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Short Communication

Identification and molecular characterization of novel and divergent HoBi-like pestiviruses from naturally infected cattle in India

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ABSTRACT

HoBi-like pestiviruses have been sporadically reported from naturally infected cattle in South America, Asia and Europe. While the closely related bovine viral diarrhoea virus 1 (BVDV-1) and BVDV-2 have been reported from cattle in India, the prevalence and diversity of HoBi-like viruses have not yet been studied. Here we report the genetic diversity and molecular characteristics of HoBi-like viruses, through systematic surveillance in cattle ($n = 1049$) from 21 dairy farms across India during 2012–2013. On the basis of real-time RT-PCR, virus isolation and nucleotide sequencing results, of the 20 pestivirus positive cattle, HoBi-like viruses were identified in 19 cattle from four farms in three states and BVDV-1b in one cattle. Phylogenetic analysis of 5′-UTR and N^{pro} region identified the circulation of two lineages of HoBi-like viruses in India, that were distinct to those circulating globally, highlighting the independent evolution of at least three lineages of HoBi-like viruses globally. Antigenic differences were also evident between the two Indian lineages. In addition to revealing that HoBi-like virus may be more widespread in Indian cattle than previously reported, this study shows greater genetic divergence of HoBi-like viruses indicating a need for continued pestivirus surveillance in cattle.

1. Introduction

Pestiviruses are economically important pathogens of livestock. The genus *Pestivirus* in the family *Flaviviridae* consists of four approved species: Bovine viral diarrhoea virus 1 (BVDV-1), Bovine viral diarrhoea virus 2 (BVDV-2), Classical swine fever virus (CSFV) and Border disease virus (BDV). But atypical bovine pestiviruses (termed HoBi-like pestiviruses), identified in cattle and buffaloes over the last decade have not yet been assigned to species (Pletnev et al., 2011). Based on genetic and antigenic analysis, HoBi-like pestiviruses are more closely related to BVDV-1 and BVDV-2 (Schirrmeier et al., 2004; Liu et al., 2009b, 2010; Decaro et al., 2011), and therefore they have been proposed to be classified as a new species, BVDV-3 (Liu et al., 2009b; Bauermann et al., 2013). BVDVs are major pestiviruses in bovine, particularly in cattle and are prevalent worldwide. Depending on virus

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strain and host factors, the clinical manifestation of BVDV infection ranges from apparently healthy to death. The pestivirus genome is single stranded, positive sense RNA of approximately 12.3 kb in length, flanked by untranslated regions (UTR) at both ends. The single open reading frame (ORF) expressed as a polyprotein, is cleaved into four structural proteins and seven to eight non-structural proteins (Meyers and Thiel, 1996).

Since the first detection of an atypical bovine pestivirus, strain D32/00-'HoBi', in foetal bovine serum (FBS) originating from Brazil (Schirrmeier et al., 2004), genetically similar HoBi-like viruses have frequently been identified in commercial FBS batches, mostly of South American origin but also originating from Mexico, Canada and Australia and in contaminated cells (Stalder et al., 2005; Stahl et al., 2010; Xia et al., 2011; Mao et al., 2012). Although less frequently reported than BVDV-1 and BVDV-2, natural infections with HoBi-like virus cause clinical disease similar to that induced by classical BVDV-1 and BVDV-2. However, the recent association of HoBi-like viruses with severe respiratory and reproductive disease in cattle (Decaro et al., 2011, 2012) has raised concerns.

Natural infection of cattle with HoBi-like viruses has been reported since mid 2000s in Brazil, Italy, Thailand and more recently in Bangladesh (Cortez et al., 2006; Stalder et al., 2007; Stahl et al., 2007; Kampa et al., 2009; Decaro et al., 2011; Haider et al., 2014). However, there is no information on prevalence of HoBi-like virus in India, a country with the highest cattle population in the world, whereas BVDV-1 has been reported to be widely prevalent and BVDV-2 sporadically (Mishra et al., 2004; Behera et al., 2011). Surveillance for the prevalence of BVDV in dairy cattle during 2012–2013 resulted in the detection of HoBi-like viruses in 19 cattle in four farms across three states in India. Nucleotide sequencing and phylogenetic analysis of 5′-UTR and N^{pro} gene region showed that two HoBi-like virus lineages that are distinct to previously reported HoBi-like viruses are co-circulating in cattle in India indicating circulation of at least three lineages of HoBi-like viruses globally.

2. Materials and methods

2.1. Origin of samples and clinical history

Whole blood (with and without K_2 EDTA) samples from each cattle ($n = 1049$) were collected from 21 dairy farms across eight States (Punjab, Haryana, Uttar Pradesh, Andhra Pradesh, Tamil Nadu, Chhattisgarh, Maharashtra and Gujarat) in India. Approximately, 20% of the animals from each farm were randomly sampled. The animals were of 4–36 months of age and were apparently healthy or have had a history of reproductive problems (abortion, stillbirth, early embryonic death, retention of placenta, pyometra, repeat breeding), respiratory problems (coughing, nasal discharge, pneumonia) or diarrhoea. BVDV vaccination has never been employed in any of these farms. Blood and serum samples from each animal were aseptically collected from the jugular vein in sterile vacutainers using separate needles and were shipped in ice to the laboratory within 48 h.

2.2. Real Time RT-PCR for detection and differentiation of pestiviruses

A TaqMan assay (Hoffmann et al., 2006) with minor modifications (in thermal profile) was used for pestivirus RNA detection. Viral RNA was extracted from leukocytes using RNeasy mini kit (Qiagen, Germany) following manufacturer's protocols and subjected to the panpesti TaqMan assay using Light Cycler 480 (Roche, USA). The assay targeting the 5′-UTR was conducted in 25 μl reaction volume using the primers BVD190-F (Hoffmann et al., 2006), V326 (Vilcek et al., 1994), probe TQ-Pesti (Gaede et al., 2005), 2 μl of RNA and Superscript III Platinum one-step real time RT-PCR reagent set (Invitrogen, USA). The details of primers and probes used in this study are listed in the supplementary Table 1.

Blood leukocytes found positive for pestivirus RNA by the panpesti TaqMan assay were then tested by TaqMan real time RT-PCR in uniplex format using primers and probes specific to BVDV-1, BVDV-2 and HoBi-like pestivirus, Light Cycler 480 and SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen, USA) as reported earlier (Baxi et al., 2006; Liu et al., 2008). For HoBi-like pestivirus detection, 800 nM of primers T134-F and T220-R and 400 nM of probe T155r-P (Liu et al., 2008) were used.

2.3. Virus isolation

Virus isolation was carried out on Madin Darby bovine kidney (MDBK) cells maintained in EMEM (Eagle's minimal essential medium, Sigma) containing 15% horse serum (Invitrogen). After 4 days incubation at 37 °C, the cultures were frozen thawed thrice and the clarified supernatant was passaged again on MDBK cells. The viral growth was then monitored by immunoperoxidase monolayer assay (IPMA) in 96-well TC plates, as described earlier (Mishra et al., 2008), using a pool of pan-pestivirus reacting monoclonal antibodies (mAbs) WB103/WB105 (Veterinary laboratory Agency, UK). Leukocytes obtained from all the sampled in-contact animals were also subjected to virus isolation.

2.4. Virus neutralization assay

Serum samples ($n = 367$) obtained from the four pestivirus-positive cattle herds, were heat inactivated at 56 °C for 30 min and 1:5 diluted serum was subjected to virus neutralization assay as described earlier (Mishra et al., 2008) using 96-well TC plates, MDBK cells and 200 $TCID_{50}$ of BVDV-1 cattle isolate Ind S-1449 (Mishra et al., 2004), BVDV-2 cattle isolate Ind 141353 (Behera et al., 2011) and HoBi-like virus isolate IndBHA5309/12 (this study). Ten randomly selected BVDV antibody positive serum samples from each farm were further tested at serial two-fold dilutions to determine the neutralizing antibody titre against BVDV-1, BVDV-2 and HoBi-like virus.

2.5. Antigenic characterization

For antigenic typing, a virus neutralization assay was performed using 200 $TCID_{50}$ of HoBi-like virus strain

IndBHA5309/12 and serial twofold dilutions of polyclonal sera against BVDV-1, BVDV-2, HoBi-like virus and BDV. Further antigenic characterization of HoBi-like viruses was carried out using a selection of mAbs raised against BVDV-1, BVDV-2 and BDV. The antisera raised against BVDV-1 and BVDV-2 were obtained from our earlier work (Mishra et al., 2008; Behera et al., 2011) and polyclonal serum against BDV strain X818 was a kind gift from P. Becher, Institut fur Virologie, Justus-Liebig-Universitat, Giessen, Germany. Serum obtained from a cow in this study, with an antibody titre of 1:320 against HoBi-like virus (8-fold higher than BVDV-1 and BVDV-2) was used as HoBi-like virus polyclonal serum.

2.6. RT-PCR amplification

RNA isolation was carried out from 140 μl of infected MDBK cells using QIAamp viral RNA mini kit (Qiagen, Germany) following the manufacturer's protocols. Amplification of 5′-UTR (248 bp) of HoBi-like viruses was carried out for the original leucocyte samples and isolates in a single step RT-PCR using primers TF1 (Liu et al., 2009a) and 326 (Vilcek et al., 1994) using SuperScript™ III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen, USA). The primers 324/326 (Vilcek et al., 1994) were used to amplify 5′-UTR (288 bp) of the BVDV-1 genome. For amplification of the complete N^{pro} gene, the cDNA synthesis was carried out in 20 μl volume as described earlier (Mishra et al., 2008). A 1080 bp fragment (position in BVDV strain SD1: nt 368–1448) covering the entire N^{pro}, C and N-terminal part of E^{rns} was amplified with primers 390F (Nagai et al., 2004) and 1400R (Becher et al., 1997).

2.7. Cloning, nucleotide sequencing and phylogenetic analysis

The 5′-UTR RT-PCR products were purified by QIAquick gel extraction kit (Qiagen) and were directly sequenced in both directions using gene specific primers, ABI PRISM Big Dye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI 3130 genetic analyzer (Applied Biosystems, USA) following the manufacturer's instructions. The 1080 bp DNA fragment of N^{pro}-C-E^{rns} was purified, cloned, using the pGEMT Easy TA cloning kit (Promega, USA) and three independent clones of each DNA fragment were sequenced in both directions employing M13 forward and reverse sequencing primers (Promega, USA).

The corresponding overlapping sequences were assembled using SeqMan II (DNASTAR Inc., Madison, USA). Additional pestivirus sequences were retrieved from NCBI GenBank and used in subsequent analyses. Sequence analysis was carried out for 239 nt of 5′-UTR, 504 nt of N^{pro} sequence and concatenated datasets of 5′-UTR and N^{pro}. The percentage of nucleotide identity values for both 5′-UTR and N^{pro} and amino acid identity values for N^{pro} were estimated by MegAlign (DNASTAR). Multiple sequence alignments were carried out using MUSCLE (Edgar, 2004) and manually optimized. Maximum likelihood (ML) phylogenetic trees were constructed using the GTR + gamma model using RAXML (Stamatakis et al., 2006). Phylogenetic supports were estimated using 1000 ML bootstrap replications (Stamatakis, 2006) and visualized using FigTree v.1.4 (Rambaut, 2012). Nucleotide sequences of HoBi-like viruses and the BVDV-1 isolate reported in this work were submitted to GenBank under Accession numbers KM201299–KM201318 and KM261863–KM261882 (supplementary Table 2).

3. Results

3.1. Identification and isolation of HoBi-like viruses

TaqMan real-time RT-PCR results showed that 20 (1.9%) of the total 1049 cattle blood samples, were positive for pestiviruses of which 19 were positive for HoBi-like virus and one was positive for BVDV-1. Isolation of all the 20 pestiviruses in culture revealed that they were of noncytopathic biotype. The HoBi-like viruses originated from four dairy farms spread across three states in western, central and northern India: Pune ($n = 10$) and Nagpur ($n = 4$) in Maharashtra, Rajnandgaon ($n = 4$) in Chhattisgarh state and Ludhiana ($n = 1$) in Punjab (Supplementary Table 2). The lone BVDV-1 isolate originated from a 6-month-old calf in the same dairy farm in Ludhiana, Punjab, indicating the co-circulation of BVDV-1 (IndMDV18697/12) and HoBi-like virus infections. The BVDV prevalence rates in these farms varied between 2.2% and 9.1%. In the four dairy farms from which HoBi-like virus was isolated, 24 (60%) of the 40 cattle tested (10 from each farm) exhibited >4–8-fold neutralizing antibody titres to HoBi-like virus than to BVDV-1 and BVDV-2 titres, while 4 cattle had >4-fold titres to BVDV-1 compared to BVDV-2 and HoBi-like virus titres, and 12 cattle had similar antibody titre against BVDV-1, BVDV-2 and HoBi-like virus (Table 1a).

3.2. Antigenic characterization

Neutralization test using polyclonal sera showed that the HoBi-like pestiviruses were efficiently neutralized by higher dilution of HoBi-like pestivirus polyclonal serum (field cattle serum) but could not be neutralized efficiently by BVDV-1, BVDV-2 or BDV polyclonal serum (Table 1b). With this polyclonal serum, a heterologous titre of 1:20 could be determined against the BVDV-1 or BVDV-2 strains. The mAb reactivity results of HoBi-like viruses (representative of one isolate from each farm) demonstrated that there are two mAb binding patterns, one for the viruses obtained from three farms (BHA, NAR and MDV) and the other for the viruses obtained from the fourth (ABI) farm (Table 2). Failure of reactivity with BVDV-1 specific and BDV specific mAbs and moderate reactivity with BVDV-2 specific mAb BA2 were more closely related to the reaction pattern of BVDV-2 strains.

3.3. Genetic characterization in 5′-UTR

The primer pairs, 324 and 326 (Supplementary Table 1) failed to detect the HoBi-like viruses in this study, although the primers TF1 and 326 could detect all of them. Sequencing of three independently amplified 5′-UTR amplicons, two from the original blood samples and one

Table 1a
Comparative virus neutralization titres of 40 cattle sera from four HoBi-like virus positive dairy farms.

Farm	Sera	Pestivirus strain		
		BVDV-1[a]	BVDV-2[b]	HoBi-like[c]
A	1	10	10	80
	2	80	20	10
	3	10	20	160
	4	40	40	320
	5	20	10	80
	6	10	10	320
	7	40	40	40
	8	20	20	20
	9	40	40	320
	10	80	40	320
B	1	40	40	40
	2	10	10	160
	3	160	40	20
	4	20	10	80
	5	80	80	80
	6	20	20	20
	7	40	80	640
	8	20	10	320
	9	20	20	20
	10	20	40	320
C	1	40	10	320
	2	160	80	1280
	3	80	80	640
	4	10	20	80
	5	40	40	40
	6	10	10	10
	7	40	40	640
	8	20	20	20
	9	20	10	80
	10	20	40	160
D	1	40	40	40
	2	640	80	80
	3	320	20	10
	4	20	40	1280
	5	80	40	320
	6	10	10	320
	7	20	10	80
	8	160	160	160
	9	10	10	10
	10	80	160	640

[a] Strain Ind S-1449.
[b] Strain Ind 141353.
[c] Strain IndBHA5309/12.

Table 1b
Neutralization titre of heterologous antiserum against HoBi-like virus from India.

Antiserum against pestivirus strain	HoBi-like virus (IndBHA5309/12)
BVDV-1 (Ind S-1449)	10
BVDV-2 (Ind 141353)	10
BDV (X818)	<10
HoBi-like virus[a]	320

[a] Serum from a field cattle in one of the HoBi-like virus infected farms.

from the pestivirus isolates showed all three were almost identical with a difference of only one or two nucleotides. Phylogenetic analysis of the 5′-UTR sequences of 19 newly identified HoBi-like viruses and representatives of major lineages identified five clusters within the HoBi-like virus clade with two co-circulating clusters in India, one

Table 2
Monoclonal antibody (mAb) reactivity pattern of the four representative HoBi-like virus isolates from India.

mAbs	Specificity	IndBHA 5309/12	IndNAR 108/12	IndMDV 18963/12	IndABI 15385/12
103/105	Panpestivirus	+++	+++	+++	+++
348	BVDV-1/BVDV-2	++	++	++	–
BA2	BVDV-2	++	++	++	++
WB166	BVDV/BDV	++	++	++	–
WB160	BVDV-1/BDV	++	++	++	++
157	BVDV-1	–	–	–	–
WS363	BDV	–	–	–	–
WS371	BDV	–	–	–	–
BA29	BVDV-2	–	–	–	–
WS538	BVDV-2	–	–	–	–

+++: Strong reaction; ++: Moderate reaction; –: No reaction.

containing all four isolates from a farm in Chhattisgarh state (IndABI15385/12, IndABI15987/12, IndABI16020/12, IndABI16023/12) and another consisting of all remaining HoBi-like isolates from two farms in Maharashtra state (e.g. IndBHA5296/12 and IndNAR0040/12) and from the farm in Punjab state (e.g. IndMDV18963/12 (data not shown). All the previously reported HoBi-like viruses from South America, Europe and Australia were grouped in a separate cluster. The Thai strain Th/04_Khonkaen and Bangladesh strain BGD/ZS5 were grouped into the fourth cluster, while the strains BGD/ZS1 and BGD/ZS3 from Bangladesh formed the fifth cluster. Indian HoBi-like virus isolates within the same cluster shared 99–100% sequence identity while the isolates between the clusters (IndBHA5309/12 cluster and IndABI15385/12 cluster) shared only 86.2% nucleotide sequence identity (Table 3). Surprisingly, viruses of the group represented by IndBHA5309/12 were the most divergent HoBi-like strains reported so far. The lone BVDV-1 isolate identified in this study was typed as BVDV-1b and showed highest (99.2%) nucleotide sequence identity with the Chinese cattle BVDV-1b strain T-482/99.

3.4. Genetic characterization of N^{pro} and concatenated datasets of 5′-UTR and N^{pro}

Additional phylogenetic analysis was carried out for full length N^{pro} gene sequences (data not shown) and for the concatenated datasets of 5′-UTR and N^{pro} (Fig. 1). Since N^{pro} gene sequences of HoBi-like viruses from Bangladesh have not yet been determined, they could not be included in this analysis. The results (Fig. 1) demonstrate that within the HoBi-like virus clade, the two novel Indian HoBi-like virus groups formed distinct phylogenetic groupings to each other and to all other previously reported HoBi-like viruses with strong support (98–100%). Overall, three HoBi-like virus lineages could be identified: the first lineage included previously reported HoBi-like viruses from South America, Europe, Australia and South East Asia, the second lineage included IndABI15385/12 group of viruses and the third lineage encompassed IndBHA5309/12 group of viruses from India. The nucleotide sequence identity with previously reported HoBi-like viruses in the N^{pro} was in the range of 79–80% for IndBHA5309/12 group of viruses and 84.3–85.1% for IndABI15385/12 group of

Table 3
Percentage nucleotide similarity between selected Indian HoBi-like viruses with other HoBi-like viruses and reference pestiviruses in 5′-UTR.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	
–	74.4	69.7	72.9	68.2	70.6	72.7	71.8	71.8	66.4	71.0	74.7	70.6	69.1	**1. NADL (BVDV-1)**
	–	79.9	70.3	80.1	77.8	72.8	79.5	80.8	77.8	77.8	66.2	77.8	78.0	**2.890 (BVDV-2)**
		–	71.2	90.7	86.2	76.0	91.3	90.9	90.5	86.2	68.4	86.2	90.0	**3. IndABI15385/12**
			–	69.9	72.9	73.7	71.6	72.0	70.8	72.9	81.8	72.9	71.6	**4. Alfort (CSFV)**
				–	86.4	75.4	99.2	99.6	93.6	86.4	67.4	86.4	90.0	**5. B1-Au (HoBi-like)**
					–	74.9	88.3	88.3	87.4	100	71.7	100	83.0	**6. IndBHA5309/12**
						–	76.0	76.0	75.2	74.9	72.6	74.9	74.8	**7. Girraffe-1 (Giraffe)**
							–	99.6	93.8	86.6	67.5	86.6	88.0	**8. D32/00_HoBi (HoBi-like)**
								–	93.4	88.3	67.1	88.3	90.0	**9.Italy 83-10ncp (HoBi-like)**
									–	87.0	67.9	68.4	91.0	**10. KhonKaen (Hobi-like)**
										–	71.3	100	83.1	**11. IndNAR108/12**
											–	71.7	67.0	**12. X818 (BDV)**
												–	83.0	**13. IndMDV18963/12**
													–	**14. BGD/ZS5 (HoBi-like)**

Indian HoBi-like viruses are labelled in bold italics. Nucleotide sequences were obtained from GenBank as provided in the legend to Fig. 1.

viruses (Table 4). The nucleotide sequence identity between the two Indian lineages was 79.6%, while the predicted amino acid sequence identity was 83.9%. Alignment of the predicted amino acid sequences of whole length N^{pro} (168 amino acids) with other pestiviruses revealed that instead of six cysteine residues found conserved for all other pestiviruses (BVDV-1, BVDV-2, BDV, CSFV, Giraffe), HoBi-like viruses contained five cysteine residues and a **C**106**S** substitution. Additionally, of the seven C-terminal amino acids of N^{pro} (**PIWVASC**) that were conserved across all previously reported HoBi-like viruses, **PIWVTSC** were found conserved in Indian HoBi-like pestiviruses.

4. Discussion

Here, we described the first identification and molecular characterization of two divergent lineages of HoBi-like viruses originating from naturally infected cattle in India. HoBi-like viruses were identified in four premises within three states; two in the west, one in the central and another in the north of India, while BVDV-1b was detected in one premise. Previous studies showed that BVDV-1 is predominantly prevalent and BVDV-2 occurs sporadically in Indian cattle (Mishra et al., 2004; Behera et al., 2011). Predominant detection of emergent HoBi-like viruses over BVDV-1 in this study is rather surprising. Although such a situation is not unlikely, the most realistic explanation of this observation can be attributed to the lack of continuous well-designed BVDV surveillance data in India for comparison. The majority of animals from which HoBi-like viruses were detected in this study, had history of reproductive disease, respiratory disease or diarrhoea, while some animals were apparently healthy consistent with previous reports (Cortez et al., 2006; Decaro et al., 2011, 2012).

It is interesting that two novel and divergent groups of HoBi-like viruses are co-circulating in India. A single lineage of HoBi-like viruses originating from different

Table 4
Percentage nucleotide and amino acid (bold and italics) similarities between selected HoBi-like strains from India with other HoBi-like strains and reference pestiviruses in the entire N^{pro} gene.

1	2	3	4	5	6	7	8	9	10	11	
–	66.7 (67.9) –	67.3 (70.8) 68.7 (69.6) –	63.5 (64.9) 79.6 (83.9) 66.9 (69.6) –	67.3 (68.5) 84.3 (88.7) 68.1 (69.0) 80.0 (85.7) –	66.9 (67.3) 67.1 (66.1) 67.1 (68.5) 63.1 (63.1) 66.3 (64.9) –	66.5 (67.9) 84.5 (90.5) 68.5 (69.6) 79.4 (86.3) 95.8 (96.4) 66.5 (64.9) –	67.1 (68.5) 84.3 (89.3) 68.3 (69.6) 79.6 (86.9) 96.0 (96.4) 65.7 (65.5) 96.2 (98.2) –	66.3 (69.0) 85.1 (88.7) 67.7 (69.6) 79.0 (85.1) 90.1 (92.3) 66.3 (64.9) 91.1 (94.6) 90.1 (94.0) –	68.8 (72.0) 66.5 (67.3) 67.1 (70.2) 66.3 (64.9) 66.9 (66.7) 69.2 (69.0) 66.3 (66.1) 67.5 (67.3) 67.7 (65.5) –	69.0 (69.0) 68.3 (68.5) 72.2 (73.8) 67.3 (66.7) 66.7 (67.9) 66.9 (70.2) 67.3 (67.9) 68.1 (68.5) 68.5 (69.6) 68.5 (70.2) –	**1. 890 (BVDV-2)** 2. **IndABI15385/12** **3. Alfort (CSFV)** **4. IndBHA5309/12** **5. Brz buf 9 (HoBi-like)** **6. Girraffe-1 (Giraffe)** **7. D32/00_HoBi** **8. Italy-83/10ncp (HoBi-like)** **9. Th/04KhonKaen (HoBi-like)** **10. NADL (BVDV-1)** **11. X818 (BDV)**

Indian HoBi-like viruses are labelled bold and underlined.

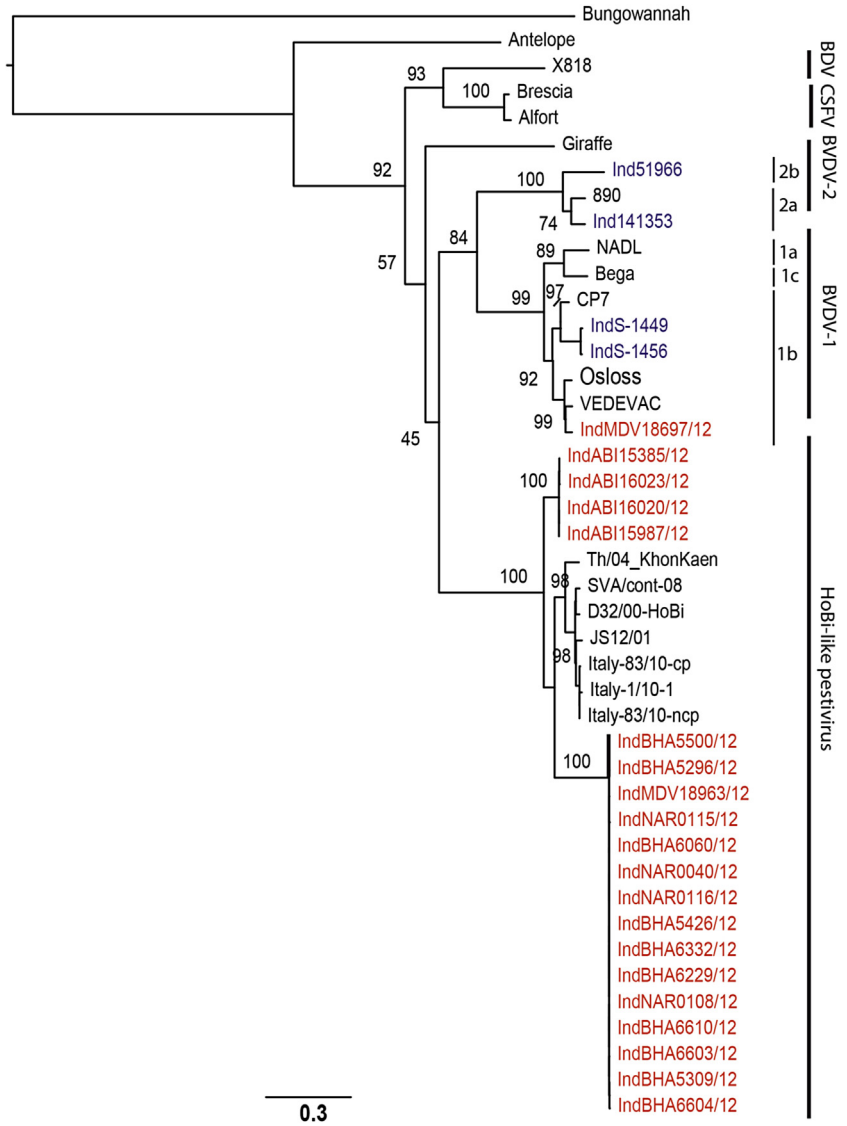

Fig. 1. Phylogenetic tree based on the combined datasets of 5′-UTR and N^{pro} sequences of HoBi-like pestiviruses analyzed in this study and other pestiviruses. The maximum likelihood tree was generated using concatenated datasets of 5′-UTR and N^{pro} under the GTR + gamma substitution model in RAXML (Stamatakis et al., 2006). Numbers indicate the percentage of 1000 bootstrap replicates that support each phylogenetic branch. Reference 5′-UTR-N^{pro} sequences and their GenBank accession numbers: HoBi-like pestivirus strains: Italy-1/10-1 (HQ231763), Italy-83/10ncp (JQ612704), Italy-83/10cp (JQ612705), JS12/01 (JX469119), Th/04_KhonKaen (FJ040215), SVA/cont-08 (FJ232693), D32/00_HoBi (AY735486), BVDV-1 strains: NADL (M31182), Osloss (M96687), CP7 (U63479), VEDEVAC (KC695814), Bega (AF049221), Ind S-1449 (AY911670), Ind S-1456 (AY911671); BVDV-2 strains: 890 (U18059), Ind 141353 (HQ444199), Ind 51966 (EU371402); BDV strains: X818 (AF037405), CSFV strains: Alfort (J04358), Brescia (M31768); Pestivirus of giraffe H138 (AF144617); Pronghorn antelope (AY781152); Bungowannah (DQ901403). The isolates obtained in this study are labelled red and previously reported Indian isolates are labelled blue.

farms in western and northern India were phylogenetically closely related (99–100% sequence identity) and clustered into a single group indicating links between three dairy farms. Although this could not be proved directly due to lack of data regarding movement/purchase of animals between the farms, and these farms were located 500–2000 km apart, a common source of infection is likely. Whereas the HoBi-like viruses originating from another herd in central India were phylogenetically divergent from the other group suggesting two separate introductions of HoBi-like viruses in India. The phenomenon of clustering of BVDV strains on the same farm or in different farms has been noticed previously (Ridpath et al., 2006). Although the source of introduction into India is unknown, on the basis of 5′-UTR sequences, one group of Indian HoBi-like viruses were found more closely related to the Th/04_KhonKaen strain from Thailand and the strains from Bangladesh indicating probable introduction from these two countries. However, independent evolution of HoBi-like viruses in India is also likely, since another group of viruses were found to be the most highly divergent strains identified so far.

It has been recommended that, at least two regions of the BVDV genome (5′-UTR and N^{pro} or E2) should be analyzed and agreement sought between them in order to define the relationships of isolates investigated with some certainty (Becher et al., 1997, 1999). Since phylogenetic trees based upon the entire N^{pro} sequences or the datasets combining 5′UTR and N^{pro} genetic regions of pestiviruses provide increased statistical support, this region has been found useful for subtype classification (Becher et al., 1997; Liu et al., 2009b). Till date, all the previously reported HoBi-like viruses except the Thai and Bangladesh strains are very closely related genetically (Liu et al., 2009b; Bauermann et al., 2013; Haider et al., 2014). Based on the results of this study, and the subtype assignment criteria of Becher et al. (1999), we propose that HoBi-like viruses can be classified into three subtypes (a, b and c), subtype 'a' consisting of all the previously reported HoBi-like strains including the strain Th/04_Khonkaen, subtype 'b' consisting of four Indian strains (Ind IndABI15385/12 group) and subtype 'c' encompassing fifteen Indian strains (IndBHA5309/12 group). This division is also supported by their antigenic reactivity patterns. Moderate reactivity with the BVDV-1/BDV specific mAb WB160 found for Indian HoBi-like strains was in contrast to the lack of reactivity with the reference strain D32/00_HoBi noticed earlier (Schirrmeier et al., 2004), which indicates differences in the mAb reactivity pattern among HoBi-like viruses.

In conclusion, this study extends our knowledge on the epidemiology and genetic heterogeneity among the HoBi-like viruses, highlighting the global circulation and independent evolution of at least three groups of HoBi-like viruses with two groups in India. The increased and improved surveillance for HoBi-like viruses around the world will undoubtedly reveal additional genetic diversity in future. The increasing reports of HoBi-like viruses from cattle in the field suggest that natural infection of cattle with HoBi-like virus may be more widespread than previously thought. Considering the growing evidence of occurrence of HoBi-like pestiviruses in geographically distant cattle populations, these emerging viruses present considerable risk to the cattle health and management and BVD control programmes. The identification of HoBi-like virus in Indian cattle emphasizes the need for continued monitoring besides determining the extent of economic losses it can cause in dairy farming.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2014.09.017.

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