Identification of bovine viral diarrhoea virus type 1 in Indian buffaloes and their genetic relationship with cattle strains in 5′ UTR

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Bovine viral diarrhoea (BVD) caused by either bovine viral diarrhoea virus type 1 (BVDV 1) or BVDV 2 is responsible for significant economic losses in cattle and buffaloes. We recently established prevalence of BVDV 1b viruses in Indian cattle. Detection and typing of BVDV in Indian buffaloes is yet to be reported, though buffaloes and cattle contribute equally to livestock production systems in India. In this study, we report identification of BVDV 1 in Indian buffaloes and their genetic relationship with cattle strains. The 5′ untranslated region (UTR) of 15 BVDVs amplified directly from clinical samples of buffaloes and cattle by RT–PCR during 2001–05 was sequenced. Phylogenetic analysis revealed that 11 samples from cattle and three from buffaloes clustered with BVDV 1b, whereas one buffalo sample clustered with BVDV 1c, which may be its true representative. The study established prevalence of BVDV 1b and 1c subtypes in Indian buffaloes and close relationship among cattle and buffalo 1b viruses. Detection of BVDV 1b in cattle serum used as nutrient component recognized the need for mandatory testing of bovine sera before using for cell culture and manufacturing of veterinary vaccines.

Keywords: Bovine viral diarrhoea, buffaloes, cattle, genetic typing.

THE genus *Pestivirus* in the family Flaviviridae comprises four accepted species: Bovine viral diarrhoea virus 1 (BVDV1), Bovine viral diarrhoea virus 2 (BVDV2), Border disease virus (BDV) and Classical swine fever virus (CSFV)[1]. Bovine viral diarrhoea (BVD) caused by either BVDV 1 or BVDV 2 is an infectious, multi-symptomatic disease which is responsible for significant economic losses in cattle and buffaloes[2].

The genome of BVDV consists of a single-stranded positive-sense RNA, about 12.3 kb long, which contains one reading frame coding for a polyprotein of about 3900 amino acids and is flanked at the 5′ and 3′ termini by untranslated regions (5′ UTR, 3′ UTR)[3]. For BVDV types 1 and 2, the length of the 5′ UTR varies between 372 and 385 nt, whereas it is 185–226 nt for the 3′ UTR. The 5′ UTR contains an internal ribosome entry site responsible for cap-independent initiation of viral protein synthesis. The 5′ UTR is highly conserved and is usually used in studying the differences between and within pestivirus species and genotypes, while the 3′ UTR is highly variable[4–7]. On the basis of the 5′ UTR sequence, BVDV 1 was initially divided into two major groups[4], BVDV 1a and BVDV 1b, and more extensive analysis later divided them into 11 groups[6]. Moreover, there was agreement between groupings[8–10] on the basis of 5′ UTR and other coding regions such as N^{pro} and E2.

Though BVDVs have been reported in various species of animals, and antibodies to pestiviruses have been detected in more than 40 ruminant species[11], information on pestivirus infection and genetic typing in buffaloes is limited, barring a single report[12]. Serological evidence of BVDV infection in buffaloes has been reported from Brazil[13], Pakistan[14], Egypt[15] and India[16]. We recently isolated BVDVs from Indian cattle[8,10] and genetically typed them as BVDV 1b. However, detection and typing of BVDV in Indian buffaloes is yet to be reported, though buffaloes and cattle contribute equally to livestock production systems in India.

Detection of BVDVs directly in clinical samples by RT–PCR and their subsequent typing is a useful and rapid approach as many cell lines get adventitiously contaminated with them[17]. Considering the importance of genetic heterogeneity in diagnosis, epidemiology and control of BVD in this study, we report the prevalence of BVDV 1 in buffaloes and their genetic relationship with cattle strains directly in clinical samples collected during the period 2001–05 from different geographical locations in India.

Blood samples from 219 cattle and 183 buffaloes obtained during 2001–05 from Orissa, Gujarat, Maharashtra, Uttar Pradesh and Punjab, were initially tested for BVDV using an antigen capture ELISA (Institut Porquier, France), four buffalo samples and eleven cattle samples were found positive. The animals were either apparently healthy or had a history of respiratory disorders, diarrhoea and reproductive problems. The list of samples analysed in this study is given in Table 1. Total RNA was extracted either from serum samples using QIAamp viral RNA kit (Qiagen) or from leukocytes using SV Total RNA isolation system (Promega), following manufacturer's recommendations. The cDNA synthesis was carried out using random hexamer primers (Promega) and the Superscript II reverse transcriptase (Invitrogen). A 288 bp DNA product was amplified from the 5′ UTR using primers 324 and 326 (ref. 18) and (Invitrogen) PCR reagents. The PCR products were recovered from agarose using the QIAquick gel extraction kit (Qiagen). The purified PCR products were cloned into pGEMT Easy vector (Promega) and three recombinant plasmids for each sample were sequenced in both strands using the *fmol* cycle sequencing kit (Promega) and SQ3 sequencer (Hoefer)[8].

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Table 1. Characteristics and origin of BVDV strains

Sample number	Species	Sex	Clinical sample	Place of origin
S-1315	Buffalo	Bull	Serum	Sabaramati, Gujarat
S-1335	Buffalo	Bull	Serum	Sabaramati, Gujarat
S-1773	Cattle	Bull	Serum	Sabaramati, Gujarat
S-1445	Cattle	Bull	Serum	Chipilima, Orissa
S-1453	Cattle	Bull	Serum	Chipilima, Orissa
4951	Cattle	Bull	Serum used for cell culture	Pune, Maharashtra
S-1783	Cattle	Bull	Serum	Anand, Gujarat
S-1448	Cattle	Bull	Serum	Chipilima, Orissa
S-2092	Buffalo	Bull	Serum	Salon, Uttar Pradesh
685	Cattle	Bull	Blood leukocytes	Bareilly, Uttar Pradesh
S-7156	Cattle	Cow	Serum	Mathura, Uttar Pradesh
S-7176	Cattle	Bull	Serum	Mathura, Uttar Pradesh
S-7180	Cattle	Bull	Serum	Agra, Uttar Pradesh
S-7199	Cattle	Cow	Serum	Agra, Uttar Pradesh
M 2014	Buffalo	Bull	Blood leukocytes	Ludhiana, Punjab

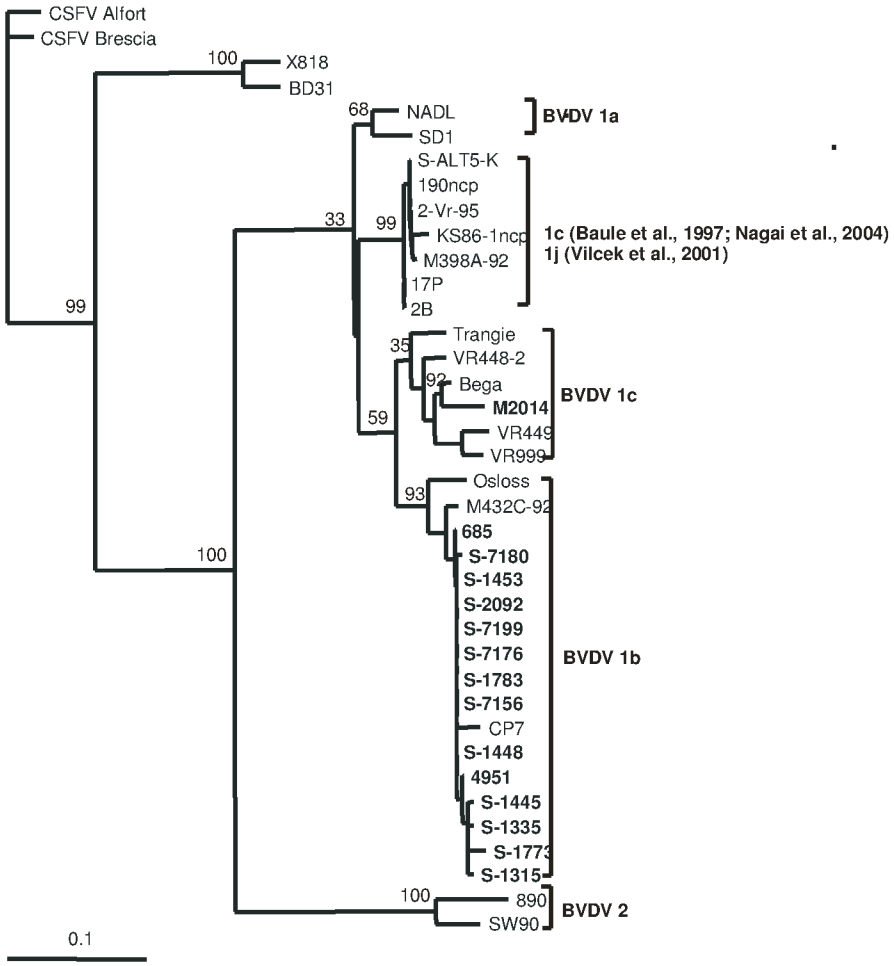

Figure 1. Neighbour joining tree of BVDV strains from Indian buffaloes and cattle in 5′ UTR. The distance was calculated using Kimura two-parameter method using transition/transversion ratio of 2.0. CSFV Alfort was used for rooting the tree. Numbers at the nodes represent bootstrap confidence values based on 1000 replicate datasets. The sequences reported in this study are available on request and labelled bold. Sequences of other strains were taken from GenBank with the following accession numbers: CSFV Alfort–J04358; CSFV Brescia–M31768; X818–AF037405; BD31–U70263; NADL–M31182; SD1–M96751; S-ALT5-K–U97474; 190ncp–AB019679; 2-Vr-95–AJ293594; KS86-1ncp–AB042713; M398A-92–U97444; 17P–AF244954; 2B–AF244957, Trangie–AF049222; VR448-2–AY763035; VR449–AY763034; VR999–AY762998; Osloss–M96687; M432C-92–U97447; CP7–U63479; 890–U18059, and SW90–AB003622.

The nucleotide sequences were assembled and proofread using the SEQMAN program of the DNASTAR computer program package (DNASTAR Inc., USA). The percentage of nucleotide identity was calculated using the MEGALIGN program of the DNASTAR package. For phylogenetic analysis, nucleotide sequences using 239 bp of the 5′ UTR (132–370 nt NADL) flanked by primers 324 and 326 were aligned utilizing the CLUSTAL X program[19]. Additional sequences from representative isolates of BVDV 1a, 1b, 1c, BVDV2, BDV and CSFV obtained from the NCBI database were included in the analysis. The phylogenetic tree was constructed using the NEIGHBOUR program from the PHYLIP package[20] based on Kimura two-parameter method using a transition/transversion ratio of 2.0. Statistical analysis of phylogenetic tree was carried out by bootstrap analysis on 1000 replicates using SEQBOOT and CONSENSE programs[20].

In this study, among the four buffalo strains three were typed as BVDV 1b and one as BVDV 1c (Figure 1). The Indian buffalo strain M2014 grouped with recently identified Australian cattle strains[7], VR448-2, VR449, VR999 and earlier strains of Bega and Trangie in the BVDV 1c branch. Nucleotide sequence comparison (Table 2) revealed that the M2014 strain showed maximum homology (93.7%) with Bega followed by VR448-2 (90.8%) and VR449 (89.1%). Homology with other three buffalo strains (1b) was about 80% and with cattle strains (1b) in the range of 79.8–81.9%. Homology between buffalo strains S-1315 and S-1335 from Sabaramati, Gujarat and S-2092 from Salon, Uttar Pradesh ranged between 98.7 and 99.2%, though they were from distant geographical locations; this suggests a common origin.

All the cattle strains obtained directly from clinical samples were typed as BVDV 1b (Figure 1), similar to our previous report[8] on BVDV isolates. Though they were collected from different geographical locations in India, there was little variation in the 5′ UTR and highest homology was observed with CP7 strain (97.1–98.3%) followed by M432C-92 strain from Mozambique. Strain 4951 was identified from cattle serum used for cultivation of cells in manufacturing veterinary vaccines. This stresses the need for mandatory testing of bovine serum in India before being used for cell cultivation. The adventitious contamination of cell lines and vaccines with BVDV and their transmission in causing outbreaks has been reported earlier[17,21].

The 5′ UTR-based studies provide meaningful phylogenetic results similar to coding region analysis[6,9]. Besides, it gets amplified efficiently from clinical samples. When the 5′ UTR sequences were analysed, Japanese strains KS86-1ncp and 190ncp[9] formed a cluster with South African strains M398A-92 and S-ALT5-K as BVDV 1c[5] along with 17P, 2B isolates (acc. nos AF244934 and AF244957) from Argentina and 2-Vr-95 isolate (acc. no. AJ293594) from Italy. Strains M065B and M1515A (similar to M398A-92 and S-ALT5-K) earlier[5] grouped in 1c were later[6] typed as 1j. The grouping of these strains in a cluster was also evident in our analysis, but was different from cluster 1c representing Indian buffalo strain. Our analysis grouped Bega and Trangie strains as subtype 1c similar to a recent study[7], in contrast to an earlier report[9] which classified them as 1a′. Our results support the nomenclature of BVDV 1 isolates previously described[6] and propose that Australian 1c isolates along with Indian buffalo strain M2014 are true representatives of 1c subtype and the strains earlier typed[5,9] as 1c actually belong to 1j or 1e.

Despite the limited number of samples studied here, the 5′ UTR phylogenetic analysis revealed that the cattle strains were of BVDV 1b subtype, whereas buffalo strains were of 1b and 1c, suggesting more heterogeneity. Interestingly, Indian buffalo strain M2014 showed closest relationship with Australian 1c strains, and Indian 1b strains from cattle and buffaloes showed maximum similarity with German strain CP7, suggesting that these viruses might have been introduced from these countries

Table 2. Percentage of 5′ UTR nucleotide sequence identity between Indian BVDV strains and other BVDVs strains using MegAlign program

	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
VR448-2 (1)	90.8	89.5	85.4	86.0	86.6	86.1	86.1	85.3	84.5	64.4	88.1	88.7	95.8	93.3	100
VR449 (2)	89.1	88.7	82.0	83.1	83.6	83.2	81.5	82.4	81.5	62.8	87.3	87.0	93.7	100	
Bega (3)	93.7	89.5	84.5	86.4	87.0	86.6	84.9	85.7	84.9	63.2	88.1	87.9	100		
190ncp (4)	82.0	89.5	87.9	86.9	87.4	87.0	85.3	86.1	85.3	66.5	99.6	100			
2B (5)	82.2	89.0	88.1	86.4	87.7	87.3	85.6	86.4	85.6	66.1	100				
890 (6)	60.3	65.3	64.9	65.7	65.1	64.7	62.6	63.9	62.6	100					
S-1773 (7)	79.8	84.9	92.4	96.6	97.9	98.3	96.2	98.3	100						
S-1335 (8)	80.7	85.7	93.7	97.5	98.7	99.2	97.1	100							
CP7 (9)	81.5	84.9	92.9	97.0	98.3	97.9	100								
4951 (10)	81.5	86.6	94.5	98.3	99.6	100									
S-7176 (11)	81.9	87.0	95.0	98.7	100										
685 (12)	81.4	86.4	94.5	100											
Osloss (13)	79.1	85.8	100												
NADL (14)	84.5	100													
M2014 (15)	100														

having long trade links with India. The only earlier report[12] of buffalo A1 isolate originating from Australia has also been typed as BVDV 1c. Though it could not be included in our analysis due to availability of only 131 nt of its 5′ UTR sequence, the probability of introduction of 1c viruses from Australia is more likely.

In conclusion, from this study we have established the prevalence of BVDV 1b and 1c subtypes in Indian buffaloes. There was a close relationship among 1b strains of cattle and buffaloes. Detection of BVDV directly in clinical samples and 5′ UTR sequence analysis may be a quick and easy method for identification of types and subtypes.

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