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Research Article

PREVALENCE OF BOVINE VIRAL DIARRHOEA VIRUS IN WEST BENGAL, INDIA

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ABSTRACT: Bovine Viral Diarrhea (BVD) is one of the most economically important diseases in cattle. The present study was undertaken to diagnose the persistently infected (PI) animals by Antigen-ELISA and Reverse Transcriptase PCR using serum samples from organized farms as well as rural areas of West Bengal. The results showed that out of 964 serum samples tested 07 (0.73%) was positive for BVDV by Antigen-ELISA. For further confirmation, RNA was extracted from the positive samples and RT-PCR was performed with 5' UTR specific primers which showed 294 bp amplicons. This finding showed circulation of BVDV in cattle in West Bengal, India.

Key words: Bovine virus diarrhea virus, Prevalence, Persistent infection.

INTRODUCTION

Bovine Viral Diarrhoea (BVD) is a common viral disease of cattle, sheep, other even-toed ungulates and is caused by bovine viral diarrhea virus (BVDV), a member of *Pestivirus* under the family Flaviviridae (Becher and Thiel 2011, Simmonds *et al.,* 2012). BVDV causes serious alimentary, respiratory and reproductive ailments like diarrhea, pneumonia or abortion in cattle and small ruminants, beside hemorrhagic syndromes and thrombocytopenia (Baker 1987).

The genome of BVDV consists of a single stranded, plus-sense RNA molecule of approximately 12300 bases in length in non-cytopathic strains (Collett *et al.,* 1988a). BVD viruses are further classified into two genotypes: BVDV-1 and BVDV-2 (Pellerin *et al.,* 1994). Both the strains may exist as one of two biotypes, cytopathic (cp) and non-cytopathic (ncp), depending on cytopathic ability of the strain in cell cultures (Gillespie *et al.,* 1961, Ridpath 2002).

The open reading frame (ORF) of BVDV can be divided into distinct regions that encode the individual viral proteins. The viral proteins are coded in the order of NH_2-N^{pro}-Capsid-E^{rns}-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-

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COOH (Collett *et al*., 1988b). The E^{rns} protein, present as homodimer, is a heavily glycosylated and is both associated with the virus particle as well as secreted in soluble form in infected cells. Some antibodies produced against E^{rns} are neutralizing (Fetzer *et al.,* 2005). This protein has a unique feature in that it possesses RNase activity capable of degrading both single stranded and double stranded RNA (Hausmann *et al.,* 2004). This function is thought to be important in limiting the host's innate immune response to double-stranded RNA, an indicator of viral infection (Matzener *et al.,* 2009).

BVDV can spread horizontally by direct contact with persistently infected animals. Vertical infection of the foetus during the early pregnancy, before it reaches immunocompetency may result in persistent infection (PI) of newborns calves (Brownlie 1991). PI animals shed virus in high concentrations throughout their lives without mounting an antibody response to the virus (Paton *et al.,* 1999). Most PI animals develop mucosal disease after super infection with a homologous cytopathic BVDV strain and die within 2 years of age (Brownlie *et al.,* 1987). Thus, successful eradication of BVDV requires detection and elimination of PI animals prior to breeding seasons (Zimmer *et al.,* 2004). Currently, methods for detecting PI animals include antigen-captured ELISAs and RT-PCR from pooled samples (Dubovi 2013).

In India, the first serological evidence of BVDV infection in cattle was reported from Chiplima farm and agricultural university in Odisha by Nayak *et al.* (1982). Subsequently, serological examination of BVDV of 40.60% was reported from Gujarat (Mukherjee *et al.,* 1989). An overall BVD sero-prevalence rate of 30% was detected in cattle and buffalo from 14 states of India during 1999-2004 (Sood *et al.,* 2007). In another study, 37.6% of Indian cattle were found positive for BVDV antibodies indicating widespread infection in the country (Bhatia *et al.,* 2008).

Though the earlier studies indicated serological evidence of BVDV infection, the confirmatory evidence of BVD in Indian cattle was confirmed by phylogenetic analysis (Mishra *et al.,* 2004). The genetic monitoring of presence of BVDV has been investigated by 5' UTR specific primers sequence in cattle (Mishra *et al.,* 2005) and in Indian buffalo (Mishra *et al.,* 2007) for identification of types through construction of phylo-genetic tree.

RT-PCR technique has been adopted for detection of BVD viral RNA for diagnosis purpose (Hamel *et al.,* 1995). This technique is sensitive enough to detect persistently infected lactating cows in a herd of up to 100 animals (Drew *et al.,* 1999). The virus has also been detected from pooled sera samples of cattle (Bedekovic *et al.,* 2012). Considering the persistently infected animals as main source of virus spread, the virus is detected within couple of days of infection depending upon the biotypes (Houe 1999). The infection has been detected from blood, sera or buffy coat by RT-PCR (Goyal 2005).

Bull used for semen production should be screen at regular interval to detect PI animals for controlling the disease spread by RT-PCR that detects the PI animals at 12-14 days of post infection and also detects 100-103 folds lower virus level than virus isolation techniques (Drew *et al.,* 1999). Again cow and calves housed at different farms are analyzed by real time-PCR for detection PI animals for eradication program of BVDV (Mahlum *et al.,* 2001). The present study has been envisaged to identify the

persistently infected animals of BVDV from organized farm and rural areas.

MATERIALS AND METHODS

Collection of sample: Blood samples were collected aseptically in evacuated collection tubes (VAKU-8) from different age group of bovine species from different organized farms and rural farmers of West Bengal. We used single container for single animal to prevent cross contamination. After collection blood samples were kept for 3 h in room temp followed by overnight refrigeration at 4°C before serum separation. Finally serum samples were preserved at -24°C before further studies. Serum samples were kept at 4°C for few days before ELISA. BVD virus stays stable for virus isolation at temperature of 4°C for couple of weeks. However, considering that this is a RNA virus, the genome is easily destroyed and such samples can become less suitable even for RT-PCR testing. So the positive serum sample by Ag-ELISA was further kept at - 40°C for isolation of viral RNA. Serum samples were collected from three different corners located at different districts of West Bengal *i.e.* Paschim Medinipur, Nadia and Murshidabad.

Antigen-ELISA of serum samples: Antigen ELISA was performed using a commercially available kit following the manufacture's protocol (IDEEX KIT, Switzerland). First, the antibody coated plate and all the reagents were brought to room temperature (~ 25°C). Then sample positions were recorded in the X Chek software using relevant template. Thereafter 50 µl of the detection antibodies was added to each well by pipette along with 50 µl negative control and 50 µl positive controls in duplicate in appropriate wells. Fifty microlitre volume of samples, in duplicate, were added in to rest of the wells. Then the plate was incubated for 2 h at 37°C under tightly sealed condition in a shaking incubator to avoid evaporation. After that each well was washed with 300 µl of wash solution for five times and the plate was firmly tapped onto absorbent material to remove residual wash fluid from each well. Thereafter, 100 µl of conjugate was dispensed into each well and incubated for 30 minutes at room temperature. The wells were then washed with 300 µl of wash solution as mentioned above. Then 100 µl of TMB substrate solution was dispensed into each well and the plate was incubated for 10 min at room temperature in dark. Then 100 µl of stop solution was dispensed into each well to stop the reaction and the absorbance was measured at 450 nm. The result was analyzed using X Chek software.

Extraction and Quantification of viral RNA: The Viral RNA was extracted as per manufacturers' protocol (Qaigen, Germany) from 140 µl serum samples which were positive in Antigen-ELISA. Firstly, 560 µl prepared buffer was mixed with AVL containing carrier RNA in a 1.5 ml micro-centrifuge tube. Then, 140 µl serum was mixed to AVL carrier RNA in the micro-centrifuge tube and mixed by pulse-vortexing for 15 sec. The mixture was incubated at room temperature for 10 min. The content was briefly centrifuged to pool droplets from inside of the lid. Then, 560 µl of ethanol (96-100%) was added to the sample and mixed by pulse-vortexing for 15 sec. followed by brief centrifugation. Then, 630 µl of sample solution was charged onto the QIA amp mini column without wetting the rim. After closing the cap, tubes were centrifuged at 8000 rpm for one min. The QIA amp mini column was placed into a clean 2 ml collection tube and free-flow was discarded for two times. After that 500 µl of

buffer AW1 was added on to the QIA amp mini column and centrifuged at 8000 rpm for 1 min. Then, 500 µl of buffer AW2 was added and centrifuged at 14000 rpm for 3 min. Finally, the RNA was eluted with 60 µl of AE buffer and equilibrated at room temperature, incubated at room temperature and centrifuged at 8000 rpm for 1 min. Ten microlitre RNA preparation was mixed with 990 µl DEPC treated water/ nuclease free water. OD was taken in UV-VIS spectrophotometer at 260 nm and 280 nm and concentration to check purity of the extract.

Reverse Transcriptase-PCR

BVDV 5' UTR specific primers (Table 1) were synthesized from a private firm. The master mixture was prepared in a micro centrifuged tube adding the following components one by one on an ice cooler.

Parameter	Volume Taken
5 X Qaigen RT PCR buffer	10 µl.
Forward primer (100 µM)	0.3 µl
Revese primer (100 µM)	0.3 µl
dNTP mix (10mM)	2 µl
RT-PCR enzyme	2 µl
Template (RNA sample)	10 µl
Nuclease free water	25.4 µl
Total	50 µl

The mixture was briefly centrifuged and reverse transcription was done at 55^{0}C for 30 min in the thermo-cycler machine. This was followed by PCR amplification of the desired gene using the following steps: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final Extension-72°C for 10 min.

The PCR amplicons were electrophoresed in 1% agarose gel at 100 V for 1 h and viewed in gel documentation system.

RESULTS AND DISCUSSION

A total of 964 serum samples were collected and tested by Antigen-ELISA. Out of this, only 07 samples, *i.e.* 7 animals were positive for BVDV antigen and 957 animals were negative (Table 2). The test showed that presence of BVD viral antigen in animal serum samples was very low in West Bengal (Fig.2). For further confirmation we extracted total RNA from sample which is positive by ELISA and PCR amplified a gene of BVDV. The Glycoprotein E^{rns} is a major polypeptide of BVD virus which is used for screening of BVD by ELISA. After gel electrophoresis of PCR product 294 base pair (bp) amplified products were observed in all serum samples positive by antigen ELISA. There was no non-specific amplification in the reaction (Fig.1). This indicated that the infection was due to BVD virus. The investigation of 964 serum samples tested by Antigen-ELISA detected 0.73% positivity showing circulation of BVDV in the bovine species in West Bengal, although not at an alarming level.

Table 1. 5 'UTR specific primers used for RT-PCR of BVDV.

Sl. No.	Primers name	Sequence (5'-3')	Tm (°C)	Amplicon length (bp)
1.	5' UTR Forward Primer	GCTAGCCATGCCTTAGTAGGACT	60.1	294
2.	5' UTR Reverse Primer	CAACTCCATGTGCCATGTACAGCA	59.7	

Table 2. Distribution of animals tested in West Bengal and prevalence of BVDV.

Sl. No.	Particulars	Total no of serum sample collected	Total no of Positive samples by Ag-ELISA	Reactor Percentage (%)
1.	Organised Farm-1	529	06	1.34
2.	Organised Farm-2	110	00	00
3.	Organised Farm-3	139	00	00
4.	Organised Farm-4	106	00	00
5.	Organised Farm-5	30	00	00
6.	Rural area	50	01	02
	Grand Total	964	07	0.73

Eradication of Bovine Viral Diarrhea virus is based on the identification and elimination of PI animals. The rapid identification and removal of such animals minimize the risk of BVDV transmission to the herd, particularly in pregnant animals. The intensity of virus shedding by PI animals into the surroundings are so high that even short contact with such type of animals may lead to the infection (Moen *et al.,* 2005). A failure to detect PI animals may have serious problem in cattle health. Most population of BVDV infection is characterized by a high sero-prevalence which is reflected by the presence of antibody in the colostrums transferred to the newborns after parturitions. The maternal antibody generally disappears from most of the young calves by 90 days of age (Coria *et al.,* 1978). The development of test which identifies PI animals regardless of the presence of maternal antibodies should be in priorities when planning for BVDV eradication program. The World Organization for Animal Health (OIE) has recommended several different methods for the etiological diagnosis of BVD/MD. Due to its simplicity, price and rapid performance, the commercially available Antigen-ELISA is very frequently used.

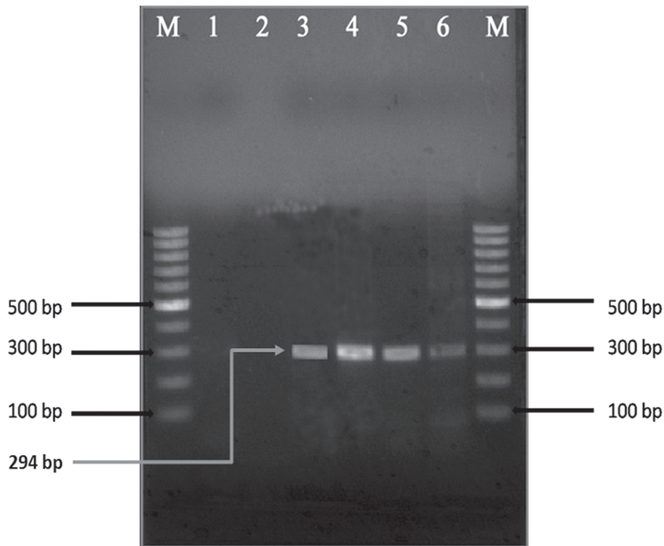

Fig. 1. Gel Electrophoresis of 5' UTR of BVDV genome amplified by RT-PCR.

M- 100 bps DNA ladder
1,2 - Negative control; 3, 4, 5 & 6- 294 bps amplification by 5' UTR

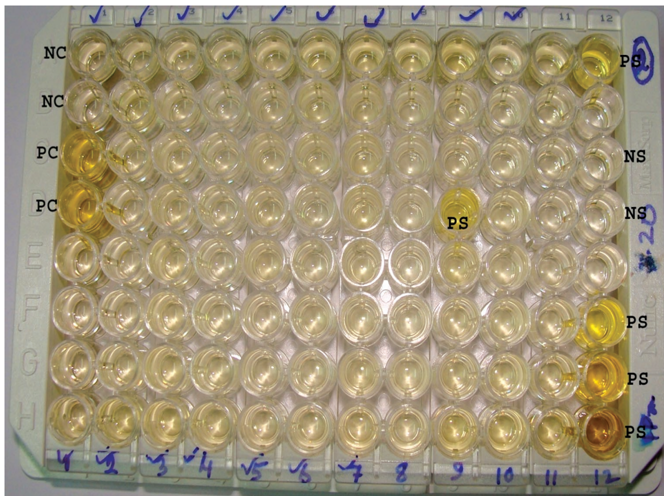

Fig. 2. Antigen-ELISA of BVDV from serum sample for sero-surveillance.

NC- Negative Control; PC- Positive Control; PS- Positive Sample; NS-Negative Sample.

Six samples, out of 529, were positive both in Ag-ELISA and RT-PCR from one farm of Nadia. In another case only one was positive out of 50 sera samples from rural area of Paschim Medinipur. This finding supported the finding of Mishra *et al.* (2007) on distribution of BVDV in other states of India. The incidence of BVDV clearly indicated that geographical distribution of the virus is present in the animal population in different districts of West Bengal. BVDV was independently reported from India in different clusters on the basis of genetic analysis of NS3 gene (Mishra *et al.,* 2008). This widespread prevalence of virus variants may be explained by the intensive movement of cattle between farms within the same or different regions and by poor BVDV control procedures.

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