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RESEARCH ARTICLE
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A comparative evaluation of avidin-biotin ELISA and micro SNT for detection of antibodies to infectious bovine rhinotracheitis in cattle population of Odisha, India

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Abstract

Aim: The present study was undertaken to serologically detect Infectious Bovine Rhinotracheitis (IBR) in the cattle population of Odisha, India using micro-Serum neutralization test (micro SNT) and Avidin-Biotin Enzyme linked immuno sorbent assay (AB ELISA) and finding out their comparative efficacy to serve as a suitable diagnostic tool in field condition.

Materials and Methods: The study was carried out using serum samples (n=180) collected randomly from cattle populations of nine districts of Odisha. Similarly vaginal swabs (n=26) from cattle having history of repeat breeding, abortion, vulvo-vaginitis and nasal swabs (n=8) from calves with respiratory symptoms and nasal discharge were collected aseptically, to ascertain the circulation of virus among the cattle population.

Results: Virus isolation by cell culture and subsequent confirmation by polymerase chain reaction confirmed four isolates. Screening of serum samples revealed 9.44% and 12.22% samples positive for IBR antibodies in micro SNT and AB ELISA respectively. The sensitivity and specificity of AB ELISA test was found to be 88.23% and 95.70% respectively taking micro SNT as gold standard and the kappa value between the two tests was 0.75.

Conclusion: Screening of serum samples revealed 9.44% and 12.22% samples positive for IBR antibodies in micro SNT and AB ELISA respectively, thus highlighting the circulation of virus among the livestock population of Odisha and that AB ELISA could be more efficiently applied for the sero-diagnosis of IBR virus infections at field conditions, with demand for more study on faster, efficient and large scale screening of the infected animals.

Keywords: avidin-biotin ELISA, bovine herpes virus -1, cattle, infectious bovine rhinotracheitis, isolation, serum neutralization test, Odisha.

Introduction

Infectious bovine rhinotracheitis (IBR) is caused by Bovine Herpes Virus-1(BHV-1), a member of the genus Varicellovirus in the sub-family Alphaherpes-virinae, family Herpesviridae and order Herpesvirales. Virions are enveloped and variably sized (approximately120–250 nm in diameter), containing an icosahedral nucleocapsid composed of 162 capsomers. Genome is linear double-stranded DNA, 125–290 kbp in size [1].The virion contains about 70 proteins, of which 11 are envelope glycoproteins. Glycoprotein gB, gC and gD are of major importance followed byadditional glycoproteins (gE, gI, gH, gL, gG, gK and gM), thymidine kinase. BHV-1 virus has been divided into subtypes like BHV-1.1, BHV-1.2, and BHV-1.3. Among them, BHV- 1.1 is mostly related to the respiratory syndrome, BHV- 1.2 to genital infections, and BHV-1.3/BHV-5 is associated with neurological disorders of the cattle. However, all subtypes were found to be antigenically similar[2].

IBR has proved to be a serious threat to the bovine population, associating itself with variety of clinical syndromes involving ocular, respiratory and genital tract. Secondary bacterial infections, latency and poor prevention measures further adds to the sequelae of the disease, thus complicating recovery and generating an eminent threat of spontaneous reoccurrence.The productivity and re-productivity of the animals is greatly decreased as an outcome of the disease [3]. After being reported for the first time in India in the year 1976 the disease has shown many occurrences in various regions of the country with severe economic damage to the dairy industry [4].

It is one of the most widespread respiratory/ reproductive viral diseases of bovines in India [5] and prompt detection and control measures are essential to check the transmission and propagation of the virus. An infection normally elicits immune response within

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Table-1: Details of the primer used in the present study for molecular characterization of BHV-1 isolates.

Name of the primer	Primer Sequence (5'- 3')	Nucleotide position (Genbank Accession No. AJ004801)	Expected product size	Reference
gBF	TGGTGGCCTTYGACCGCGAC	56085-56104	293bp	[13]
gBR	GCTCCGGCGAGTAGCTGGTGTG	56373-56357		
gCF	TGTGACTTGGTGCCCATGTCGC	17434-17455	173bp	[14]
gCR	CTGCTGTTCGTAGCCCACAACG	17606-17585		
gDF	CGGCCGCTGTACTACATGGA	119241-119260	343bp	[14]
gDR	GATACGTCACGGCGCAGAACC	119583-119564		

7–10 days which is presumed to persist for life, even though it may fall below the detection limit of some tests. Inspite of the fact that large number of serological tests like Serum neutralization test (SNT), Indirect Haemagglutination (IHA) and Complement fixation test (CFT) etc. has been used by several workers, there is necessity to develop and use more sensitive and specific test for serodiagnosis of IBR infection. Since ELISA is a more sensitive and specific test in terms of detection of low level of antibody for several viral diseases, this has been extensively used in recent past by various authors to monitor the seroprevalence of IBR infection of cattle population. There are many reports regarding use of various types of ELISA and neutralization tests to detect BHV-1 antibody in serum samples of cattle. These tests can also be used to detect acute, convalescent, and latent stages of disease [3].

To identify infected cattle, the use of very sensitive serological tests to detect specific antibodies is of critical importance. The availability of a reliable diagnostic tool at field conditions is important for implementing a successful control programme as it requires quick detection of IBR positive animals and their segregation from the uninfected herd thus preventing the further transmission of the virus. Therefore, this pilot study was undertaken to isolate and identify BHV-1 from the cattle population of Odisha, India. Secondly to find out the apparent seroprevalence using two tests (micro SNT and avidin-biotin ELISA (AB ELISA)) and finding out their comparative efficacy, this will aid in determining a suitable diagnostic tool to be effectively used at field conditions for diagnosis of IBR.

Materials and Methods

Ethical approval: This study was conducted after approval by the research committee and Institutional animal ethics committee.

Samples: Serum samples (n=180) were collected randomly from cattle populations of nine districts of Odisha during November, 2010 to April, 2011. Similarly vaginal swabs (n=26) from cattle having history of repeat breeding, abortion, vulvo-vaginitis and nasal swabs (n=8) from calves with respiratory symptoms and nasal discharge were collected aseptically. The serum samples were transported in ice. All sera samples were heat-inactivated at 56°C for 30 minutes and stored at -20°C until tested. The vaginal swabs and nasal swabs were collected aseptically and transported in Hanks balanced salt solution (HBSS) in ice.

Isolation of IBR virus: The vaginal and nasal swabs after being syringe filtered (milipore, 0.45μm) were subjected to log dilution at 10^{-1} and infected on to the Madin Deby bovine kidney (MDBK) cell line (obtained from National Centre for Cell Science, Pune, Maharastra). Daily observation was carried out at 24, 48, 72, 96 and 120 h for development of CPE under inverted microscope (10×). Likewise three blind passages of vaginal and nasal samples in MDBK cells were carried out which was followed by three cycles of alternate freezing and thawing. The cell culture fluid was harvested aseptically in sterile Mc Carteny's vial for further molecular characterization. Detailed study of the microscopic changes was done after staining with haematoxylin and eosin. Following the development of characteristic CPE, the virus samples were subjected to titration in MDBK cell grown in 96 well flat bottomed tissue culture plates with cover by the method of Reed and Muench [6] using positive reference serum and the tissue culture infective dose ($TCID_{50}$) was calculated.

Molecular characterization

Polymerase chain reaction: Viral DNA was extracted of from cell culture supernatant by using Viral DNA extraction kit [QIAGEN, Germany] as per manufacturer's instructions and stored at -40^0C. Molecular characterization of the isolates was carried out by PCR amplification of gB, gC and gD genes. The details of the primers used and their respected product size are given (Table-1).

micro SNT: Micro SNT was carried out as per the methodology of Tongaonkar *et al*. [7]. The seventh passaged reference IBR virus (Colorado strain) in MDBK (Madin Darby bovine kidney) cell line was used in the test and the serum titre was calculated. The test was standardized using known 5 positive and 2 negative IBR serum and the neutralizing antibody titre was fixed at 1:16. The reference sera used in the test was provided by Project Directorate on Animal disease monitoring and surveillance (PD_ADMAS, India) (now it is known as National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI)).

AB ELISA: The AB ELISA is a type of indirect ELISA and was conducted using AB ELISA kit at PD_ ADMAS as per methodology of Suresh *et al*. [8]. The result of test serum was expressed as percent positivity (PP) values calculated as follows. Serum samples showing PP $\geq$ 45% considered as positive.

PP (%) = Mean OD of the sample / Mean OD of the strong positive serum X 100.

Figure-1: MDBK cells Healthy 10X, unstained.

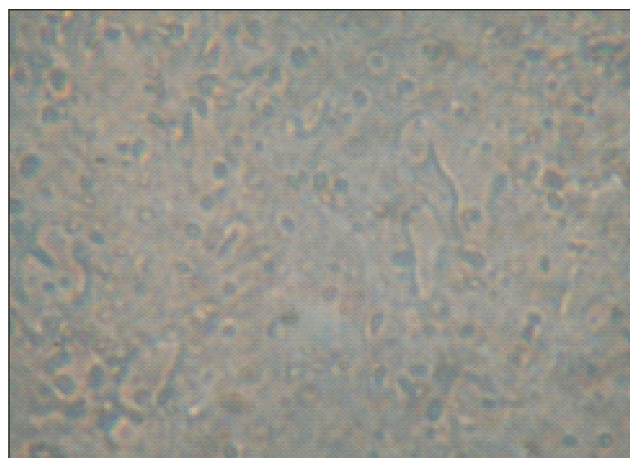

Figure-2: MDBK cells infected with BHV-1 virus at 96 h post infection showing CPE. (rounding, syncitia and vacuolation) 10X, unstained.

Figure-3: Cover slip preparation of MDBK cell infected with BHV-1 virus showing CPE (cell lysis, Cowdry type A intranuclear inclusion bodies) H&E stain.

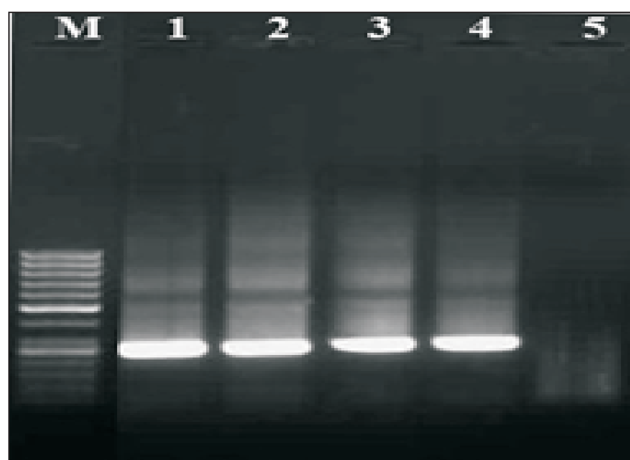

Figure-4: PCR amplification of gC gene of BHV-1
Lane M: 50 bp molecular size marker
Lane 1- 4 : 293 bp amplicon of gB region (4 isolates)
Lane 5: Negative control

(OD = Optical density)

Statistical analysis: The statistical analysis used in the study was carried out using medcalc software.

Results and Discussion

Isolation of BHV-1 virus from clinical samples: In the present study attempt was made to isolate the virus from clinical samples like vaginal swabs and nasal swabs from animals with history of repeat breeding, abortion, pustular vulvovaginitis and animals with nasal lesions. For isolation of IBR strain MDBK cell monolayers (Figure-1) were infected with 10^{-1} diluted sample. After 24 h of incubation few cells were found to be rounded whose number increased after 48 hours and after 72 h syncitia development showing "bunch of grapes" appearance of virus infected cells was observed which was in correlation with previous BHV-1 isolation studies [9]. At 96 h more than 90 % cell showed CPE (Figure-2). Following three blind passages two more passages were given in MDBK cells to increase the titer of the virus. The stained coverslip preparation revealed characteristic CPE like rounding of cells with intense stain, syncitia development, cytoplasmic and nuclear vaculation and characteristic Cowdry typeA intranuclear inclusions (Figure-3). Such CPE has already been reported by many workers who got similar CPE using MDBK, bovine turbinate cells and Vero cells [10,11]. The virus titer was found to be $10^{5.6}$ $TCID_{50}$/0.1ml.

Preliminary identification of isolates were carried out in serological test (micro-SNT), using known positive IBR reference serum, which revealed complete inhibition of CPE with titer 1:32, there by confirming them to be IBR isolates. In this way altogether four isolates of IBR were identified. Use of micro-SNT for confirmation of isolates has already been reported [12].

Confirmation of the BHV-1 isolates: In order to confirm the isolates, PCR was carried out using gene specific primers [13,14] (Table-1) and the amplified PCR products were found to be 293 bp, 173 bp and 343 bp in agarose gel for gB, gC and gD genes respectively (Figure-4). However Various PCR assays for the direct detection of BHV-1 from clinical samples have been described by many authors, using the primers for the gB gene, gC gene and gD gene [11,15,16].

Table-2: Comparison of AB ELISA with Micro SNT on cattle serum samples.

AB-ELISA test	Micro SNT		Total	Sensitivity (%)	Specificity (%)
	Positive	Negative			
Positive	15	07	22	88.23	95.70
Negative	2	156	158		
Total	17	163	180		

Serological evidence of BHV-1 antibodies by micro SNT and AB ELISA: The serum samples collected from infected and animals in same and near habitat were subjected to micro SNT and AB ELISA . In micro SNT 17 (9.44%) serum samples were found positive for BHV-1 antibodies with titer ranging from 1:16 to 1:128.which was at par with the finding observed by Bauer *et al*., [17]. SNT is considered to be gold standard test for the detection of antibodies against BHV-1 and Office International des Epizooties (OIE) has recommended its use in international trade of animals. It detects only neutralizing antibodies against BHV-1. Lower seroprevalence value of 3.7% and 5.75% by employing microSNT has also been observed in Bali and turkey respectively [18,19].Tongaonker *et al*. [7] carried out seroprevalence study of IBR by micro SNT for the first time In India, and reported 42.9% prevalence in cattle, which was higher than that found in the present study. A number of independent studies have been carried out by various workers, with prevalence rates ranging from 32.34% to 52.3% have been reported [20, 21].

ELISA is now being used frequently for either detection of antigen or antibody because of its high sensitivity and specificity with various modifications. Results of AB ELISA that was used in the current study revealed that 22 (12.22%) of serum samples were positive for antibodies against BHV-1. During the study positive antibodies were also found in animals from which the virus was isolated. Renukaradhya *et al*. [22] developed AB ELISA for the serological survey of IBR in cattle and buffalo in India and reported prevalence of 50.9% in cattle by screening serum samples of three southern states (Karnataka, Tamil Nadu, Andhra Pradesh) of India. After that a number of independent studies have been carried out in almost all states of India and a prevalence rate between 9% to 89% has been reported [23-26]. Such wide variation in the prevalence rate among different states of India could be due to factors like method of sampling, bias in source samples, agroecological and climatological factors, intensity of dairy farming, intermixing of animals, unrestricted movement of infected animals, lack of quarantine, poor education of farmers etc [24].The low prevalence rate observed in this study could be due to the fact that samples were randomly collected from different villages (unorganized sectors). It has been previously reported by various workers that animals in organized farms are more susceptible to IBR infection than unorganized farms [27]. As IBR vaccination is not practiced in the state of Odisha, the above found prevalence of IBR antibodies could be presumed to be an outcome of past or ongoing infection.

Determination of sensitivity and specificity of AB ELISA: The sensitivity and specificity of AB-ELISA was found to be 88.23% and 95.70% (Table-2). Moreover absence of neutralizing antibody does not rule out the presence of non-neutralizing binding antibody of BHV-1 which can still be detected by AB ELISA. Similarly Boelaert *et al.* [28] also obtained sensitivity of 70-90% and 90-99% using AB ELISA and SNT respectively which was in correlation with the finding of present study. In a likely fashion Bandopadhya *et al.* [3] found the relative sensitivity and specificity of the AB-ELISA were found to be 100% for detecting BHV-1 specific antibodies in studies involving the yak population of Arunachal Pradesh and concluded that that the AB-ELISA, which is a simpler test than the virus neutralization test, can effectively be used in future. The kappa value between the two tests in the present study was found to be 0.75.This suggests that the agreement between the two test is good (kappa value 0.6 to 0.8) and AB ELISA is able to detect additional seropositive reactors compared to micro-SNT.

Conclusion

The present study suggests that AB ELISA could be more efficiently applied for the sero-diagnosis of IBR virus infections as compared to micro SNT. Furthermore isolation of IBR virus from clinical cases and its identification provides a concrete diagnosis and endorses presence of IBR virus in cattle population of the state. The result of this pilot study carried out warrants future studies with an aim to effectively screen the larger population of cattle in India, characterization of the isolates to understand the molecular mechanism of pathogenesis, development of suitable vaccine and subsequent steps to eradicate the virus from the population are straight way need.

Authors' contributions

NNM, PD and HKP have conceived, planned and designed the study. SiR recorded and analysed the data. SR provided technical support. Manuscript was drafted and revised by NNM, PD, LNS under the guidance of HKP. 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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