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Evaluation of currently available bovine viral diarrhoea virus (BVDV) and HoBi-like pestivirus (HoBiPeV) specific diagnostic tests in detection of highly divergent HoBiPeVs in cattle

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ABSTRACT

The emergence of novel and divergent HoBi-like pestivirus (HoBiPeV) strains in cattle in Asia recently has raised concerns with regard to their reliable and accurate diagnosis. Hence, the aim of this study was to evaluate currently available BVDV diagnostic tests and HoBiPeV-specific diagnostic tests in detection of genetically divergent strains of HoBiPeV. One strain each of HoBiPeV-c and d were subjected to two BVDV diagnostic RT-PCR tests, one HoBiPeV specific RT-PCR test, three BVDV diagnostic qRT-PCR tests, one HoBiPeV specific qRT-PCR test and two BVDV antigen capture ELISAs. Archived cattle sera (n = 41) from farms with reports of HoBiPeV natural infection were assessed for detection of HoBiPeV antibodies by VNT and two commercial BVD antibody ELISA kits. BVDV diagnostic qRT-PCR tests had better sensitivity than BVDV diagnostic RT-PCR tests, while majority of them except a commercial kit showed a lower sensitivity for HoBiPeV-d strain. The HoBiPeV specific qRT-PCR test was found more sensitive than HoBiPeV specific RT-PCR but both had lower sensitivity for HoBiPeV-d strain, as displayed by primer/probe sequence mismatches. The BVDV E^{rns} antigen ELISA detected both the strains of HoBiPeV, but with a lower sensitivity for HoBiPeV-d strain, whereas BVDV NS3 antigen ELISA failed to detect them even at a high HoBiPeV titre. Compared to VNT, commercial BVDV antibody ELISA showed low to moderate sensitivity in detection of HoBiPeV antibodies, with a failure rate of 31.25% for the whole virus antigen based ELISA and a failure rate of 56.25% for NS3 antibody ELISA. The present study demonstrated new challenges in HoBiPeV diagnosis indicating a need in improvement of both HoBiPeV specific diagnostic RT-PCR and qRT-PCR for better utility in HoBiPeV epidemiology and biological product safety. Although more studies are required, this study reinforces that combined use of BVDV E^{rns} and NS3 antigen ELISA may have some utility in preliminary differentiation between HoBiPeV and BVDV infection in PI cattle. Additionally, we show that the comparative VNT has a better sensitivity in detection of HoBiPeV exposure and there is a need of robust antibody ELISA for reliable detection of antibodies against this emerging bovine pestivirus.

1. Introduction

Bovine pestiviruses are important pathogens of cattle and have significant economic impact on dairy cattle production worldwide. This group of viruses within the family *Flaviviridae*, genus *Pestivirus* have recently been classified into three of the eleven officially recognized pestivirus species, *Pestivirus A,* comprising of bovine viral diarrhea virus 1 (BVDV-1), *Pestivirus B,* comprising of bovine viral diarrhea virus 2 (BVDV-2) and *Pestivirus H,* comprising of HoBi-like pestivirus (HoBiPeV) (Smith et al., 2017). While the species demarcation criteria include differences in nucleotide and deduced amino acid sequence relatedness, antigenic relatedness and host origin, these bovine pestiviruses cause subclinical to severe acute disease called bovine viral diarrhea (BVD) and highly fatal mucosal disease (MD) in cattle.

Similar to other pestiviruses, HoBiPeV genome consists of a single-stranded positive-sense RNA of about 12.3 kb in length. A single open reading frame codes for four structural proteins (C, E^{rn}s, E1 and E2) and seven to eight non-structural proteins (N^{pro}, p7, NS2-3, NS4A, NS4B, NS5A,NS5B), and is flanked by 5′- and 3′-untranslated regions (UTR) (Meyers and Thiel, 1996). Although related to certain extent, significant genetic and antigenic differences exist among HoBiPeV, BVDV-1 and BVDV-2. BVDV-1 has been further divided into 21 genotypes (Yesilbag

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et al., 2017), while BVDV-2 has been classified into three genotypes (Neill et al., 2019), and HoBiPeV into 3–4 genotypes (Liu et al., 2009; Mishra et al., 2014; Giammarioli et al., 2015).

Since the first detection of strain D32/00-'HoBi', in foetal bovine serum (FBS) originating from Brazil (Schirrmeier et al., 2004), HoBiPeV has frequently been identified in commercial FBS batches, mostly of South American origin but also originating from Mexico, Canada and Australia and in contaminated cells (Xia et al., 2011; Mao et al., 2012). Natural HoBiPeV infection in cattle has so far been reported in Brazil, Italy, Thailand, India and Bangladesh (Cortez et al., 2006; Stahl et al., 2007; Kampa et al., 2009; Decaro et al., 2011; Haider et al., 2014; Mishra et al., 2014), while HoBiPeV is the most prevalent ruminant pestivirus in Northeastern Brazil (Silveira et al., 2018). Natural infection has also been reported in buffaloes in Brazil (Cortez et al., 2006) and in sheep and goats in China (Shi et al., 2016). The association of HoBiPeV with reproductive disease (Decaro et al., 2012a), severe respiratory disease (Decaro et al., 2011, 2012b, 2013), gastroenteritis and economic losses in cattle farms (Decaro et al., 2016) and mucosal disease in cattle (Decaro et al., 2014; Weber et al., 2016; Cruz et al., 2018) and respiratory disease in small ruminants (Shi et al., 2016) has raised further concerns.

The clinical outcome following infection with field strains of classical BVDV-1, BVDV-2 or HoBiPeV varies vastly and include enteric, respiratory and reproductive disease (Evans et al., 2019). Hence, these pestiviruses cannot be differentiated on the basis of only clinical presentation and requires laboratory diagnostic tests. A TaqMan assay for specific detection of HoBiPeV has been developed earlier (Liu et al., 2008), while TaqMan assays able to detect HoBiPeV (Losurdo et al., 2015) and discriminate them from extant bovine pestiviruses (Mari et al., 2016) have recently been developed. Although it was initially thought that HoBiPeV are less diverse genetically, marked genetic diversity has been identified recently, with reports of novel and divergent HoBiPeV lineages circulating in cattle in India and Bangladesh (Mishra et al., 2014; Haider et al., 2014; Giammarioli et al., 2015). Previous studies have shown that the diagnostic tests designed for classical BVDV-1 and BVDV-2 either fail to detect HoBiPeV or detects HoBiPeV with reduced sensitivity (Schirrmeier et al., 2004; Peletto et al., 2012; Bauermann et al., 2013; Larska et al., 2013). However, these BVDV diagnostic tests or currently available HoBiPeV specific tests have not been evaluated for their ability in detection of novel and highly divergent HoBiPeV, which is not only important for HoBiPeV free countries, but also for the countries where these viruses currently circulate. Hence, the objective of this study is to evaluate currently available BVDV diagnostic tests and HoBiPeV-specific diagnostic tests in detection of genetically divergent HoBi-like pestiviruses circulating in cattle.

2. Materials and methods

2.1. Viruses and cells

HoBi-like pestivirus strains, Ind BHA5309/12 (GenBank Acc.: KM201300 - 5′-UTR, KM261864 - N^{pro}) and Ind ABI15383/12 (GenBank Acc.: KM201313 – 5′-UTR, KM261878 - N^{pro}) (Mishra et al., 2014), BVDV-1b strain Ind S-1449 (GenBank Acc: AY911670) (Mishra et al., 2004) and BVDV-2a strain Ind 141353 (GenBank Acc: HQ444199) (Behera et al., 2011), obtained from the pestivirus repository of ICAR-National Institute of High Security Animal Diseases, Anand Nagar, Bhopal, India were propagated in BVDV free Madin-Darby Bovine Kidney (MDBK) cells obtained from Cell Culture Collection of Veterinary Medicine, Friedrich-Loeffler Institute, Island of Riems, Germany. MDBK cells were propagated in Eagle's Minimum Essential Medium (EMEM; Sigma) supplemented with 15% horse serum (Gibco-BRL Life Technologies), while the virus strains were propagated using EMEM supplemented with 7.5% horse serum. After 4 days, the infected cultures were frozen and thawed thrice and the clarified supernatants were subjected to immuno peroxidase monolayer assay (IPMA) using pan-pestivirus (WB103/105) monoclonal antibodies as described earlier (Mishra et al., 2008). The virus titres were determined using the previously described method (Reed and Muench, 1938) and stored at −80 °C until used.

2.2. RNA extraction

Viral RNA was extracted from 140 µl infected cell culture supernatants from serial 10-fold dilutions of HoBiPeV, BVDV-1 and BVDV-2 strains of known titre or uninfected cell culture supernatants by QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA extraction from pestivirus free cattle serum spiked with known concentrations of HoBiPeV/BVDV-1 was conducted using the same protocol. The RNA was recovered in 30 µl of RNase-free water and stored at −80 °C until used.

2.3. Evaluation of BVDV diagnostic and HoBiPeV specific conventional RT-PCR

A comparative study was taken up to determine the detection limit of BVDV diagnostic (BVDV-1 and BVDV-2) and HoBiPeV specific conventional RT-PCR using RNA extracted from HoBiPeV strains, IndBHA5309/12 and Ind15385/12. For comparative purposes, BVDV-1 strain Ind S-1449 and BVDV-2 strain Ind 14135 were also used. The list of primers used here is described in Table 1. The relationship between the genetically divergent HoBiPeV strains used here and other HoBiPeV strains and reference pestiviruses is shown in Fig. 1. The BVDV diagnostic RT-PCR included widely used pestivirus primer sets, 324/326 (Vilcek et al., 1994), and the primer sets, BVD190-F (Hoffmann et al., 2006) and 326 (Vilcek et al., 1994). The reactions were performed in 25 µL volume using 2 µL of template RNA and SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen, Carlsbad, USA) with the following thermal reaction conditions: one cycle at 60 °C for 30 min (RT step), one cycle at 94 °C for 2 min (initial denaturation of DNA) and 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 68 °C for 25 s. Final extension was done for 5 min at 68 °C followed by a soak cycle at 4 °C.

For HoBiPeV specific RT-PCR, previously reported primers, N2 and R5 (Bauermann et al., 2014a), which have been employed for HoBiPeV surveillance in FBS and detection of PI cattle were used. Briefly, the reactions were carried out in 25 µL volume using 2 µL of template RNA and SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen, Carlsbad, USA) with the same thermal profile described above for 324/326 RT-PCR. The RT-PCR products were run on agarose gel and the results were recorded in Geldoc.

2.4. Evaluation of BVDV diagnostic real time RT-PCR and HoBiPeV specific real time RT-PCR

To evaluate ability of BVDV diagnostic qRT-PCR assays in detection and limits of detection of highly divergent HoBiPeV, we used one

Table 1
Primers and probes used in RT-PCR and qRT-PCR assays for detection of highly divergent HoBiPeV strains.

Primer/ Probe	Primer/Probe Sequence	Reference
BVD190-F	GRAGTCGTCARTGGTTCGAC	Hoffmann et al., 2006
324	ATGCCCATAGTAGGACTAGCA	Vilcek et al., 1994
326/V326	TCAACTCCATGTGCCATGTAC	Vilcek et al., 1994
TQ-Pesti	TGCYAYGTGGACGAGGGCATGC	Gaede et al., 2005
N2	TCGACGCATCAAGGAATGCCT	Bauermann et al., 2014a,b
R5	TAGCAGGTCTCTGCAACACCCTAT	Bauermann et al., 2014a,b
T134-F	GACTAGTGGTGGCAGTGAGC	Liu et al., 2008
T220-R	GAGGCATTCCTTGATGCGTC	Liu et al., 2008
T155r-P	ACTCGGGGCTTCGGTGATCCAGGG	Liu et al., 2008

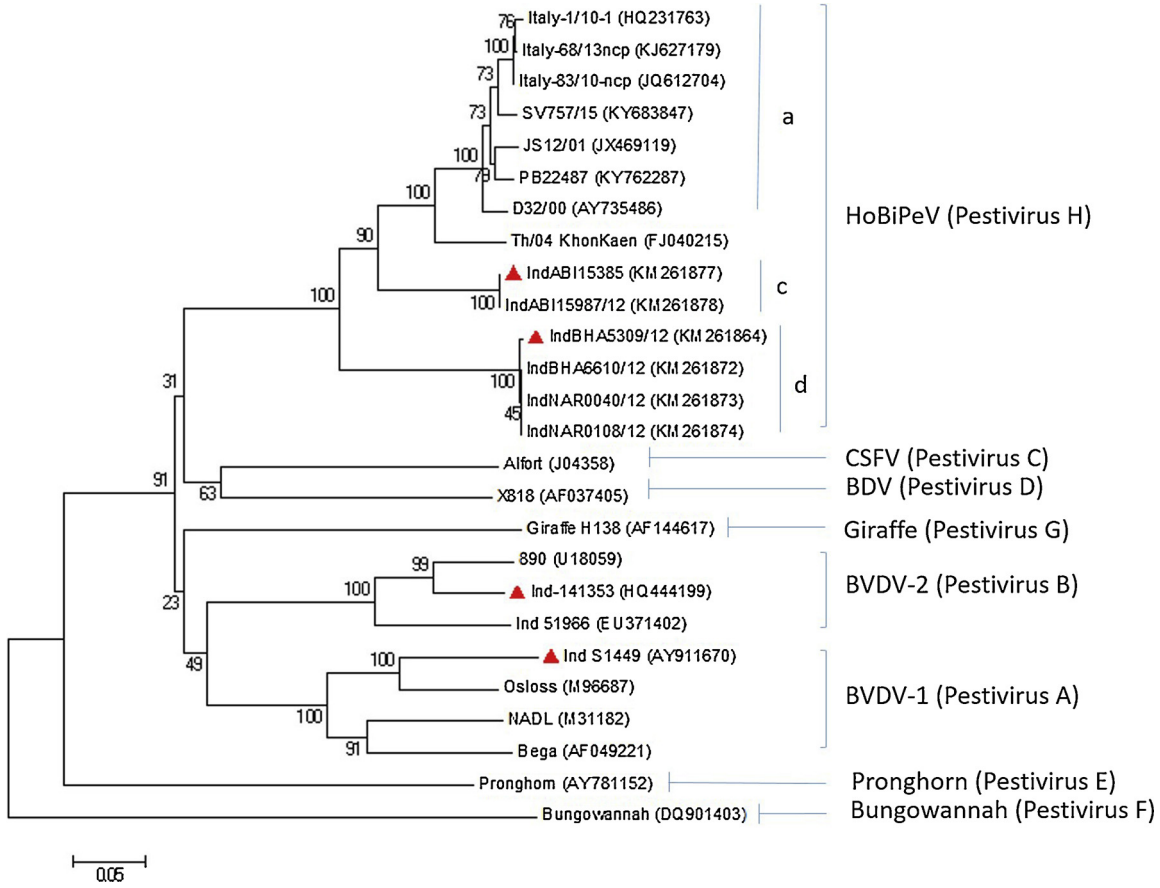


Fig. 1. Genetic relationship between divergent HoBiPeV strains and other bovine pestivirus strains used in this study and reference pestivirus strains. The phylogenetic tree was constructed based on entire N^{pro} gene sequences and performed with MEGA6 using NJ method. The pestivirus strains evaluated in the study are labelled as red triangles with GenBank Accessions provided in brackets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

previously reported pestivirus generic qRT-PCR assay targeting at 5′-UTR (Hoffmann et al., 2006) and two commercially available qRT-PCR assays, VetMax-Gold-bovine virus diarrhea RNA test kit (Applied Biosystems, Life Technologies, Austin, TX, USA) and *virotype* BVDV RT-PCR Kit (Qiagen, Labor Diagnostik Leipzig GmbH, Leipzig, Germany), and these assays are hereafter referred to as BVDV diagnostic qRT-PCR. However, these two commercial kits do not disclose about the target region or the primers/probes employed. For evaluation of HoBi-PeV specific qRT-PCR, we used a previously reported TaqMan assay (Liu et al., 2008). Serial tenfold dilutions of RNA extracted from HoBiPeV strains, IndBHA5309/12, Ind15385/12, BVDV-1 strain Ind S-1449 and BVDV-2 strain Ind 141353 were used as template in 25 µL reactions and amplified in CFX96 (CFX96, Bio-Rad Corporation, Madison, USA). The primers and probes used in BVDV diagnostic qRT-PCR and HoBiPeV-specific qRT-PCR assays are shown in Table 1.

2.4.1. BVDV diagnostic qRT-PCR

Briefly, the BVDV diagnostic qRT-PCR assay (Hoffmann et al., 2006) was conducted using the primers BVD190-F (Hoffmann et al., 2006), V326 (Vilcek et al., 1994), probe TQ-Pesti (Gaede et al., 2005) and Superscript III Platinum one-step real time RT-PCR reagent set (Invitrogen, USA) in 25 µl reaction volume, consisting of 12.5 µl of Reaction mix (2X), 0.5 µl of Rox, 0.5 µl of Taq Mix, 0.5 µl (10 pmol) each of forward and reverse primers, 2 µl of probe (2.5 pmol), 6.5 µl of nuclease free water and 2 µl of RNA. The thermal profile consisted of reverse transcription at 50 °C for 30 min, RT inactivation/ initial denaturation at 95 °C for 5 min, and 40 amplification cycles of denaturation at 95 °C for 30 s and annealing/extension at 60 °C for 1 min.

2.4.2. VetMax-Gold BVDV RNA detection kit

The assay using VetMAX-Gold BVDV PI detection Kit, was carried out as per the manufacturer's protocol in a total reaction volume of 25 µL, which consisted of 12.5 µL of 2X RT-qPCR buffer, 1 µL of 25X BVDV primer-probe mix, 1 µL of 25X RT-qPCR enzyme mix, 2.5 µL of nuclease free water and 8 µL RNA template. The thermal profile was programmed as follows: reverse transcription at 45 °C for 10 min, RT inactivation/ initial denaturation at 95 °C for 10 min, and 40 amplification cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 45 s. The interpretations of test results were as per the protocol supplied by the manufacturer.

2.4.3. Virotype BVDV RNA detection kit

The assay using *virotype* BVDV RT-PCR test kit was performed following the manufacturer's protocol in a 25 µL reaction volume, consisting of 19.75 µL of RT-qPCR mix, 0.25 µL of enzyme mix and 5 µl of RNA template using following thermal cycling conditions: reverse transcription at 50 °C for 20 min, RT inactivation at 95 °C for 15 min, followed by 40 amplification cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 45 s and extension at 68 °C for 45 s. Interpretation of results was as per the protocol of manufacturer.

2.4.4. HoBi-PeV specific qRT-PCR

The HoBi-PeV specific qRT-PCR assay (Liu et al., 2008) was conducted using SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen, USA), in a total reaction volume of 25 µL, containing 800 nM of primers T134-F and T220-R and 400 nM of probe T155r-P (Liu et al., 2008), and 2 µL of RNA template. The thermal

Table 2
Detection limits of BVDV diagnostic RT-PCR and HoBiPeV-specific RT-PCR for detection of highly divergent HoBiPeV strains and BVDV-1/BVDV-2 in supernatants of infected cells.

Pestivirus genotype (Isolate)	Titre ($TCID_{50}$/mL)	BVDV diagnostic RT-PCR		HoBi specific
		BVD-190 F/ V326	324/326	N2/R5
HoBiPeV-c (Ind ABI15385/12)	$10^{5.6}$	+	−	+
	$10^{4.6}$	+	−	+
	$10^{3.6}$	+	−	+
	$10^{2.6}$	+	−	+
	$10^{1.6}$	+	−	+
	$10^{0.6}$	−	−	−
	$10^{0.06}$	−	−	−
HoBiPeV-d (Ind BHA5309/12)	$10^{8.3}$	+	+	+
	$10^{7.3}$	+	+	+
	$10^{6.3}$	+	+	+
	$10^{5.3}$	+	+	+
	$10^{4.3}$	+	−	+
	$10^{3.3}$	+	−	+
	$10^{2.3}$	−	−	−
	$10^{1.3}$	−	−	−
	$10^{0.3}$	−	−	−
BVDV-1b (Ind S-1449)	$10^{6.5}$	+	+	−
	$10^{5.5}$	+	+	−
	$10^{4.5}$	+	+	−
	$10^{3.5}$	+	+	−
	$10^{2.5}$	+	+	−
	$10^{1.5}$	+	−	−
	$10^{0.5}$	+	−	−
	$10^{0.05}$	+	−	−
BVDV-2a (Ind 141353)	$10^{6.6}$	+	+	−
	$10^{5.6}$	+	+	−
	$10^{4.6}$	+	+	−
	$10^{3.6}$	+	+	−
	$10^{2.6}$	+	+	−
	$10^{1.6}$	+	−	−
	$10^{0.6}$	+	−	−
	$10^{0.06}$	−	−	−

(+: positive, -: negative).

profile consisted of reverse transcription at 50 °C for 30 min, RT inactivation/ initial denaturation at 95 °C for 5 min, followed by 40 amplification cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min.

2.5. Evaluation of RT-PCR in bovine serum spiked with known concentrations of highly divergent HoBiPeV and BVDV-1

To ascertain ability of RT-PCR in detection of co-circulating HoBiPeV and BVDV-1, pestivirus free bovine serum samples were spiked with known concentrations (10^5 $TCID_{50}$/mL to 10^1 $TCID_{50}$/mL) of HoBiPeV strain IndBHA5309/12 and BVDV-1 strain Ind S-1449 in various combinations (Table 2). Viral RNA from spiked serum samples was extracted as described above and was subjected to BVDV diagnostic RT-PCR (324/326) and HoBiPeV specific RT-PCR (N2/R5) assays as described earlier in this study. To confirm the results, a selected number of amplicons (one from each group) were directly sequenced using the same primer sets used for amplification, ABI PRISM Big Dye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI 3130 genetic analyzer (Applied Biosystems, USA).

2.6. Evaluation of BVDV antigen capture ELISA on known concentrations of HoBiPeV

To determine whether currently available BVDV antigen capture ELISA kits are suitable in detecting the highly divergent HoBiPeV strains, two commercially available ELISA kits were evaluated. The first one, IDEXX BVDV Ag/Serum Plus (IDEXX, USA) is an E^{rns} antigen capture ELISA, intended to detect BVDV E^{rns} antigen in cattle serum, plasma, blood and ear notch samples, while the second one, INGEZIM BVD DAS (Ingenasa, Spain) is a NS3 antigen capture ELISA intended to detect BVDV NS3 antigen. HoBiPeV strains (Ind BHA5309/12 and Ind ABI15385/12), BVDV-1 strain Ind S-1449 and BVDV-2 strain Ind 141353 of known virus titres were diluted 10-fold in pestivirus free bovine serum and tested in duplicate wells using IDEXX BVDV Ag/ Serum Plus kit and INGEZIM BVD DAS kit according to the instructions of the manufacturer.

For IDEXX BVDV Ag/Serum Plus kit, the assay was carried out using 50 µl of samples and 2 h of incubation time with anti E^{rns} mAb coated plate. The OD values were obtained in ELISA reader (Merilyser) at 450 nm. The negative control mean, positive control mean and S-N (Sample - Negative) values were calculated. The samples with S-N values of > 0.3 were classified as positive and S-N values of < 0.3 were classified as negative for BVDV antigen.

The assay using INGEZIM BVD DAS kit was performed using 100 µl of samples and 18 h of incubation time with anti NS3 mAb coated plate. The OD values were measured at 450 nm and cut off values were calculated [Corrected cut off (positive) = cut off of positive control + 15% of cut off of positive control; Corrected cut off (negative) = cut off of positive control - 15% of cut off of positive control] as per manufacturer protocol. Samples with OD greater than corrected positive cut off are considered positive, while samples with OD lesser than corrected negative cut off were considered negative for BVDV antigen.

2.7. Virus neutralization test (VNT)

To detect and quantify neutralizing antibodies, virus neutralization test was employed for a selected number of archived BVDV/HoBiPeV neutralizing antibody positive cattle sera (n = 41) obtained from our previous work (Mishra et al., 2014), which belonged to four different dairy farms in India with reports of circulation of two genetically highly divergent HoBiPeV strains (Mishra et al., 2014). The sera were classified into four groups (Farm 1–4), of which sera belonging to group 1 originated from cattle in a farm with circulation of HoBiPeV-c genotype, while sera from groups 2, 3 and 4 were from cattle in farms with circulation of HoBiPeV-d genotype.

The serial two-fold heat inactivated sera samples (starting dilution 1:5) were subjected to VNT using 96-well TC plates, MDBK cells, 200 $TCID_{50}$ of HoBiPeV isolates, Ind BHA5309/12 or Ind 15385/12 (as per the sera groups), BVDV-1 cattle isolate Ind S-1449 (Mishra et al., 2004), BVDV-2 cattle isolate Ind 141353 (Behera et al., 2011), antiserum against BVDV (VMRD, Pullman, USA) and immuno-peroxidase monolayer assay (IPMA) as previously described (Mishra et al., 2008). The antibody titres were expressed as the reciprocal value of the highest serum dilution that completely inhibited virus replication in 50% of wells. The virus neutralizing antibody titre of 1:10 or above was considered as positive and as an indicator of seroconversion.

2.8. Evaluation of BVD antibody ELISA kits in detection of antibodies against HoBiPeV and comparison with VNT results

To evaluate ability of commercial BVD antibody ELISA kits in detection of HoBiPeV antibodies in narturally infected cattle, the field sera (n = 41) subjected to VNT as described above, were tested using IDEXX BVDV Total Ab kit (IDDEX, USA), and INGEZIM BVD Compac ELISA kit (INGENASA, Spain).

The IDEXX BVDV Total Ab ELISA is based on whole virus antigen and has high sensitivity and specificity in detection of antibodies against BVDV-1 and BVDV-2. The assay was performed according to the protocol of the manufacturer using 1:5 serum dilution and 1.5 h incubation time of serum with antigen-coated plate. The results were expressed as corrected optical density (COD) values calculated by subtracting COD of the test positive control from the COD value of the

sample tested. The COD ≥ 0.3 was interpreted as positive for BVDV-1/BVDV-2 antibodies.

The INGEZIM BVD Compac ELISA kit is based on a competitive ELISA format using recombinant p80 (NS3) antigen and mAbs specific to pestivirus (BVDV/BDV) p80 antigen and is intended to detect p80 (NS3) antibodies in cattle, sheep and goats. The assay was performed using 1:5 serum dilution and 1 h of incubation with NS3 antigen coated plates and the assay was performed as per the manufacturer's instructions. The cut-off [Cut off (positive) = 0.5 X (OD of negative control); Cut off (negative) = 0.55 X (OD of negative control] values were calculated as per the instructions of the manufacturer. Samples with values of > positive cut off were interpreted as positive, while samples with values of < negative cut off were interpreted as negative for anti BVDV antibodies.

Additionally, to determine the ability of commercial BVD antibody ELISA kits in detection and quantification of levels of antibodies against HoBiPeV, one representative sample from each of the four group of sera having highest homologous neutralizing antibody titre against HoBiPeV and showing higher antibody levels in ELISA formats (in terms of OD values) were selected for further comparative analysis. Two-fold diluted (starting from 1:5 dilution) sera samples were tested by BVD antibody ELISA kits as described above and the results were compared with the VNT results.

3. Results

3.1. Detection limit of BVDV diagnostic and HoBiPeV specific RT-PCR

The results of one of the commonly used BVDV diagnostic RT-PCR, using primers BVD-190 F/326 (Table 2) showed that although the assay could detect strains of highly divergent HoBiPeV strains, it showed less sensitivity in detection of HoBiPeV-d genotype ($10^{3.3}$ $TCID_{50}$/ml) than HoBiPeV-c genotype ($10^{1.6}$ $TCID_{50}$/ml). In contrast, evaluation of another routinely used BVDV diagnostic RT-PCR, using primers 324/326 showed that the primers failed to detect the strain of HoBiPeV-c genotype even at high virus titre ($10^{5.6}$ $TCID_{50}$/ml), while it could detect the strain of HoBiPeV-d genotype but with a reduced detection limit ($10^{5.3}$ $TCID_{50}$/ml) (Table 2). The panpestivirus primers used in these assays however displayed high sensitivity for both BVDV-1 and BVDV-2.

The results of HoBiPeV specific RT-PCR (Table 2) using primers N2/R5 showed that although the assay was able to detect highly divergent strains of HoBiPeV, the primer sets had a better sensitivity in detection of HoBiPeV-c strain ($10^{1.6}$ $TCID_{50}$/ml) than HoBiPeV-d strain ($10^{3.3}$ $TCID_{50}$/ml). However, the assay was found to be HoBiPeV specific, since no amplification was observed with higher concentrations of BVDV-2 ($10^{5.6}$ $TCID_{50}$/ml) and BVDV-1 ($10^{6.5}$ $TCID_{50}$/ml).

3.2. Performance of BVDV diagnostic qRT-PCR assays

The OIE recommended BVDV diagnostic qRT-PCR assay (Hoffmann et al., 2006) was able to detect the strains of highly divergent HoBiPeV strains, but had a lower detection limit for the HoBiPeV-d strain ($10^{2.3}$ $TCID_{50}$/ml) than for the HoBiPeV-c strain ($10^{0.6}$ $TCID_{50}$/ml) (Table 3, Fig. 2a, b). The commercial VetMax-Gold qRT-PCR assay displayed similar results with higher detection limit for the HoBiPeV-c strain ($10^{0.6}$ $TCID_{50}$/ml) than the HoBiPeV-d strain ($10^{2.3}$ $TCID_{50}$/ml) (Table 3, Fig. 3a, b). The commercial virotype qRT-PCR assay showed better sensitivity in detection of highly divergent HoBiPeV strains with high detection limits for both HoBiPeV-c ($10^{0.6}$ $TCID_{50}$/ml) and HoBiPeV-d ($10^{0.3}$ $TCID_{50}$/ml) strains (Table 3, Fig. 4a, b). Altogether, the results showed that all the three BVDV diagnostic qRT-PCR assays have similar sensitivity in detection of HoBiPeV-c strain, while the virotype qRT-PCR assay is 100-fold more sensitive in detection of HoBiPeV-d strain than the other two assays. However, all the three BVDV diagnostic qRT-PCR assays showed high sensitivity in detection of BVDV-1 and BVDV-2 strains studied here.

Table 3
Comparison of sensitivity of BVDV diagnostic qRT-PCR assays and HoBiPeV specific qRT-PCR assay in detection of highly divergent HoBiPeV strains and BVDV-1/BVDV-2.

Pestivirus genotype (Isolate)	Titre ($TCID_{50}$/ml)	Commercial kits		BVDV qRT-PCR (Hoffmann et al., 2006)	HoBiPeV qRT-PCR (Liu et al., 2008)
		VetMAX Gold	Virotype		
HoBiPeV-c (Ind ABI15385/12)	$10^{5.6}$	+	+	+	+
	$10^{4.6}$	+	+	+	+
	$10^{3.6}$	+	+	+	+
	$10^{2.6}$	+	+	+	+
	$10^{1.6}$	+	+	+	+
	$10^{0.6}$	+	+	+	+
	$10^{0.06}$	–	–	–	–
HoBiPeV-d (Ind BHA5309 12)	$10^{8.3}$	+	+	+	+
	$10^{7.3}$	+	+	+	+
	$10^{6.3}$	+	+	+	+
	$10^{5.3}$	+	+	+	+
	$10^{4.3}$	+	+	+	+
	$10^{3.3}$	+	+	+	+
	$10^{2.3}$	+	+	+	+
	$10^{1.3}$	–	+	–	–
	$10^{0.3}$	–	+	–	–
BVDV-1b (Ind S-1449)	$10^{6.5}$	+	+	+	–
	$10^{5.5}$	+	+	+	–
	$10^{4.5}$	+	+	+	–
	$10^{3.5}$	+	+	+	–
	$10^{2.5}$	+	+	+	–
	$10^{1.5}$	+	+	+	–
	$10^{0.5}$	–	+	+	–
BVDV-2a (Ind 141353)	$10^{6.6}$	+	+	+	–
	$10^{5.6}$	+	+	+	–
	$10^{4.6}$	+	+	+	–
	$10^{3.6}$	+	+	+	–
	$10^{2.6}$	+	+	+	–
	$10^{1.6}$	+	+	+	–
	$10^{0.6}$	+	+	+	–
	$10^{0.06}$	–	+	+	–

(+ positive, - negative).

3.3. Performance of HoBiPeV-specific qRT-PCR assay

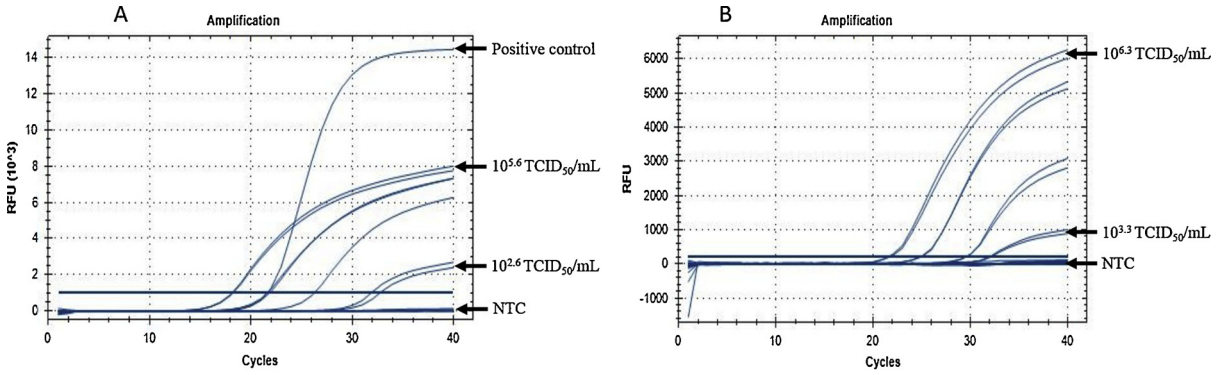
Evaluation of HoBiPeV specific qRT-PCR assay demonstrated higher detection limit for HoBiPeV-c strain ($10^{0.6}$ $TCID_{50}$/ml) than HoBiPeV-d strain ($10^{2.3}$ $TCID_{50}$/ml) (Table 3, Fig. 5a, b). Although the assay was less sensitive than the commercial virotype kit, it showed high specificity for HoBiPeV.

3.4. HoBiPeV detection in spiked serum samples

When tested without spiking, The N2/R5 primer set amplified HoBiPeV-d strain at a virus titre of $10^{3.0}$ $TCID_{50}$ to $10^{5.0}$ $TCID_{50}$, while the 324/326 primer sets amplified it only at a titre of $10^{5.0}$ $TCID_{50}$ (Table 4). When these primers were evaluated in serum samples spiked with both HoBiPeV and BVDV-1, similar or lower detection limits were observed. No amplification with N2/R5 primer sets was observed in spiked samples having a titre of $10^{3.0}$ $TCID_{50}$ or lower for HoBiPeV-d strain, while 324/326 primer sets amplified BVDV-1 up to a titre of $10^{1.0}$ TCID50. The results showed that in case of mixed infection with highly divergent HoBiPeV and BVDV-1, N2/R5 primer set has a lower sensitivity for detection of HoBiPeV.

3.5. BVDV antigen capture ELISA

The BVDV E^{rns} antigen capture ELISA was able to detect the highly divergent HoBiPeV strains, but with variable detection limits (Table 5). The lowest virus titre detected by E^{rns} antigen ELISA was $10^{4.6}$ $TCID_{50}$/ml for HoBiPeV-c strain, while it was $10^{6.3}$ $TCID_{50}$/ml for HoBiPeV-d strain indicating significant difference in sensitivity for HoBiPeV-d



Quantitative RT-PCR assay in detection of known concentrations ($TCID_{50}$/mL) of (A) IndABI15385/12 and (B) IndBHA5309/12 using BVDV diagnostic primers and probes

Fig. 2. Analytical sensitivity of BVDV diagnostic qRT-PCR (Hoffmann et al., 2006) in detection of HoBiPeV strains of genotype c (A) and d (B).

genotype. For strains of BVDV-1 and BVDV-2, the detection limits varied from $10^{3.6}$ $TCID_{50}$/ml to $10^{4.5}$ $TCID_{50}$/ml. In contrast, the NS3 antigen capture ELISA failed to detect the highly divergent HoBiPeV strains even at high virus titre (Table 5). However, it was able to detect both BVDV-1 and BVDV-2 with similar detection limits observed for E^{rns} antigen capture ELISA. The results showed that the E^{rns} antigen capture ELISA was able to detect highly divergent HoBiPeV strains, but it had a lower sensitivity in detection of HoBiPeV-d strain.

3.6. Neutralizing activity of sera from animals of HoBiPeV infected farms

The virus neutralizing antibody activity of sera (n = 41) from cattle in four farms with reports of HoBiPeV circulation showed that 16 of the 41 cattle sera tested exhibited > 4-fold neutralizing antibody titres to HoBiPeV than to BVDV-1 and BVDV-2 titres, while 4 sera had > 2-fold titres to HoBiPeV than BVDV-1 and BVDV-2, while 10 sera had > 2-4-fold higher titre against BVDV-1/BVDV-2 than HoBiPeV and the rest had almost similar antibody titre against BVDV-1, BVDV-2 and HoBiPeV (Table 6). The distribution pattern of VN titre varied between the farms, while the VN titre against HoBiPeV in some of the field sera reached to a level as high as 2560.

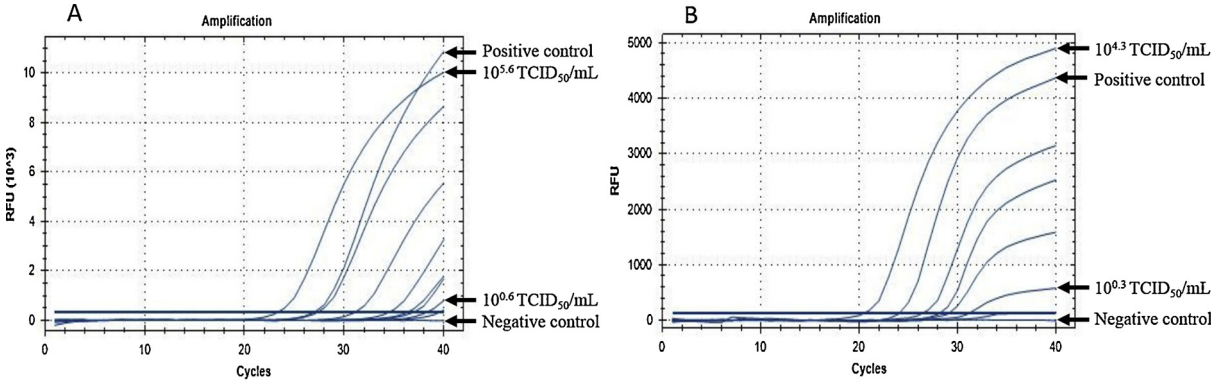
3.7. BVDV antibody ELISA in detection of antibodies against HoBiPeV

The IDEXX BVDV Total Ab ELISA detected antibodies against bovine pestiviruses in 33 (80.49%) out of 41 VNT positive serum samples, while INGEZIM BVD Antibody ELISA was able to detect antibodies only in 26 (63.41%) samples indicating low to moderate sensitivity of the antibody ELISA kits for antibodies against HoBiPeV (Table 6). When results of VNT and ELISA were compared, although the IDEXX BVDV Total Ab ELISA kit showed better correlation than the INGEZIM BVD Antibody ELISA kit, three sera having high VN titre (1280) against HoBiPeV were found negative by both the kits indicating their limited utility in detection of HoBiPeV antibodies.

When BVDV antibody ELISA kits were evaluated for quantification of levels of antibodies against HoBiPeV, the results of four sera (one from each farm) having high VN titre against HoBiPeV (> 4-16-fold titre difference with BVDV-1/BVDV-2) showed antibody ELISA positive results up to 1:40 serum dilution in three sera (corresponding to VN titres of 640 to 2560) for both the kits, while the other serum sample was found positive only in case of undiluted sample (Table 7). Both the antibody ELISA kits failed to detect antibodies in a serum displaying a VN titre of 20 against BVDV-1 or BVDV-2 and 320 against HoBiPeV. The results indicate low sensitivity of BVDV antibody ELISA kits used here either in detection or accurate quantitation of levels of antibodies against HoBiPeV.

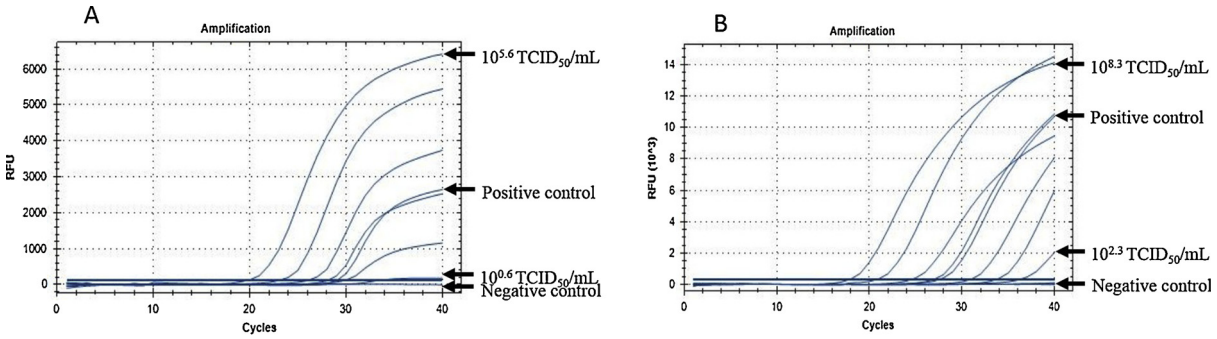
4. Discussion

Recent reports of natural infections with HoBiPeV in cattle associated with respiratory, gastrointestinal and reproductive diseases in different parts of world (South America, Europe and Asia) and identification of novel and divergent HoBiPeV strains in Indian subcontinent (Decaro et al., 2011, 2012a,b, 2016; Haider et al., 2014; Mishra et al., 2014; Weber et al., 2016; Silveira et al., 2018; Cruz et al., 2018) have raised concerns regarding the ability of the commonly used BVDV diagnostic tests and HoBiPeV-specific diagnostic tests in detection of highly divergent HoBiPeV strains. Additionally, natural HoBiPeV



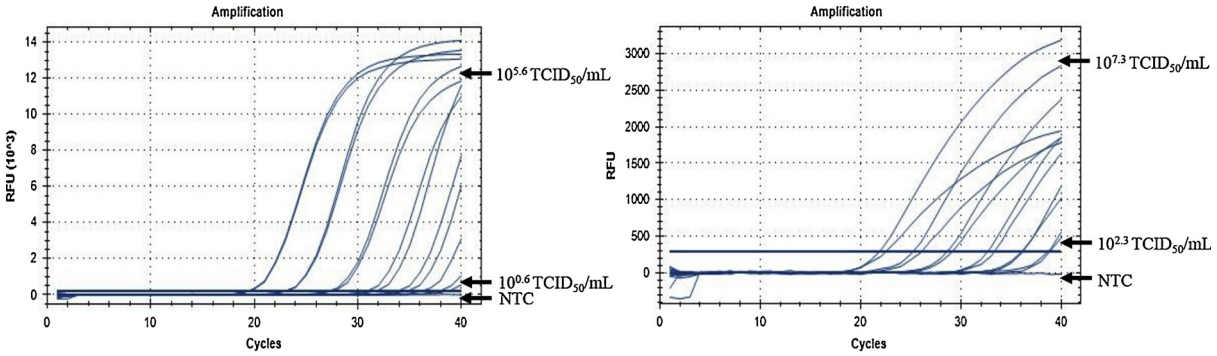
Quantitative RT-PCR assay in detection of known concentrations ($TCID_{50}$/mL) of (A) IndABI15385/12 and (B) IndBHA5309/12 using VetMAX™Gold BVDV PI Detection Kit

Fig. 3. Analytical sensitivity of commercial VetMAX-Gold BVDV qRT-PCR in detection of HoBiPeV strains of genotype c (A) and d (B).



Quantitative RT-PCR assay in detection of known concentrations (TCID$_{50}$/mL) of (A) IndABI15385/12 and (B) IndBHA5309/12 using Virotype® BVDV RT-PCR Kit

Fig. 4. Analytical sensitivity of commercial virotype BVDV qRT-PCR in detection of HoBiPeV strains of genotype c (A) and d (B).



Quantitative RT-PCR assay in detection of known concentrations (TCID$_{50}$/mL) of (A) IndABI15385/12 and (B) IndBHA5309/12 using HoBiPeV specific primers and probes

Fig. 5. Analytical sensitivity of HoBiPeV specific qRT-PCR (Liu et al., 2008) in detection of HoBiPeV strains of genotype c (A) and d (B).

Table 4
Testing of bovine serum spiked with known concentrations of HoBiPeV-d strain and BVDV-1b strain in RT-PCR using primers 324/326 and N2/R5.

Ind S1449 (BVDV-1) + IndBHA5309/12 (HoBiPeV-d) (TCID$_{50}$/ml)		RT-PCR primers used	
BVDV-1b	HoBiPeV-d	324/326*	N2/R5*
10^5	10^5	BVDV-1	HoBiPeV
10^5	10^3	BVDV-1	No amplification
10^5	10^1	BVDV-1	No amplification
10^3	10^5	BVDV-1	HoBiPeV
10^3	10^3	BVDV-1	No amplification
10^3	10^1	BVDV-1	No amplification
10^1	10^5	No amplification	HoBiPeV
10^1	10^3	No amplification	No amplification
10^1	10^1	No amplification	No amplification
10^5	–	BVDV-1	No amplification
10^3	–	BVDV-1	No amplification
10^1	–	No amplification	No amplification
–	10^5	No amplification	HoBiPeV
–	10^3	No amplification	No amplification
–	10^1	No amplification	No amplification

-: No addition of virus; *: Sequencing was done using same primers used for amplification.

Table 5
Sensitivity of BVDV E^{rns} antigen ELISA and BVDV NS3 antigen ELISA in detection of known concentrations (TCID$_{50}$/ml) of highly divergent HoBiPeV strains and BVDV-1/ BVDV-2 in serum.

Pestivirus genotype (Isolate)	Titre (TCID$_{50}$/mL)	E^{rns} Ag ELISA (Idexx)	NS3 Ag ELISA (Ingenasa)
HoBiPeV-c (Ind ABI15385/12)	$10^{5.6}$	+	–
	$10^{4.6}$	+	–
	$10^{3.6}$	–	–
HoBiPeV-d (Ind BHA5309/12)	$10^{8.3}$	+	–
	$10^{7.3}$	+	–
	$10^{6.3}$	+	–
	$10^{5.3}$	–	–
BVDV-1b (Ind S-1449)	$10^{6.5}$	+	+
	$10^{5.5}$	+	+
	$10^{4.5}$	+	+
	$10^{3.5}$	–	–
BVDV-2a (Ind 141353)	$10^{6.6}$	+	+
	$10^{5.6}$	+	+
	$10^{4.6}$	+	+
	$10^{3.6}$	+	+
	$10^{2.6}$	–	–

(+ positive, - negative).

infection occurs in other ruminant species, such as buffalo (Stalder et al., 2005) and sheep and goats (Shi et al., 2016). Moreover, HoBiPeV has frequently been detected in contaminated bovine serum in several counties (Bauermann and Ridpath, 2015) with implications of safety of biological products. Availability and use of appropriate diagnostic tests in detection of HoBiPeV are hence important not only in limiting the spread of this pestivirus in case of outbreaks or during import of live animals and products through international trade, but also due to the risks imposed on BVDV eradication programs. So far, four genotypes of HoBiPeV (a–d) have been reported (Mishra et al., 2014; Haider et al., 2014; Giammarioli et al., 2015), and the BVDV or HoBiPeV diagnostic tests have been evaluated mostly for the strains of HoBiPeV-a genotype, which include all the HoBiPeV isolated from samples collected outside the Indian subcontinent. In this study, we report evaluation of BVDV diagnostic tests and HoBiPeV specific diagnostic tests for their ability in detection of recently identified highly divergent strains of HoBiPeV-c and HoBiPeV-d genotype circulating in India, which has significant implications on HoBiPeV diagnosis and BVD control.

Table 6
Comparison of commercial BVDV antibody ELISA and virus neutralization test in detection of antibodies against HoBiPeV or BVDV-1/BVDV-2 in sera of cattle from four farms with HoBiPeV natural infection.

	Animal ID	IDEXX Ab ELISA	Ingenasa Ab ELISA	VNT titre with		
				BVDV-1	BVDV-2	HoBiPeV (c/d)
Farm 1	1a	P	P	1:640	1:160	1:2560
	1b	N	P	1:10	1:20	1:320
	1c	P	P	1:320	1:160	1:640
	1d	P	P	1:10	1:10	1:320
	1e	P	P	1:20	40	1:160
	1f	P	P	1:20	1:20	20
	1g	P	P	10	1:20	40
	1h	P	P	1:10	1:10	10
	1i	P	P	1:320	1:160	80
Farm 2	2a	P	N	1:80	1:40	1:640
	2b	P	P	1:20	1:20	1:1280
	2c	P	P	1:80	1:80	1:160
	2d	P	P	1:10	1:20	1:1280
	2e	N	N	1:10	1:10	1:1280
	2f	P	N	1:320	1:160	20
	2g	N	N	1:20	1:10	1:1280
	2h	N	N	1:40	1:80	10
	2i	P	P	1:160	1:20	1:640
	2j	P	P	1:40	1:10	10
	2k	P	P	1:20	1:20	10
	2l	P	N	1:20	1:10	20
Farm 3	3a	N	N	1:20	1:20	1:1280
	3b	P	P	1:20	1:10	1:320
	3c	P	P	1:40	1:20	1:160
	3d	P	P	1:20	1:320	1:640
	3e	P	N	1:20	1:10	1:640
	3f	P	P	1:80	1:40	1:80
	3g	N	N	1:10	1:160	160
	3h	P	N	1:10	1:10	1:640
	3i	P	P	1:10	1:20	10
	3j	P	P	1:20	1:2560	1280
Farm 4	4a	N	P	1:320	1:80	1:10
	4b	N	N	1:160	1:80	640
	4c	P	P	1:320	1:80	1:160
	4d	P	P	1:160	1:40	160
	4e	P	N	1:20	1:80	1:160
	4f	P	P	1:320	1:20	1:80
	4g	P	N	1:160	1:80	1:20
	4h	P	P	1:160	1:40	1:640
	4i	P	N	1:80	1:20	1:20
	4j	P	N	1:80	1:10	1:10
Total	41	33 positive (80.49%)	26 positive (63.41%)	41	41	41

P – positive, N – negative.

Evaluation of two commonly used BVDV diagnostic RT-PCR tests showed that one of the test (Vilcek et al., 1994) failed either completely or partially in detection of highly divergent HoBiPeV strains, while the other one (Hoffmann et al., 2006) was able to detect them but with reduced sensitivity. Our results corroborated the findings of previous studies which showed that the pestivirus generic primer pairs 324/326, which is commonly used for BVDV surveillance are not reliable in detection of HoBiPeV, due to the presence of mismatch at the 3′-end of 324 primer sequence (Decaro et al., 2012a; Bauermann et al., 2014a). The lack of detection for HoBiPeV-c strain and a very low detection limit of 324/326 RT-PCR assay for HoBiPeV-d strain observed in this study compared to the detection limit reported for HoBiPeV-a strain (Bauermann et al., 2014a) has shown its further limitations in detection of HoBiPeV. This can be explained by the fact that there was three mismatches within 324 primer sequence and one mismatch within 326 primer sequence for the HoBiPeV-c strain Ind ABI15385/12 (GenBank Acc. KM201313), while four mismatches, including one at 3′-end of 324 primer sequence were found for the HoBiPeV-d strain Ind BHA5309 (GenBank Acc. KM201300). The RT-PCR assay (Hoffmann et al., 2006) which uses the OIE prescribed pestivirus generic primer sets 190-F/326 and of late is being used for BVDV screening could detect the divergent HoBiPeV strains of genotype c and d indicating some utility in HoBiPeV detection. However, a reduced sensitivity ($10^{3.3}$ $TCID_{50}$) was observed for strain of HoBiPeV-d genotype when compared to the sensitivity for HoBiPeV-c genotype that may affect detection of acutely infected animals following infection with strains of HoBiPeV-d.

Since these two BVDV diagnostic RT-PCR assays are unable to type the bovine pestiviruses, we evaluated the performance of a previously reported HoBiPeV specific RT-PCR (Bauermann et al., 2014a). The RT-PCR assay using N2/R5 primers was found to be HoBiPeV specific without showing any cross reactivity with BVDV-1/BVDV-2 corroborating previous findings (Bauermann et al., 2014a,b). Detection limit of this assay has been reported to be $10^{1.6}$ $TCID_{50}$ for HoBiPeV-a strain D32/00 (Bauermann et al., 2014a). However, a lower sensitivity ($10^{3.3}$ $TCID_{50}$) of this assay for HoBiPeV-d genotype was observed in this study that may lead to failure in detection of infected animals having a low HoBiPeV-d virus titre, which is common in most of the acute infections of HoBiPeV. It may be noted that when compared with N2 primer sequences, four mismatches were found for HoBiPeV-c strain and three mismatches were found for HoBiPeV-d strain, while two mismatches within R5 primer sequences were observed for HoBiPeV-c strain. Moreover, in the cattle serum spiked study, it was observed that in case of mixed infection with highly divergent HoBiPeV-d and BVDV-1, N2/R5 primer set has a lower sensitivity for detection of HoBiPeV, indicating a requirement in refinement of HoBiPeV specific primers for RT-PCR.

In contrast to the results of BVDV diagnostic RT-PCR, a higher sensitivity in detection of HoBiPeV strains of genotype c and d was found using the commercial BVDV diagnostic qRT-PCR tests (VetMax Gold and Virotype) or the previously reported BVDV diagnostic qRT-PCR (Hoffmann et al., 2006). The commercial Virotype qRT-PCR test showed a higher sensitivity in detection of both the highly divergent HoBiPeV strains, compared to the other two qRT-PCR tests evaluated, which showed a lower sensitivity ($10^{2.3}$ $TCID_{50}$) in detecting HoBiPeV-d strain. A previous study has shown better rates (100%) of detection by using Virotype qRT-PCR in serum, buffy coat and ear notch samples in HoBiPeV PI animals than that found (83% for both serum and buffy coat) using VetMAX Gold qRT-PCR (Bauermann et al., 2014b). The differences in sensitivity of these tests may be partly due to the mismatches in primer or probe sequences with HoBiPeV genomic

Table 7
Evaluation of BVDV antibody ELISA kits for quantification of levels of HoBiPeV antibodies.

Ab ELISA	Serum dilutions used in Ab detection ELISA																	
	Serum 4h					Serum 2b			Serum 3b					Serum 1a				
	1:10	1:20	1:40	1:80	1:160	1:10	1:20	1:40	1:10	1:20	1:40	1:80	1:160	1:10	1:20	1:40	1:80	1:160
IDEXX	+	+	+	–	–	–	–	–	+	+	+	–	–	+	+	+	–	–
INGENASA	+	+	+	–	–	–	–	–	+	+	+	–	–	+	+	+	–	–
VN Titre (homologous)	640					320				320				2560				

+: Positive; -: Negative.

sequences. However, it could not be ascertained due to lack of information on the nucleotides and probe sequences employed in both the two commercial BVDV diagnostic qRT-PCR tests, while one mismatch with primer 190 F (towards 3′-end) was found for the HoBiPeV-d strain and one mismatch with probe sequence (towards 3′-end) was found for HoBiPeV-c strain. The lower sensitivity of some of the BVDV diagnostic qRT-PCR tests for HoBiPeV-d strain is not unexpected, since strains of this genotype are distantly related to BVDV-1 or BVDV-2 and are the most highly divergent HoBiPeV strains reported so far (Mishra et al., 2014; Giammarioli et al., 2015).

As BVDV qRT-PCR tests have limited utility in surveillance and epidemiology of HoBiPeV due to their lack of ability in differentiation of BVDV-1, BVDV-2 and HoBiPeV infection, HoBiPeV specific qRT-PCR test (Liu et al., 2008) was evaluated here for detection of highly divergent HoBiPeV strains. This test has been used in successful detection of HoBiPeV in other studies (Bauermann et al., 2014a,b; Decaro et al., 2014; Mishra et al., 2014), although a detection rate of 83% has been found in PI animals (Bauermann et al., 2014b) and some cross reactivity with BVDV-2 strains has been reported (Decaro et al., 2013). Despite the test was found HoBiPeV specific and was able to detect the strains of both HoBiPeV-c and d genotype, lower sensitivity ($10^{2.3}$ $TCID_{50}$) was observed for the HoBiPeV-d strain. Mismatches within T134 F and T220R primer sequences were found for both the strains, while within T155 r probe sequences, five mismatches including one towards 3′-end were found for HoBiPeV-d strain (KM201300), compared to only one mismatch found for HoBiPeV-c strain (KM201313). Lower sensitivity in detection of HoBiPeV-d strain may be partly due to high mismatches found specifically in the probe sequences. Since differentiation between HoBiPeV and BVDV-1/BVDV-2 is critical in bovine pestivirus epidemiology, HoBiPeV-specific qRT-PCR test has been found useful for surveillance and monitoring of HoBiPeV. However, in view of the lower sensitivity for HoBiPeV-d strain in this study, further improvement of HoBiPeV specific qRT-PCR test is required. Recently, a two-step real-time RT-PCR assay has been developed for detection of bovine pestiviruses, including HoBiPeV (Losurdo et al., 2015), while a two-step multiplex real-time RT-PCR assay has shown promising results for simultaneous detection and differentiation of BVDV-1, BVDV-2 and HoBiPeV (Mari et al., 2016). However, these new tests need to be validated in detection of highly divergent HoBiPeV strains. Moreover, specificity, reproducibility and other diagnostic parameters of HoBiPeV-specific or pestivirus specific tests should be evaluated in future.

Both E^{rns} based and NS3 based BVDV antigen ELISA kits are routinely used for detection of BVDV PI animals. The BVDV NS3 antigen ELISA used in this study failed to detect both the highly divergent strains of HoBiPeV even at a high virus titre corroborating findings of previous studies (Schirrmeier et al., 2004; Bauermann et al., 2012, 2014b). The failure in detection may be due to lack of cross reactivity between HoBiPeV and BVDV within the epitopes of NS3 mAb used in this commercial kit or due to high divergence found between HoBiPeV and BVDV NS3 in previous studies (Schirrmeier et al., 2004; Bauermann et al., 2012). In contrast, the BVDV E^{rns} antigen ELISA was able to detect both the highly divergent strains of HoBiPeV in serum but with differences in sensitivity. Successful detection of HoBiPeV by employing this antigen ELISA has been reported in several studies earlier (Stahl et al., 2007; Bauermann et al., 2012, 2014b; Haider et al., 2014). However, the same test either failed to detect HoBiPeV isolate IZSPLV_To (Peletto et al., 2012) or showed low COD levels in serum of cattle infected experimentally with HoBiPeV strain Th/04_Khonkaen (Larska et al., 2012) or failed in detection of some of the experimentally generated HoBiPeV PI calves at birth and one week after birth (Bauermann et al., 2014b). In a previous study, it has been found that this test has almost similar sensitivity in detection of BVDV-1, BVDV-2 and HoBiPeV (Bauermann et al., 2012). However, a lower sensitivity for HoBiPeV-d strain was observed in this study, which may be due to probable divergence between HoBiPeV and the E^{rns} mAb 15c5 epitope. This indicates that it may fail in detection of some of the HoBiPeV PI animals having a low virus titre, since the virus titre in serum of PI animals has been reported to range between $10^{2.3}$ and $10^{6.0}$ $TCID_{50}$ (Bolin et al., 1985). Hence, further studies using a broader selection of HoBiPeV isolates and field samples representing different geographical areas are required to determine the suitability of this test in surveillance of HoBiPeV. Nevertheless, in agreement with a previous study, our results indicate that during preliminary screening of PI animals use of both E^{rns} antigen ELISA and NS3 antigen ELISA together may be able to differentiate between HoBiPeV infection and BVDV infection.

Although VNT is the gold standard for detection of specific antibodies against HoBiPeV, antibody ELISA is most commonly used as an alternative method for detection of pestivirus antibodies in cattle. Evaluation of commercial BVDV antibody ELISA kits in detection of HoBiPeV antibodies in cattle from farms with circulation of highly divergent strains HoBiPeV infection showed low to moderate sensitivity of the tests used. Compared to the VNT, the whole virus antigen based ELISA showed a sensitivity of 80.49%, while the NS3 antigen based kit displayed a sensitivity of 63.41% in detection of pestivirus antibodies. But they failed in detecting antibodies in 5 of 16 (31.25%) and 9 of 16 (56.25%) animals respectively, which had high (> 4-fold) HoBiPeV specific neutralizing antibodies. Variation in sensitivity of BVDV antibody ELISA kits for detection of antibodies against HoBiPeV and their correlation with VNT has been reported in previous studies (Decaro et al., 2011; Larska et al., 2013; Bauermann et al., 2012, 2014a). Antibodies against HoBiPeV were not detected by the whole virus antigen based BVDV antibody ELISA, until 4 weeks post infection (Larska et al., 2012) or until 6 weeks post infection, where it detected 77% of VN positive samples (Bauermann et al., 2012). Delay in detection of antibodies against HoBiPeV and preferential detection of BVDV-1 antibodies by the commercial antibody ELISA kits has also been noticed in another study (Larska et al., 2013). In consistent with our results, a lower sensitivity of NS3 antigen based ELISAs (indirect and blocking) has been found for detection of antibodies against HoBiPeV with a report of sensitivity as low as 33.3% (Bauermann et al., 2012; Larska et al., 2013). This may be partly due to the differences found between HoBiPeV and BVDV NS3 amino acid sequences (Schirrmeier et al., 2004). Due to low sensitivity and late detection of seroconversion, BVDV NS3 antibody ELISAs may have limited utility in detection of antibodies against HoBiPeV.

Failure of BVDV antibody ELISA in detection of considerable number of sera having HoBiPeV neutralizing titre of below 256 has been reported (Bauermann et al., 2012). Surprisingly, both the BVDV antibody ELISA kits used here failed to detect antibodies in three samples, which had very high HoBiPeV neutralizing antibody titres (1280) indicating their limited utility in detection of HoBiPeV antibodies. Despite the fact that E2 protein is responsible for generation of neutralizing antibodies and the antibody ELISA used are based on whole virus antigen or NS3 antigen, with some degree of sequence conservation anticipated, the reasons are not clearly known. Failure of antibody ELISAs in quantification of levels of HoBiPeV antibodies is another limitation. Considering the occurrence of low antibody titres following natural infection with bovine pestiviruses and low to moderate sensitivity of commercial BVDV antibody ELISA kits for HoBiPeV exposure found earlier and in this study, there is a need of robust antibody ELISA kits for reliable detection of antibodies against all the three bovine pestiviruses.

The present study demonstrated newer challenges in HoBiPeV diagnosis indicating a need in improvement of both HoBiPeV specific diagnostic RT-PCR and qRT-PCR and development of robust antibody ELISA for better utility in HoBiPeV epidemiology. The results suggest that use of pestivirus generic qRT-PCR followed by HoBiPeV-specific qRT-PCR may have some utility in detection of highly divergent HoBiPeV strains. The use of multiple tests and need of constant updating of diagnostic tests for accurate and reliable detection of HoBiPeV and its differentiation from BVDV-1/BVDV-2 is envisaged.

Declaration of Competing Interest

All the authors declare no competing interest.

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References

Bauermann, F.V., Flores, E.F., Ridpath, J.F., 2012. Antigenic relationships between bovine viral diarrhea virus 1 and 2 and HoBi virus: possible impacts on diagnosis and control. J. Vet. Diagn. Invest. 24, 253–261.

Bauermann, F.V., Ridpath, J.F., Weiblen, R., Flores, E.F., 2013. HoBi-like viruses: an emerging group of pestiviruses. J. Vet. Diagn. Invest. 25, 6–15.

Bauermann, F.V., Flores, E.F., Falkenberg, S.M., Weiblen, R., Ridpath, J.F., 2014a. Lack of evidence for the presence ofemerging HoBi-like viruses in North American fetal bovine serum lots. J. Vet. Diagn. Invest. 26, 10–17.

Bauermann, F.V., Falkenberg, S.M., Vander ley, B., Decaro, N., Brodersen, B.W., Harmon, A., Hessman, B., Flores, E.F., Ridpath, J.F., 2014b. Generation of calves persistently infected with HoBi-like pestivirus and comparison of methods for detection of these persistent infections. J. Clin. Microbiol. 52, 3845–3852.

Bauermann, F.V., Ridpath, J.F., 2015. HoBi-like viruses- the typical atypical bovine pestivirus. Anim. Health Res. Rev. 16, 64–69.

Behera, S.P., Mishra, N., Vilcek, S., Rajukumar, K., Nema, R.K., Prakash, A., Kalaiyarasu, S., Dubey, S.C., 2011. Genetic and antigenic characterization of bovine viral diarrhoea virus type 2 isolated from cattle in India. Comp. Immunol. Microbiol. Infect. Dis. 34, 189–196.

Bolin, S.R., McClurkin, A.W., Coria, M.F., 1985. Frequency of persistent bovine viral diarrhea virus infection in selected cattle herds. Am. J. Vet. Res. 46, 2385–2387.

Cortez, A., Heinemann, M.B., De Castro, A.M.M.G., Soares, R.M., Pinto, A.M.V., Alfieri, A.A., Flores, E.F., Leite, R.C., Richtzenhain, L.J., 2006. Genetic characterization of Brazilian bovine viral diarrhea virus isolates by partial nucleotide sequencing of the 5′-UTR region. Pesq. Vet. Bras. 26, 211–216.

Cruz, R.A.S., Rodrigues, W.B., Silveira, S., Oliveira, V.H.S., Campos, C.G., Leite Filho, R.V., Boabaid, F.M., Driemeier, D., Canal, C.W., Alfieri, A.A., Pescador, C.A., Colodel, E.M., 2018. Mucosal disease-like lesions caused by HoBi-like pestivirus in Brazilian calves in 2010–2011: clinical, pathological, immunohistochemical and virological characterization. Res. Vet. Sci. 119, 116–121.

Decaro, N., Lucente, M.S., Mari, V., Cirone, F., Cordioli, P., Camero, M., Sciarretta, R., Losurdo, M., Lorusso, E., Buonavoglia, C., 2011. Atypical pestivirus and severe respiratory disease in calves. Europe. Emerg. Infect. Dis. 17, 1549–1552.

Decaro, N., Lucente, M.S., Mari, V., Sciaretta, R., Pinto, P., Buonavoglia, D., Martella, V., Buonavoglia, C., 2012a. Hobi-like pestivirus in aborted bovine fetuses. J. Clin. Microbiol. 50, 509–512.

Decaro, N., Mari, V., Pinto, P., Lucente, M.S., Sciarretta, R., Cirone, F., Colaianni, M.L., Elia, G., Buonavoglia, C., 2012b. Hobi-like pestivirus: both biotypes isolated from diseased animal. J. Gen. Virol. 93, 1976–1983.

Decaro, N., Mari, V., Lucente, M.S., Sciarretta, R., Elia, G., Ridpath, J.F., Buonavoglia, C., 2013. Detection of a Hobi-like virus in archival samples suggests circulation of this emerging pestivirus species in Europe prior to 2007. Vet. Microbiol. 167, 307–313.

Decaro, N., Lanave, G., Lucente, M.S., Mari, V., Varello, K., Losurdo, M., Larocca, V., Bozzetta, E., Cavaliere, N., Martella, V., Buonavoglia, C., 2014. Mucosal disease-like lesions in a calf persistently infected by Hobi-like pestivirus. J. Clin. Microbiol. 8, 2946–2954.

Decaro, N., Lucente, M.S., Losurdo, M., Larocca, V., Elia, G., Occhiogrosso, L., Marino, P.A., Cirone, F., Buonavoglia, C., 2016. HoBi-like pestivirus and its impact on cattle productivity. Transbound. Emerg. Dis. 63, 469–473.

Evans, C.A., Pinior, B., Larska, M., Graham, D., Schweizer, M., Guidarini, C., Decaro, N., Ridpath, J., Gates, M.C., 2019. Global knowledge gaps in the prevention and control of bovine viral diarrhoea (BVD) virus. Transbound. Emerg. Dis. 66, 640–652.

Gaede, W., Reiting, R., Schirrmeier, H., Depner, K.R., Beer, M., 2005. Detection and species-specific differentiation of pestiviruses using real-time RT-PCR. Berl. Munch. Tierarztl. Wochenschr 118, 113–120.

Giammarioli, M., Ridpath, J.F., Rossi, E., Bazzuchi, M., Casciari, C., De Mia, G.M., 2015. Genetic detection and characterization of emerging HoBi-like viruses in archival foetal bovine serum batches. Biologicals 43, 220–224.

Haider, N., Rahman, M.S., Khan, S.U., Mikolon, A., Gurley, E.S., Osmani, M.G., Shanta, I.S., Paul, S.K., Macfarlane-Berry, L., Islam, A., Desmond, J., Epstein, J.H., Daszak, P., Azim, T., Luby, S.P., Zeidner, N., Rahman, M.Z., 2014. Identification and epidemiology of a rare HoBi- like pestivirus strain in Bangladesh. Transbound. Emerg. Dis. 61, 193–198.

Hoffmann, B., Depner, K., Schirrmeier, H., Beer, M., 2006. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J. Virol. Methods 136, 200–209.

Kampa, J., Alenius, S., Emanuelson, U., Chanlun, A., Aiumlamai, S., 2009. Bovine herpesvirus type 1 (BHV-1) and bovine viral diarrhoea virus (BVDV) infections in dairy herds: self clearance and the detection of seroconversions against a new atypical pestivirus. Vet. J. 182, 223–230.

Larska, M., Polak, M.P., Riitho, V., Strong, R., Belak, S., 2012. Kinetics of single and dual infection of calves with an Asian atypical bovine pestivirus and a highly virulent strain of bovine viral diarrhoea virus 1. Comp. Immunol. Microbiol. Infect. Dis. 35, 381–390.

Larska, M., Polak, M.P., Liu, L., Alenius, S., Uttenthal, A., 2013. Comparison of the performance of five different immunoassays to detect specific antibodies against emerging atypical bovine pestivirus. J. Virol. Methods 187, 103–109.

Liu, L., Xia, H., Belak, S., Baule, C., 2008. A TaqMan real-time RT-PCR assay for selective detection of atypical bovine pestiviruses in clinical samples and biological products. J. Virol. Methods 154, 82–85.

Liu, L., Xia, H., Wahlberg, N., Belak, S., Baule, C., 2009. Phylogeny, classification and evolutionary insights into pestiviruses. Virology 385, 351–357.

Losurdo, M., Mari, V., Lucente, M.S., Colaianni, M.L., Padalino, I., Cavaliere, N., Buonavoglia, C., Decaro, N., 2015. Development of a TaqMan assay for sensitive detection of all pestiviruses infecting cattle, including the emerging HoBi-like strains. J. Virol. Methods 224, 77–82.

Mao, L., Li, W., Zhang, W., Yang, L., Jieyuan, Jiang., 2012. Genome sequence of a novel Hobi-like pestivirus in China. J. Virol. 86, 124–144.

Mari, V., Losurdo, M., Lucente, M.S., Lorusso, E., Elia, G., Martella, V., Patruno, G., Buonavoglia, D., Decaro, N., 2016. Multiplex real-time RT-PCR assay for bovine viral diarrhea virus type 1, type 2 and HoBi-like pestivirus. J. Virol. Methods 229, 1–7.

Meyers, G., Thiel, H.J., 1996. Molecular characterization of pestiviruses. Adv. Virus Res. 47, 53–118.

Mishra, N., Pattnaik, B., Vilcek, S., Patil, S.S., Jain, P., Swamy, N., Bhatia, S., Pradhan, H.K., 2004. Genetic typing of bovine viral diarrhea virus isolates from India. Vet. Microbiol. 104, 207–212.

Mishra, N., Rajukumar, K., Vilcek, S., Tiwari, A., Satav, J.S., Dubey, S.C., 2008. Molecular characterization of bovine viral diarrhea virus type 2 isolate originating from a native Indian sheep (*Ovies aries*). Vet. Microbiol. 130, 88–98.

Mishra, N., Rajukumar, K., Pateriya, A., Kumar, M., Dubey, P., Behera, S.P., Verma, A., Bhardwaj, P., Kulkarni, D.D., Vijaykrishna, D., Reddy, N.D., 2014. Identification and molecular characterization of novel and divergent HoBi-like pestiviruses from naturally infected cattle in India. Vet. Microbiol. 174, 239–246.

Neill, J.D., Workman, A.M., Hesse, R., Bai, J., Porter, E.P., Meadors, B., Anderson, J., Bayles, D.O., Falkenberg, S.M., 2019. Identification of BVDV2b and 2c subgenotypes in the United States: genetic and antigenic characterization. Virology 528, 19–29.

Peletto, S., Zuccon, F., Pitti, M., Gobbi, E., Marco, L.D., Caramelli, M., Masoero, L., Acutis, P.L., 2012. Detection and phylogenetic analysis of an atypical pestivirus strain IZSPLV_To. Res. Vet. Sci. 92, 147–150.

Reed, L., Muench, H., 1938. A simple method of estimating fifty percent end points. Am. J. Hyg. 18, 493–494.

Schirrmeier, H., Strebelow, G., Depner, K., Hoffman, B., Beer, M., 2004. Genetic and antigenic characterization of an atypical pestivirus isolate, a putative member of a novel pestivirus species. J. Gen. Virol. 85, 3647–3652.

Shi, H., Kan, Y., Yao, L., Leng, C., Tang, Q., Ji, J., Sun, S., 2016. Identification of natural infections in Sheep/Goats with HoBi-like pestiviruses in China. Transbound. Emerg. Dis. 63, 480–484.

Silveira, S., Baumbach, L.F., Weber, M.N., Mósena, A.C.S., Da Silva, M.S., Cibulski, S.P., Borba, M.R., Maia, R.D., Coimbra, V.C.S., De Moraes, G.M., Ridpath, J.F., Canal, C.W., 2018. HoBi-like is the most prevalent ruminant pestivirus in Northeastern Brazil. Transbound. Emerg. Dis. 65, e113–e120.

Stahl, K., Kampa, J., Alenius, S., Wadmsn, A.P., Baule, C., Aiumlamai, S., Belak, S., 2007. Natural infection of cattle with an atypical 'HoBi'-like pestivirus – implication for BVD control and safety of biological products. Vet. Res. 38, 517–523.

Stalder, H.P., Meier, P., Pfaffen, G., Canal, C.W., Rüfenacht, J., Schaller, P., Bachofen, C., Marti, S., Vogt, H.R., Peterhans, E., 2005. Genetic heterogeneity of pestiviruses of ruminants in Switzerland. Prev. Vet. Med. 72, 37–41.

Smith, D.B., Meyers, G., Bukh, J., Gould, E.A., Monath, T., Muerhoff, S., Pletnev, A., Rico-Hesse, R., Stapleton, J.T., Simmonds, P., Becher, P., 2017. Proposed revision to the taxonomy of the genus *Pestivirus*, family *Flaviviridae*. J. Gen. Virol. 98, 2106–2112.

Vilcek, S., Herring, A.J., Herring, J.A., Nettleton, P.F., Lowings, J.P., Paton, D.J., 1994. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch. Virol. 136, 309–323.

Weber, M.N., Mosena, A.C.S., Simões, S.V.D., Almeida, L.L., Pessoa, C.R., Budaszewski, R.F., Silva, T.R., Ridpath, J.F., Riet-Correa, F., Driemeier, D., Canal, C.W., 2016. Clinical presentation resembling mucosal disease associated with HoBi-like pestivirus in a field outbreak. Transbound. Emerg. Dis. 63, 92–100.

Xia, H., Vijayaraghavan, B., Belak, S., Liu, L., 2011. Detection and identification of the atypical bovine pestiviruses in commercial foetal bovine serum batches. PLoS One 6, e28553.

Yesilbag, K., Alpay, G., Becher, P., 2017. Variability and Global distribution of subgenotypes of bovine viral diarrhea virus. Viruses 9.