

## Genetic variety of bovine viral diarrhea virus 1 strains isolated from sheep and goats in India

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**Summary.** – Antigenic and genetic typing of pestiviruses isolated from Indian sheep and goats was carried out. Testing of 1777 sheep and 1026 goat blood samples collected between 2004 and 2008 resulted in isolation of twelve pestiviruses, seven from sheep and five from goats. All of them were antigenically typed as bovine viral diarrhea virus 1 (BVDV-1). Both the partial 5'-UTR and entire non-structural autoprotease (N<sup>pro</sup>) gene of the pestiviruses were amplified by RT-PCR and sequenced. The phylogenetic analysis confirmed all twelve sheep and goat pestiviruses as BVDV-1 and they were further classified into two subtypes, BVDV-1b (seven) and BVDV-1c (five). This is for the first time that BVDV-1c was detected in sheep and goats. However, no association between the subtype and geographic area of origin was observed. Although closely related, BVDV-1b and BVDV-1c isolates of sheep and goats were placed in a different clade than previously reported Indian BVDV-1b/BVDV-1c isolates. This study confirmed widespread prevalence of BVDV-1 in Indian sheep and goats that has significance in the epidemiology of bovine viral diarrhea.

**Keywords:** bovine viral diarrhea virus; BVDV-1; goat; N<sup>pro</sup>; genetic typing; sheep; 5'-UTR

### Introduction

Pestivirus infections in small ruminants can cause variety of clinical syndromes, including reproductive failure, abortion, still birth, respiratory disease, poor growth rate, diarrhea, neurological signs and muscular tremor, resulting in considerable economic losses (Nettleton *et al.* 1998). The genus *Pestivirus* belongs to the family *Flaviviridae* and comprises four accepted species: *Bovine viral diarrhea virus 1 (BVDV-1)*, *Bovine viral diarrhea virus 2 (BVDV-2)*, *Border disease virus (BDV)*, *Classical swine fever virus (CSFV)* and a tentative species, *Pestivirus of Giraffe* (Thiel *et al.*, 2005). Based on the cytopathic effect in cultured cells, pestiviruses

appear as two biotypes, non-cytopathic (ncp) and cytopathic. Only the ncp strains have the remarkable capability to establish lifelong persistent infections, which is of crucial importance for pestivirus maintenance and transmission. Historically, all pestivirus isolates originating from sheep and goats were referred as BDV. But now it is a fact that border disease (BD) in sheep and goats can be caused by BVDV-1, BVDV-2, and BDV (Paton *et al.*, 1995; Sullivan *et al.*, 1997; Pratelli *et al.*, 2001; Valdazo-Gonzalez *et al.*, 2006).

The genome of pestivirus is a single molecule of linear, positive sense RNA of approximately 12.3 kb in length flanked by UTR at the 5'- and 3'-end. A single ORF is translated into a polyprotein, which gets cleaved into four structural proteins and seven to eight non-structural proteins by cellular and viral proteases (Meyers and Thiel, 1996). The 5'-UTR is the most highly conserved region in the pestivirus genome. Hence, nucleotide sequence analysis of this region has been widely used for pestivirus diagnosis and genetic typing (Vilcek *et al.*, 1997, 2001; Valdazo-Gonzalez *et al.*, 2006; Jackova *et al.*, 2008). Additionally, N<sup>pro</sup> and E2 regions of the genome are useful for detailed phylogenetic analysis

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**Abbreviations:** BD = border disease; BDV = BD virus; BVDV = bovine viral diarrhea viruses; BVDV-1,2 = bovine viral diarrhea virus 1 and 2; IPMA = indirect immunoperoxidase monolayer assay; MAb(s) = monoclonal antibody(ies); ncp = non-cytopathic; N<sup>pro</sup> = non-structural autoprotease; SFTR = sheep fetal thymus cells

(Vilcek *et al.*, 2001; Becher *et al.*, 2003). The phylogenetic analysis of nucleotide sequences obtained from various regions of genome has led to identification of 15 subtypes within BVDV-1, 2 subtypes within BVDV-2 and at least 7 subtypes within BDV species (Vilcek *et al.*, 2001; Flores *et al.*, 2002; Jackova *et al.*, 2008; Mishra *et al.*, 2008b; Giammarioli *et al.*, 2011).

In India, sheep and goats contribute significantly to livestock economy and are reared mostly by marginal and landless farmers to sustain their livelihood. BVDV-1 is widely prevalent in Indian cattle (Mishra *et al.*, 2004). However, studies on genetic diversity of pestiviruses prevalent in small ruminants have been limited thus far, despite reports of sporadic occurrence of BVDV-2 (Mishra *et al.*, 2007, 2008b). Hence, the present study was aimed at genetic and antigenic typing of the pestivirus isolates obtained from sheep and goats collected from diverse geographical areas in India during the years 2004–2008.

### Materials and Methods

**Samples.** A total of 2803 blood and serum samples were collected between 2004 and 2008 from 1777 sheep in 92 flocks and 1026 goats in 63 flocks belonging to 13 states. The selection of flocks, sampling method and prevalence of pestivirus antibodies have previously been described (Mishra *et al.*, 2009). Seven blood samples of sheep and five blood samples of goats tested positive for pestivirus antigen by antigen capture ELISA (Institute Pourquier, France) and were processed for virus isolation and subsequent antigenic and genetic characterization. The pestivirus antigen-positive samples originated from seven states of India, six samples from northern region, two samples from southern region, one sample each from eastern and western region, and two samples from central region (Table 1).

**Virus isolation.** Three leukocyte samples and nine serum samples from antigen-positive sheep and goats were subjected to virus isolation using pestivirus-free permanent sheep cell line SFT-R, obtained from Cell Culture Collection of Veterinary Medicine, Friedrich-Loeffler Institute, Island of Riems, Germany, maintained in EMEM (Sigma) containing 10% horse serum (Invitrogen). The replication of pestivirus was demonstrated in the 2<sup>nd</sup> passage by immunoperoxidase test using a pool of pan-pestivirus reacting monoclonal antibodies (MAbs) WB103/WB105 (Veterinary laboratory Agency, UK), a peroxidase-linked secondary mouse antibody (Sigma) and substrate H<sub>2</sub>O<sub>2</sub> with 3-amino-9-ethyl carbazole (Sigma).

**Antigenic typing.** SFT-R cells were infected with the isolated pestiviruses, heat fixed and immunostained by IPMA using a selection of MAbs raised against BVDV-1, BVDV-2, and BDV as described previously (Mishra *et al.*, 2008b). BVDV-1-specific MAb 157, BVDV-2-specific MAbs BA2 and BA29 and BVDV-1 & 2 common MAb 348 were procured from VMRD, Pullman,

USA, while BDV-specific MAbs WS363 and WS371 were obtained from Veterinary Laboratory Agency, Weybridge, U.K. The staining intensity was graded as strongly reactive, weakly reactive or negative based on the reddish-brown intracellular staining of cells in the monolayer visible under the inverted microscope and from the recorded photographs.

**Extraction of viral RNA.** Viral RNA was extracted from original clinical samples (leukocyte or serum) using either RNEasy mini kit (Qiagen, Germany) or QIAamp viral RNA mini kit (Qiagen, Germany) following the manufacturer's protocols. Viral RNA from infected cell culture supernatants was extracted by QIAamp viral RNA mini kit (Qiagen, Germany). The RNA was recovered in 30 µl of RNase-free water and stored at -80°C until used.

**Amplification of 5'-UTR and N<sup>pro</sup> by RT-PCR.** Amplification of the 5'-UTR (288 bp) was carried out from the original clinical samples and isolates in one-step RT-PCR using primers 324 and 326 (Vilcek *et al.*, 1994) following our previously reported procedure (Mishra *et al.*, 2004). For amplification of complete N<sup>pro</sup> gene of the viral isolates, cDNA synthesis was carried out in 20 µl volume using random hexamer primers (Promega) and Superscript II reverse transcriptase (Invitrogen, USA). The PCR was carried out using primers 390F (5'-CTCTGC TGTACATGGCACATGGA-3'; Nagai *et al.*, 2004) and 1400R (5'-ACCAGTTGCACCAACCATG-3'; Becher *et al.*, 1997) and standard reagents (Invitrogen, USA) to amplify a 1080 bp fragment (position in BVDV strain SD1: nt 368–1448) covering entire N<sup>pro</sup>, C and a part of E<sup>ms</sup>. Amplified DNA products were detected by electrophoresis on an agarose gel stained with SYBR Safe (Invitrogen, USA). Separate rooms and dedicated materials were used for master mix preparation, RNA extraction, cDNA synthesis, DNA amplification and amplicon detection to prevent carry-over contamination.

**Nucleotide sequencing and sequence analysis.** The RT-PCR products of expected size were purified from agarose gel by QIAquick gel extraction kit (Qiagen) and directly sequenced in both directions using V.3.1 cycle sequencing kit (Applied Biosystems, USA) and ABI 3130 automatic DNA sequencer (ABI, USA), employing the same primers as used for RT-PCR. To obtain 5'-UTR sequences, RT-PCR products amplified from both the original samples and isolated viruses were sequenced. The corresponding overlapping sequences were assembled using SeqMan II program of Lasergene software (DNASTAR Inc., Madison, USA). The nucleotide sequences were submitted to GenBank and the Acc. Nos are shown in Table 1. Additional sequences were retrieved from NCBI database. Alignment of sequences for 239 bases in 5'-UTR and 504 bases in the entire N<sup>pro</sup> coding region was carried out using Clustal W program (Thompson *et al.*, 1994). Percentage of nucleotide and amino acid identity values were generated by MegAlign program of DNASTAR. Evolutionary distances were calculated employing the Kimura 2-parameter method of program DNADIST. The phylogenetic and bootstrap analyses were performed by using the NEIGHBOR and SEQBOOT programs from PHYLIP

**Table 1. History of the BVDV-1 isolates originating from Indian sheep and goats analyzed in this study**

Isolate	Species	Clinical signs	Sample	State	Year of isolation	Acc. No.	
						5'-UTR	N <sup>pro</sup>
Ind S-10241	Sheep	Pneumonia	Serum	Madhya Pradesh	2005	JQ679446	JQ710873
Ind S-11580	Goat	Healthy	Serum	Tamil Nadu	2005	JQ679447	JQ710874
Ind S-11600	Sheep	Diarrhea	Serum	Tamil Nadu	2005	JQ679448	JQ710875
Ind S/15734	Sheep	Healthy	Serum	Punjab	2006	JQ679449	JQ710876
Ind S-15815	Goat	Diarrhea	Serum	Punjab	2006	JQ679450	JQ710877
Ind S-17554	Goat	Nasal discharge	Serum	Himachal Pradesh	2006	JQ679451	JQ710878
Ind S-17555	Sheep	Nasal discharge	Serum	Himachal Pradesh	2006	JQ679452	JQ710879
Ind A12	Sheep	Abortion	Leukocytes	Maharashtra	2007	JQ679453	JQ710880
Ind 5237	Sheep	Pneumonia	Leukocytes	Uttar Pradesh	2007	JQ679454	JQ710881
Ind S-16020	Goat	Pneumonia	Serum	Uttar Pradesh	2007	JQ679455	JQ710882
Ind KG27	Goat	Nasal discharge	Leukocytes	Madhya Pradesh	2008	JQ679456	JQ710883
Ind S-18119	Sheep	Slow growth	Serum	Odisha	2005	JQ679457	JQ710884

(Felsenstein, 1993). Visualization of the tree was performed using the program TREEVIEW (Page, 1996).

## Results

### *Antigenic typing*

A total of twelve non-cytopathic pestiviruses were obtained from sheep ( $n = 7$ ) and goats ( $n = 5$ ) (Table 1). Ten viruses originated from animals showing various clinical signs such as slow growth rate, diarrhea, nasal discharge, pneumonia and abortion, while two viruses originated from apparently healthy animals. All twelve pestiviruses were recognized by a pan-pesti MAb pool (WB103/105) and reacted strongly with BVDV-1 specific MAb 157, but failed to react with BVDV-2 specific MAbs, BA2 and BA29 and BDV specific MAbs, WS363 and WS371. The reactivity with BVDV-1/2 common MAb 348 was variable to some extent. The results suggested that all the isolates belong to BVDV-1.

### *Genetic typing in 5'-UTR*

Analysis of 5'-UTR sequences obtained from the original clinical materials and the cell culture-derived isolates showed that the respective sequences were almost identical in clinical samples and virus isolates. A difference of only one or two nucleotides was observed, providing evidence that the pestivirus isolates were not a result of an adventitious contamination. Phylogenetic analysis of 239 bp 5'-UTR sequence (Fig. 1) typed all the seven sheep and five goat pestivirus isolates as BVDV-1. Of the twelve isolates, seven isolates (four from sheep and three from goats) were of BVDV-1b subtype. BVDV-1b

isolates from sheep originated from the states of Punjab (Ind S-15734), Himachal Pradesh (Ind S-17555), Uttar Pradesh (Ind 5237) and Tamil Nadu (Ind S-11600), while BVDV-1b goat isolates originated from Himachal Pradesh (Ind S-17554), Punjab (Ind S-15815) and Tamil Nadu (Ind S-11580). Though the isolates were derived from diverse geographical regions, a high level of genetic identity was observed among them. However, they were placed in a different clade from cattle BVDV-1b isolates (Fig. 1). The remaining five isolates (three from sheep and two from goats) were typed as BVDV-1c (Fig. 1). The sheep isolates originated from Maharashtra (Ind A-12), Odisha (Ind S-18119) and Madhya Pradesh (Ind S-10241), whereas goat isolates originated from Madhya Pradesh (Ind KG27) and Uttar Pradesh (Ind S-16020).

### *Genetic typing in N<sup>pro</sup>*

To confirm the genetic typing results in 5'-UTR additional genetic analysis for entire N<sup>pro</sup> gene was carried out. Similar to other pestiviruses, the N<sup>pro</sup> gene of Indian sheep and goat isolates comprised 504 nucleotides. The phylogenetic tree of the entire N<sup>pro</sup> gene (Fig 2) was supported by higher bootstrap values and revealed that all the seven sheep and five goat isolates were of BVDV-1b or BVDV-1c subtype. Highest nucleotide sequence identity in N<sup>pro</sup> was found with Indian BVDV-1b cattle isolate Ind S-1449 (95.4% to 98.2%). The BVDV-1c isolates of sheep and goats were found closer to yak BVDV-1c isolate but were placed in a different clade. The nucleotide sequence identity ranged from 95.2% to 96.4% (data not shown). When the amino acid sequence analysis of entire N<sup>pro</sup> was carried out, for BVDV-1b isolates, highest amino acid identity (>95%) was observed with Indian cattle isolate Ind S-1449, and for BVDV-1c isolates, maximum identity (94.2% to 95.8%) was

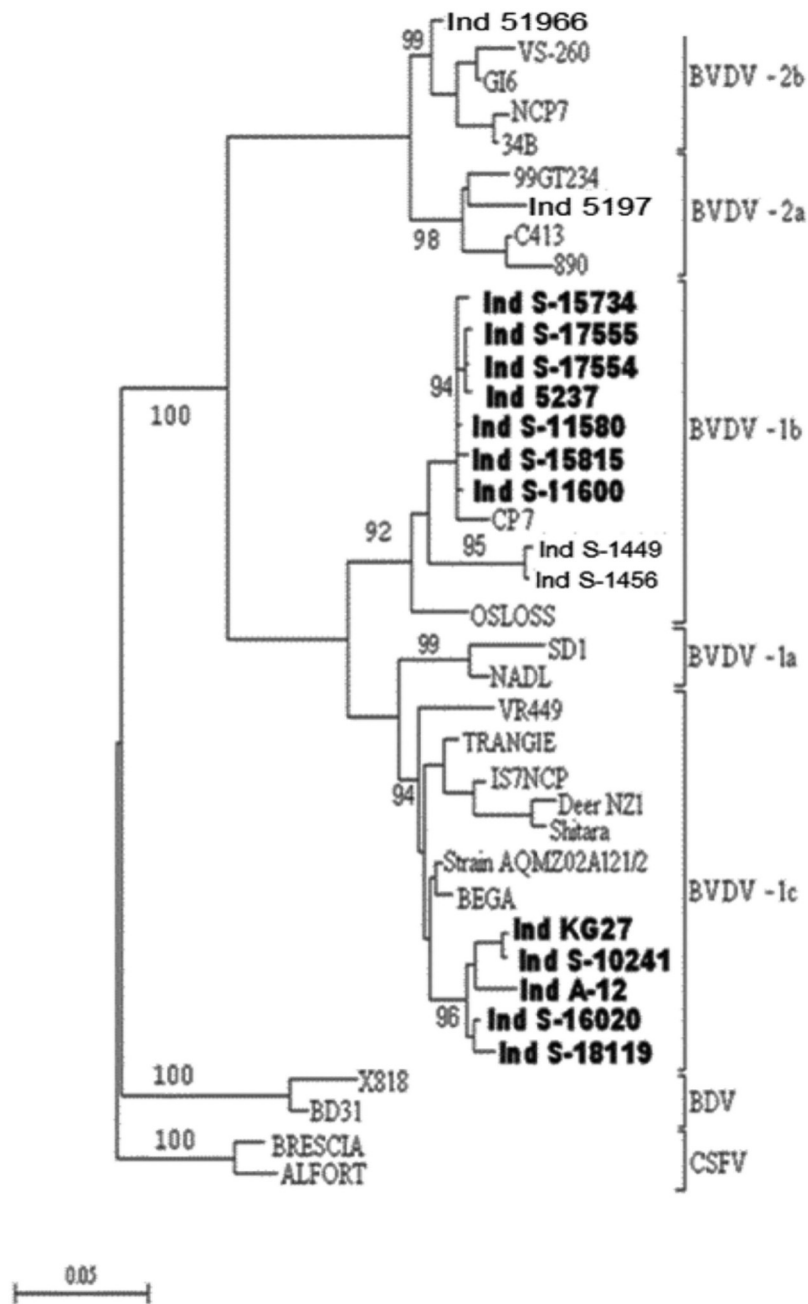


Fig. 1

## Genetic typing of Indian sheep and goat BVDV isolates in 5'-UTR

The tree was prepared using NJ method (kimura-2 parameter method). Numbers in nodes indicate the percentage of 1000 bootstrap replicates that support each group. Sequences were retrieved from the NCBI database with following Accession numbers: NADL-M31182; SD1-M96751; Osloss-M96687; CP7-U63479; Bega-AF049221; 519-AF144464; Deer NZ1-U80903; Trangie-AF049222; Shitara01/05-AB359926; IS7NCP-AB359925; Brescia-M31768; Alfort-J04358; X818-AF037405; BD31-U70263; 99GT234-DQ395297; 890-U18059; C413-AF002227; 34B-AF244952; NCP7-AY443026; GI6-AF144612; Ind S-1449-AY911670; Ind S-1456-AY911671; Ind 5197-EF547201; Ind 51966-EU371402.

observed with Yak isolate (data not shown). Alignment of N<sup>pro</sup> amino acid sequences with other pestivirus isolates revealed that all six cysteine residues were found also in

all BVDV-1b and BVDV-1c isolates of sheep and goats. In addition, similar to other pestiviruses, the putative catalytic residues E<sup>22</sup>, H<sup>49</sup>, and C<sup>69</sup> were found conserved.

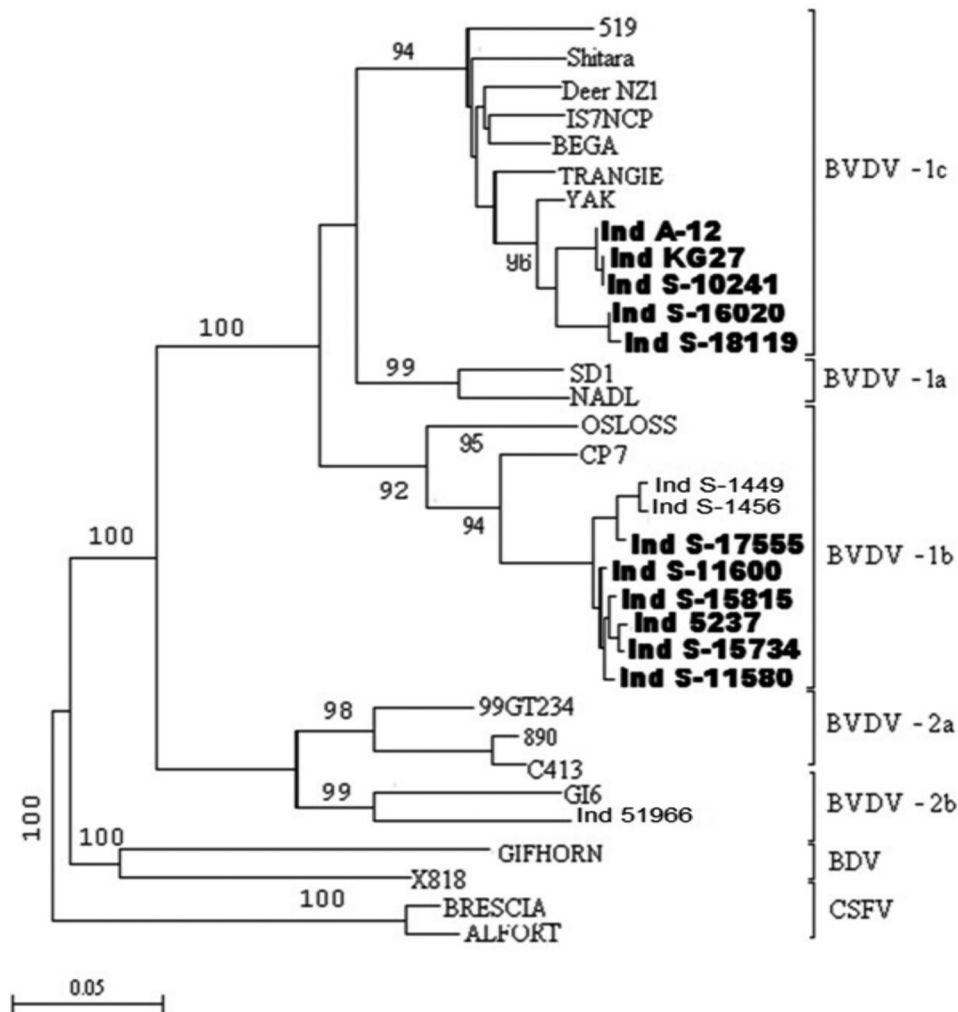


Fig. 2

Genetic typing of Indian sheep and goat BVDV isolates in entire N<sup>pro</sup> gene

The preparation of the NJ tree and sequences retrieved from NCBI database are similar as described for Fig. 1. Additional sequences are described below. Gifhorn-EU163653; Yak (Mishra *et al.*, 2008a). Due to the identical sequences obtained for Ind S-17554 and Ind S-17555, only Ind S-17555 has been shown in the figure.

## Discussion

The prevalence and economic impact of ruminant pestivirus infections in sheep and goat production is largely underestimated in India, despite their ability to cause reproductive and respiratory diseases. In this report, we determined and analyzed the nucleotide sequence of a fragment of the 5'-UTR and the entire N<sup>pro</sup> gene of twelve pestiviruses isolated from sheep and goats across diverse geographical regions during 2004–2008 in India.

The clinical signs of sheep and goats, from which pestiviruses were isolated, were diverse in agreement with those previously reported (Nettleton *et al.*, 1998; Pratelli *et al.*, 2001; Valdazo-Gonzalez *et al.* 2006). The typical clinical signs

of BD, hairy-shaker and neurological signs were however not observed, which emphasizes the difficulty of BD diagnosis based only on clinical signs. The situation gets further complicated especially in countries, where bluetongue, peste des petits ruminants, sheep pox and goat pox are endemic, as many of the clinical signs overlap.

Genetic typing of ruminant pestiviruses is important for molecular epidemiology and control programs and has been mostly based on the nucleotide sequence analysis of the 5'-UTR and N<sup>pro</sup> genomic regions (Vilcek *et al.*, 1997, 2001; Becher *et al.*, 1997, 2003; Nagai *et al.*, 2004). Earlier studies have shown sporadic occurrence of BVDV-2 in Indian goats and sheep (Mishra *et al.*, 2007, 2008b). All the seven sheep and five goat pestiviruses studied here were identified



as BVDV-1 on the basis of both 5'-UTR and N<sup>pro</sup> sequence analysis, which demonstrates that BVDV-1 infections are widespread in small ruminants. Prevalence of BVDV-1 in sheep and goats is, however, not uncommon, as ovine/caprine pestiviruses have been found to be BVDV-1 in U.K. (Vilcek *et al.*, 1997; Willoughby *et al.*, 2006), USA (Sullivan *et al.*, 1997), Italy (Pratelli *et al.*, 2001), Sweden (Vilcek *et al.*, 1997), Norway (Sandvik *et al.*, 2002) and Austria (Krametter-Froetscher *et al.*, 2010).

Of the BVDV-1 subtypes detected in sheep and goats, BVDV-1a has been found in U.K. (Vilcek *et al.*, 1997, Willoughby *et al.*, 2006), and BVDV-1b in Sweden (Vilcek *et al.*, 1997). Most (seven) of the Indian isolates analyzed in this study belong to subtype BVDV-1b. The phylogenetic analysis of sheep and goat BVDV-1b isolates suggested that in spite of their diverse geographical origins, they were genetically closely related to each other and also to the previously described BVDV-1b isolates from cattle (Mishra *et al.*, 2004). This can be explained by the fact that in India there is a frequent contact of small ruminants with cattle during farming, grazing and trade. Transmission of infection between small ruminants and cattle in both ways has been demonstrated, though usually it is from cattle to sheep or goats (Carlsson, 1991; Carlsson and Belak, 1994). The genetic typing results also revealed the first identification of BVDV-1c subtype in sheep and goats. Similar viruses at the genetic level have been detected in cattle, buffalo and deer from Germany (Becher *et al.*, 1997), in cattle from Australia (Mahoney *et al.*, 2005) and recently in yaks from India (Mishra *et al.*, 2008a). However, the origin of BVDV-1c in Indian sheep and goats is not clear, since yak BVDV-1c isolate originated from Arunachal Pradesh state in the Himalayan region, which is geographically distant from the states of Uttar Pradesh, Odisha, Madhya Pradesh and Maharashtra.

Different species of pestiviruses predominate in different countries depending on the close contact among ruminants. BD is caused by only BVDV-1 in Norway (Sandvik *et al.*, 2002) and Sweden (Vilcek *et al.*, 1997), only BDV in Australia, New Zealand and Spain (Vilcek *et al.*, 1998; Valdazo-Gonzalez *et al.* 2006), BDV and BVDV-1 in U.K. (Willoughby *et al.*, 2006) and Austria (Krametter-Froetscher *et al.*, 2010), only BVDV-2 in Korea (Kim *et al.*, 2006), BDV, BVDV-1, and BVDV-2 in USA (Sullivan *et al.*, 1997) and Italy (Pratelli *et al.*, 2001; Giammarioli *et al.*, 2011). The results provided in this study and previous reports suggest that BD is caused by both, BVDV-1 and BVDV-2 in India. Additionally, our results provide evidence of widespread prevalence of BVDV-1 in small ruminants. The identification BVDV-2 in goats and sheep earlier and BVDV-1 now warrants an intensive search for BDV.

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