188.
- Mehrotra, M.L. and Rajya, B.S. (1981) Note on seroepidemiology of infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis (IBR/IPV) virus in India. *Indian Journal of Animal Sciences* **51**, 561-562.
- Mehrotra, M.L., Rajya, B.S. and Kumar, S. (1976) Infectious bovine Rhinotracheitis (IBR) - keratoconjunctivitis in calves. *Indian Journal of Veterinary Pathology* **1**, 70-73.
- Mehrotra, M.L., Shukla, D.C. and Sharma, A.K. (1988) Evaluation of immunogenicity of a tissue culture passage bovine herpesvirus 1(BHV-1) isolate of Indian origin. *Indian Journal of Animal Sciences* **58**, 428-431.
- Misra, P.K. and Mishra, A. (1987) Infectious bovine rhinotracheitis virus infection and infertility in cows, heifers and bulls. *Indian Journal of Animal Sciences* **57**, 267 - 271.
- Mohan Kumar, K.M. and Rajasekhar, M.a.K., G. (1994) Isolation of infectious bovine rhinotracheitis virus in Karnataka. *Indian Veterinary Journal* **71**, 109-112.

- Mohan, M., Singh, B.K. and Manickam, R. (1989) Seroepidemiological studies on infectious bovine rhinotracheitis (IBR) in bulls. *Indian Veterinary Journal* **66**, 914-916.
- Penny, C.D., Howie, F., Nettleton, P.F., Sargison, N.D. and Sehock, A. (2002) Upper respiratory disease and encephalitis in neonatal beef calves caused by bovine herpesvirus type 1. *Veterinary Record* **151**, 89-91.
- Pistl, J., Bobakova, M., Mudron, P. and Pilipcinec, E. (2003) Infectious bovine rhinotracheitis- the tropical problem. *Slovensky Veterinarsky Casopis* **28**, 24-28.
- Pritchard, G., Cook, N. and Banks, M. (1997) Infectious pastular vulvovaginitis/infectious pastular balanoposthitis in cattle. *Veterinary Record* **140**, 587.
- Radostits, O.M., Gay, C.C., Blood, D.C. and Hinchcliff, K.W. (2000) *Infectious bovine Rhinotracheitis (IBR, red nose), Bovine herpesvirus -1 (BHV-1) infection*, 9th edn., W.B.Saunders, London. pp 1173 - 1184.
- Rajesh, J.B., Tresamol, P.V. and Saseendranath, M.R. (2003) Seroprevalence of infectious bovine rhinotracheitis in cattle population of Kerala. *Indian Veterinary Journal* **80**, 393-396.
- Rola, J., Larska, M. and Polak, M.P. (2005) Detection of Bovine Herpesvirus 1 from an outbreak of bovine rhinotracheitis. *Bulletin of Veterinary Institute in Pulawy* **49**, 267-271.
- Satyanarayana, K. and Suri Babu, T. (1987) Serological survey of bovine herpesvirus 1 (BHV-1) infection in bovines in Andhra Pradesh. *Indian Journal of Animal Sciences* **57**, 499-502.
- Suresh, K.B., Sudharshana, K.J. and Rajasekhar, M. (1999) Seroprevalence of infectious bovine rhinotracheitis in India. *Indian Veterinary Journal* **176**, 5- 9.
- Suri Babu, T., Mallick, B.B. and Das, S.K. (1984) Prevalence of infectious bovine rhinotracheitis virus (BHV-1) antibodies in bovines. *Indian Veterinary Journal* **61**, 195-200.
- Vaid, J., Krishna, L. and Kaushal, R.S. (1991) Seroprevalence of animal diseases in Himachal Pradesh. *Indian Veterinary Journal* **68**, 705-707.

جداسازی و تعیین خصوصیت هرپس ویروس ۱ گاوی در گاوهای منطقه بنگال غربی در هند

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پذیرش نهایی: ۱۳/۵/۱۹

دریافت مقاله: ۲۲/۹/۸۸

چکیده

تلاش برای جداسازی هرپس ویروس ۱ گاوی از سواب های بینی، جنین های سقط شده و سواب های واژن در رده سلولی *Madin Darby Bovine Kidney (MDBK)* انجام گرفت تا سویه غالب در ایالت بنگال غربی در هند مشخص گردد. ویروس تنها در یک مورد از ۶۵ سواب بینی جداسازی شد و مورد دیگری از جنین های سقط شده و سواب های واژن به دست نیامد. ویروس جداسازی شده به عنوان *BoHV-1.2* (سویه هندی شماره ۳) تعیین گردید.

واژگان کلیدی: هرپس ویروس ۱ گاوی، جداسازی، گاو، هند