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# Genetic detection and characterization of emerging HoBi-like viruses in archival foetal bovine serum batches

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ABSTRACT

Bovine viral diarrhea viruses (BVDV) are members of the *Pestivirus* genus within the family *Flaviviridae*. Based on antigenic and nucleotide differences, BVDV are classified into two recognized species, BVDV-1 and BVDV-2. More recently, a new putative pestivirus species, tentatively called "HoBi-like", has been associated with bovine viral diarrhea. HoBi-like viruses were first identified in fetal bovine serum (FBS) imported from Brazil. Subsequently, a number of HoBi-like viruses have been detected as contaminants in FBS or cell culture and in live ruminants. To further investigate the possible pestivirus contamination in commercially available FBS batches, 26 batches of FBS with various countries of origin, were tested in this study for the presence of bovine pestiviruses. All the 26 batches were positive by RT-PCR for at least one species of bovine pestiviruses. HoBi-like viruses were detected in 15 batches. Analysis of the 5′UTR and $N^{pro}$ sequences of 15 newly identified HoBi-like viruses combined with analysis of additional sequences from GenBank, identified 4 genetic groups tentatively named 3a–3d. The current study confirmed the presence of the emerging HoBi-like viruses in FBS products labeled with different geographic origins. This finding has obvious implications for the safety of biological products, such cell lines and vaccines.



## 1. Introduction

*Pestivirus* is a genus within the family *Flaviviridae*, composed of four recognized species, Bovine viral diarrhea virus 1 and 2 (BVDV-1 and BVDV-2), Classical swine fever virus (CSFV), and Border disease virus (BDV) [1]. BVDV are major pathogen of cattle, and infection results in significant economic loss worldwide. Pestivirus genomes consist of a single-stranded, positive sense RNA approximately 12.3 kb in length. A single open reading frame that encodes 11-12 structural and nonstructural proteins is flanked at either end by 5′ and 3′ untranslated region (5′UTR and 3′UTR). Of these, the non-structural amino terminal autoprotease $N^{pro}$ and the major envelope glycoprotein E2 have most commonly been used for genetic classification of new virus isolates together with the 5′UTR region which represents the most important and useful region both for diagnostic purpose and sequence analysis [2–7]. Besides the established BVDV-1 and BVDV-2 species, an additional group of unclassified pestiviruses consisting of "atypical" pestiviruses of bovine origin has been identified as a putative new species of pestivirus. Tentatively called "HoBi-like viruses" or "atypical pestiviruses", the type virus of this putative species is the strain D32/00_'HoBi', which was isolated from a batch of foetal bovine serum (FBS) imported from Brazil [8]. Subsequent to the isolation of the type virus, a number of other HoBi-like viruses have been detected as contaminants in FBS or cell culture, including CH-KaHo/cont [9], SVA/cont-08 [10], IZSPLV_To [11] and JS12/01 [12] as well as in live ruminants, such as Brz buf 9 described in a Brazilian buffalo [9] and Th/04_KhonKaen in calf in Thailand [13–15]. Natural infection in cattle with HoBi-like viral strain Italy-1/10-1 has been reported in Italy causing an outbreak of severe respiratory disease in calves. The same strain was subsequently isolated from aborted bovine foetuses collected from the same production unit [16,17]. More recently, HoBi-like viruses have been identified from naturally infected cattle also in India [18] and Bangladesh [19].

Because the clinical presentation in cattle is indistinguishable from that seen following BVDV1 and BVDV2 infection, BVDV-3 has

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been proposed as a name for this species. However, to date the International Committee on Viral Taxonomy has not yet arrived at a decision to recognize HoBi-like viruses as an official species within the Pestivirus genus. The earliest samples from which HoBi-like viruses have been isolated were collected in 2002. However, as two similar viruses were detected in aborted foetuses from Brazil in a retrospective study [20], it is likely that emergence was earlier.

Most of the HoBi-like viruses, isolated to date, originate from South America and Italy. Recently, HoBi-like viruses have been identified in the Indian subcontinent [18,19] and, in addition, two European laboratories independently detected atypical pestiviral nucleic acids in three commercial batches of FBS that were claimed to be of Australian origin, suggesting a much wider distribution than previously thought [21].

Because the HoBi-like viruses isolated from FBS, originating from South America, and the HoBi-like viruses isolated from cattle in Italy are so similar it has been proposed that HoBi-like viruses were introduced into Europe via the use of contaminated FBS.

While HoBi-like viruses are related to BVDV1 and BVDV2 at the genetic and antigenic levels, current BVDV diagnostic tests may fail to detect HoBi-like viruses or to differentiate between BVDV and HoBi-like viruses. Although the genetic diversity of HoBi-like viruses is not fully known, it is probable that, like other member of the pestivirus genus, they will be genetically diverse. The objective of this study was to test batches of commercial FBS for bovine pestiviruses and to examine the genetic diversity of HoBi-like viruses detected.

## 2. Materials and methods

The current study included samples from 26 archival batches of FBS obtained over the period 1992–2013, according to the certificate of analysis claimed by commercial undisclosed suppliers. Samples were from filtered, gamma-irradiated and non-gamma-irradiated batches of FBS.

Our genetic study was based on the 5′-UTR, supported by comparison within the $N^{pro}$ coding region. Viral RNA was extracted from 1 ml of serum using the QIAamp UltraSens Virus Kit (Qiagen). RT-PCR targeting a 221 bp segment of the 5′UTR was performed using the primer pair B5/B6 and a reaction protocol as previously described [22]. The partial $N^{pro}$ region was amplified using the primers SF1/SR1 [10] targeting a 753 bp DNA fragment. In addition to the above described methods, all the samples were screened by the one-step multiplex real-time RT-PCR using primers and type-specific (BVDV-1 and BVDV-2) TaqMan probes designed in the 5′UTR region of the pestivirus genome, as previously reported [23].

The amplicons were purified and sequenced on both strands using an ABI PRISM 3130 sequencing device (Applied Biosystems, Foster City, CA). The sequencing reads were assembled using SeqMan software within Lasergene package (DNASTAR Inc., Madison, WI). For phylogenetic analysis, additional sequences of atypical pestiviruses were retrieved from GenBank of both 5′UTR ($n = 56$) and $N^{pro}$ ($n = 37$) genomic regions. Multiple sequence alignment was done using Bioedit program, version v.7.0.5.2. Phylogenetic analysis was performed using the MEGA6 program.

## 3. Results

All 26 batches of FBS were found containing at least one species of bovine pestiviruses. Twenty batches were found positive for BVDV-1, 10 batches positive for BVDV-2, and 15 positive for HoBi-like viruses. Of these 15 batches, 7 were labeled as originating in South America and one from Australia. The country of origin was not identified for the remaining 7 batches (Table 1). The neighbour-joining analysis revealed that the 5′UTR sequences formed a well supported group together with viruses originating in cattle samples from South America and Europe or found as contaminants of FBS (Fig. 1). To confirm the grouping found in the 5′UTR, we analyzed the $N^{pro}$ region. The resulting phylogenetic tree showed that these viruses clustered in the same phylogenetic group as those seen in the 5′UTR based tree (Fig. 2). All the newly determined sequences have been deposited in GenBank (accession numbers are shown in the Table 1).

**Table 1**
The batches of foetal bovine serum tested in this study. Samples with negative results in HoBi-like virus RT-PCR are not displayed.

| Sample ID | Year | Origin | Accession no. | |
|---|---|---|---|---|
| | | | 5′UTR | $N^{pro}$ |
| 213/9SB031/10 | 2010 | South America | LN812236 | LN812251 |
| 214/9SB022/10 | 2010 | South America | LN812237 | LN812252 |
| 215/9SB025/10 | 2010 | South America | LN812238 | LN812253 |
| 216/41G655OK/06 | 2006 | Unknown | LN812239 | LN812254 |
| 217/1433088K/13 | 2013 | South America | LN812240 | LN812255 |
| 218/54040013/13 | 2013 | Unknown | LN812241 | LN812256 |
| 219/41Q1696K/09 | 2009 | South America | LN812242 | LN812257 |
| 220/41G1099K/09 | 2009 | South America | LN812243 | LN812258 |
| 221/9SB029/08 | 2008 | South America | LN812244 | LN812259 |
| 223/S0081550/08 | 2008 | Unknown | LN812245 | LN812260 |
| 224/0061162/06 | 2006 | Unknown | LN812246 | LN812261 |
| 225/0080692/08 | 2008 | Australia | LN812247 | LN812262 |
| 226/0102381/10 | 2010 | Unknown | LN812248 | LN812263 |
| 227/0071174/07 | 2007 | Unknown | LN812249 | LN812264 |
| 228/21123/92 | 1992 | Unknown | LN812250 | LN812265 |

Phylogenetic analysis of the 5′UTR sequences of 15 newly identified HoBi-like viruses combined with analysis of additional sequences from GenBank, identified 4 genetic groups tentatively named 3a-3d. Group 3a includes the type virus D32/00_'HoBi' strain together with viruses originating in cattle samples from South America and Europe or found as contaminants of FBS. Groups 3c and 3d contained viruses co-circulating in India; viruses originating from Bangladesh were mainly grouped in a separate genetic group 3b, with the exception of BGD/ZS5 strain which clustered within genetic group 3c. Sequence identity within and between genetic groups is shown in the Table 2. Interestingly, according to the criterion adopted for the segregation into genetic groups, the Thai isolate Th/04_KhonKaen did not clearly fall into any of the above mentioned groups, displaying the higher inter-group identity with groups 3a and 3c. This is even more evident in the corresponding radial tree (data not shown). Therefore, the typing of this virus is not yet clear and further analysis needs to be done. Despite the fact that 5′UTR is the most conserved region of the pestivirus genome, and fewer mutations are involved in the mathematic algorithm of the phylogenetic analysis, genetic typing using $N^{pro}$ sequences lead to identical grouping with regard to branches 3a, 3c, and 3d with similar bootstrap values. The genetic group 3b was instead not represented in the $N^{pro}$ based tree, because the corresponding sequences of isolates from Bangladesh were unfortunately not available (Fig. 2).

## 4. Discussion

FBS is used to supplement the growth media for culturing of cells used in the propagation of viruses that are employed in research and vaccines. In addition, it is frequently used in the process of embryo transplant. BVDV contamination of both animal and human vaccines has been reported [24–31]. Further, BVDV contamination of cattle vaccines has led to fetal infections resulting in persistent infection [31] and severe disease including mortality in adults [25,26]. BVDV contamination of cells used in research has

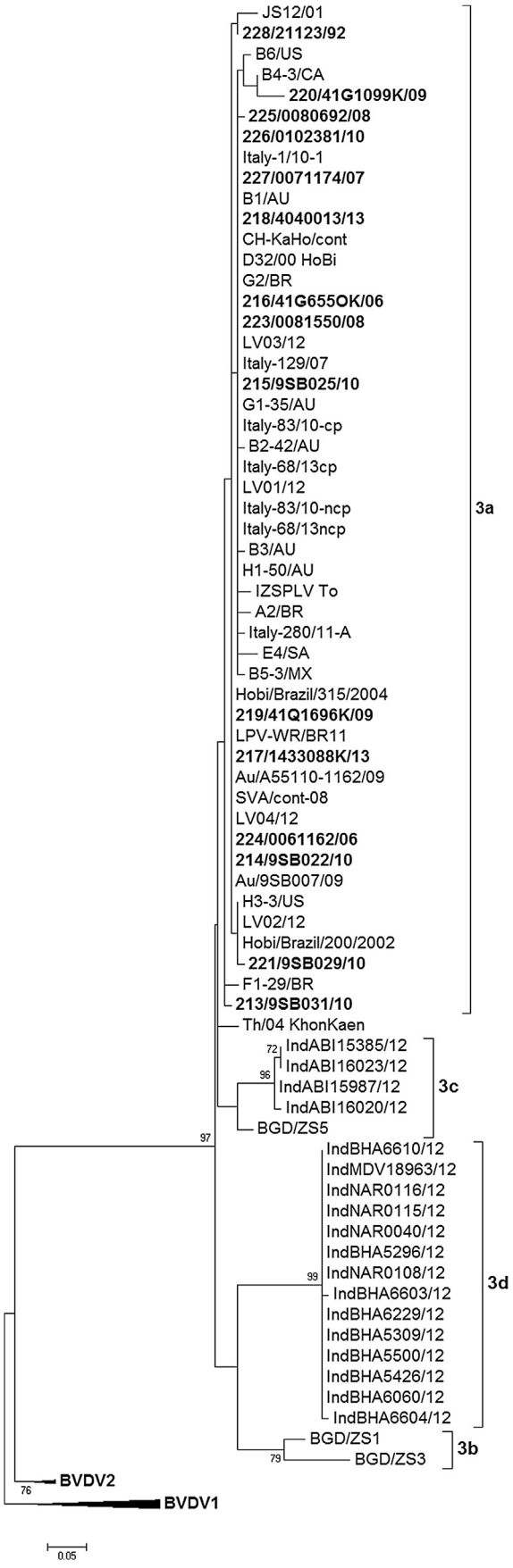


**Fig. 1.** Phylogenetic tree based on the 5′UTR sequences. Molecular evolutionary genetics analyses were performed with MEGA6 using the NJ method. Distances were computed using the Kimura two-parameter model. The numbers close to the major nodes indicate the bootstrap values (in %; 10,000 replicates). The tree describes the relationship between 5′UTR sequences retrieved from the GenBank database and the sequences analyzed in this work (labeled in bold). Bar: number of substitutions per site.

also impacted research results [32,33]. While HoBi-like viruses were first isolated from FBS, little is known about the prevalence of HoBi-like contamination of FBS. In the current study, 26 commercial batches of FBS were screened for the presence of pestiviruses. All 26 batches of FBS were found containing at least one species of bovine pestiviruses. Twenty batches having BVDV1, 10 batches having BVDV2, and 15 batches having HoBi-like viruses.

The international trade in FBS may provide a route for introduction of bovine pestivirus species and subgenotypes into new regions. Outside of some Scandinavian countries, which are free due to successful eradication programs, BVDV are endemic worldwide. However, there appears to be regional differences in the prevalence of BVDV species and subgenotypes [34–53]. In contrast to BVDV, the endemic regions for HoBi-like viruses appear to be much more restricted. Further, it appears that there may be regional differences in the presence of HoBi-like virus subgenotypes. Initial limited studies of the genetic distance of HoBi-like pestiviruses suggested the presence of an independent evolution of 3 lineages [18]. Comparison of HoBi-like virus sequences previously submitted to GenBank, including recently characterized viruses from India and Bangladesh, and HoBi-like sequences generated in this study suggest the global circulation of at least 4 genetic subgroups, tentatively named 3a-3d. As some batches of the tested FBS were combined and packages within European Union in plants that handled FBS from multiple geographic regions, there is the possibility of contaminating FBS from different sources. As a consequence we cannot speculate on the genuine country of origin. Nevertheless, the grouping obtained by our genetic analysis revealed a particular clustering pattern consisting in three distinct genetic groups originating from the Indian subcontinent (3b, 3c and 3d) and a fourth group 3a which includes all the Hobi-like viruses isolated from samples collected outside the Indian subcontinent.

The origin of HoBi-like pestivirus is unknown. Interestingly, strain 228/21123/92, compared in this study, was detected during analysis of FBS of unknown origin dating to 1992, indicating the presence of HoBi-like virus in cattle many years earlier than previously reported. It has been hypothesized that HoBi-like viruses originated in South America and were introduced into Europe (Italy) and South-East Asia (Thailand) through biological products like FBS, vaccines and semen [54]. However, the range of the genetic differences seen between South American/European isolates and those coming out of India and Bangladesh suggest that this emerging pestivirus species has been circulating in the Indian subcontinent for a significant period of time independently of the HoBi-like viruses circulating elsewhere.

In this study all of the FBS batches tested were positive for bovine pestiviruses. While irradiation of FBS is frequently used to eliminate live virus, no process is 100% effective 100% of the time. Use of FBS in growing cultured cells, particularly in bioreactors, provides an amplification system so that even a few escaping viral particles can become significant over multiple cell culture passages over time. Further, the sensitivity of HoBi-viruses to irradiation has not been established. It also should be noted that several FBS batches contaminated with HoBi-like viruses bore labels indicating that the fetal serum used originated outside of South America. There is no evidence, to date, of infection of cattle with HoBi-like viruses in North America [55] or Australia. This raises the

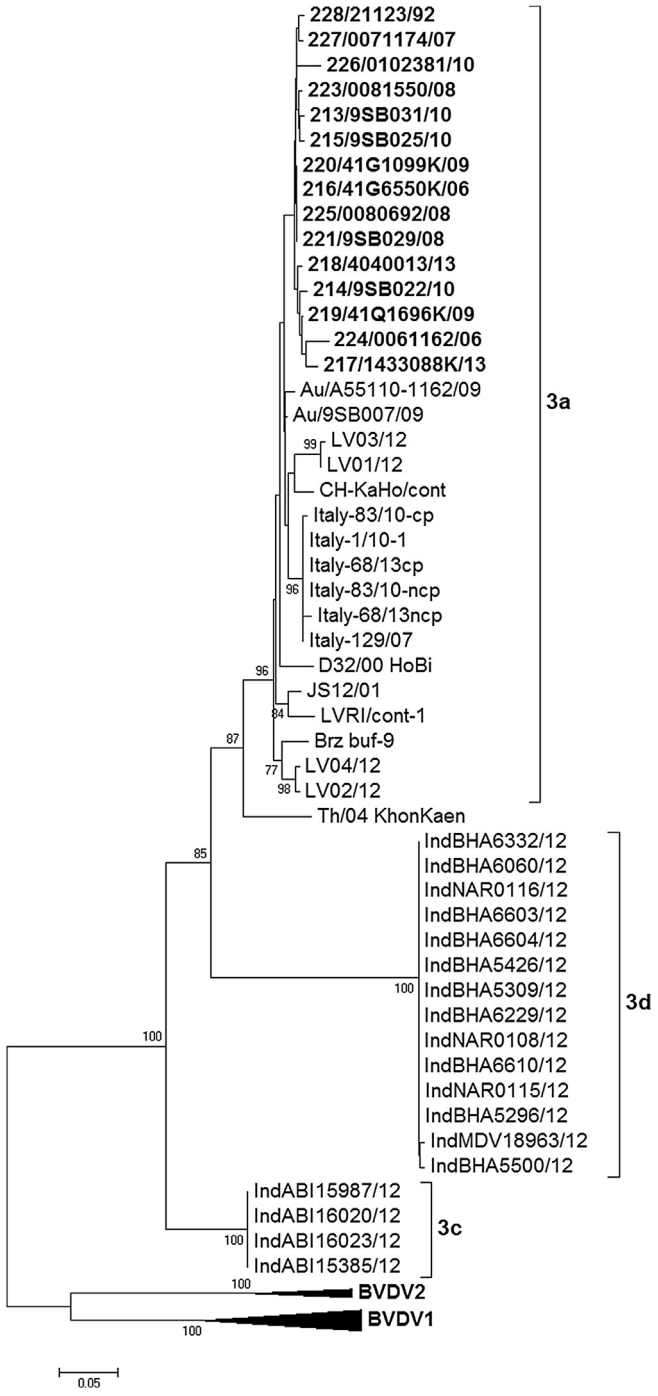


**Fig. 2.** Phylogenetic tree based on the $N^{pro}$ sequences. Molecular evolutionary genetics analyses were performed with MEGA6 using the NJ method. Distances were computed using the Kimura two-parameter model. The numbers close to the major nodes indicate the bootstrap values (in %; 10,000 replicates). The tree describes the relationship between $N^{pro}$ sequences retrieved from the GenBank database and the sequences analyzed in this work (labeled in bold). Bar: number of substitutions per site.

questions of possible contamination during processing and packaging in plants handling FBS from multiple geographic regions, mislabeling following processing or mixing of sera from different regions during processing. This is troubling because a premium is paid for FBS originating North America and Australia as these regions are free of some viral pathogens. This finding has implications for the safety of biological products, such cell lines and vaccines.

**Table 2**
Percentage of similarity of pairwise distances.

| | 3a | 3b | 3c | 3d |
|---|---|---|---|---|
| 3a | **86.50–100**<br>***92.10–100*** | 77.40–88.70<br>nd | 81.60–88.00<br>*81.90–84.70* | 80.90–84.50<br>*77.80–80.10* |
| 3b | | **89.40**<br>nd | 82.30–85.20<br>nd | 80.90–83.80<br>nd |
| 3c | | | **86.60–100**<br>***100*** | 80.20–83.80<br>*78.40–78.70* |
| 3d | | | | **99.20–100**<br>***99.50–100*** |

The table summarizes minimum and maximum identities within and between genetic groups. Calculations were performed using the alignment of the 5′UTR and $N^{pro}$ sequences used for generation of the phylogenetic trees. Values for 5′UTR are given in normal letter, $N^{pro}$ in italic. nd: not determined.
The percentage of similarity of intragenetic groups analysed in this work are labelled in bold.

The regulation and testing of FBS should be thoroughly investigated from several aspects including veterinary and human medicine. In addition, the emergence of the new pestivirus strains could represent newly recognized variables and risk factors for BVDV control programs.

## Conflict of interest

There is no conflict of interest.

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