Clinical Presentation Resembling Mucosal Disease Associated with ‘HoBi’-like Pestivirus in a Field Outbreak

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Summary

The genus Pestivirus of the family Flaviviridae consists of four recognized 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). Recently, atypical pestiviruses (‘HoBi’-like pestiviruses) were identified in batches of contaminated foetal calf serum and in naturally infected cattle with and without clinical symptoms. Here, we describe the first report of a mucosal disease-like clinical presentation (MD) associated with a ‘HoBi’-like pestivirus occurring in a cattle herd. The outbreak was investigated using immunohistochemistry, antibody detection, viral isolation and RT-PCR. The sequence and phylogenetic analysis of 5’NCR, Nprotein and E2 regions of the RT-PCR positive samples showed that four different ‘HoBi’-like strains were circulating in the herd. The main clinical signs and lesions were observed in the respiratory and digestive systems, but skin lesions and corneal opacity were also observed. MD characteristic lesions and a pestivirus with cytopathic biotype were detected in one calf. The present study is the first report of a MD like presentation associated with natural infection with ‘HoBi’-like pestivirus. This report describes the clinical signs and provides a pathologic framework of an outbreak associated with at least two different ‘HoBi’-like strains. Based on these observations, it appears that these atypical pestiviruses are most likely underdiagnosed in Brazilian cattle.

Introduction

The genus Pestivirus of the family Flaviviridae consists of four recognized 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; Simmonds et al., 2011). A tentative fifth species that was defined as a pestivirus was isolated from a giraffe in Kenya (Avalos-Ramirez et al., 2001). A tentative fifth species that was defined as a pestivirus was isolated from a giraffe in Kenya (Avalos-Ramirez et al., 2001). Besides the established species, there are three groups of recently identified, but unclassified pestiviruses consisting of atypical pestivirus of bovine origin (‘HoBi’-like pestivirus; Schirrmeier et al., 2004), as well as of non-bovine origin such as pronghorn antelope virus (Vilcek et al., 2005) and Bungowannah virus from pigs (Kirkland et al., 2007).

Pestiviruses have a single-stranded, positive-sense RNA genome that contains one open reading frame (ORF) that is flanked by non-coding regions (NCRs) at the 5’ and 3’ ends, which encodes a polyprotein that is processed into 12 polypeptides (Simmonds et al., 2011). The 5’NCR and N-terminal autoprotease (Nprotein) are the genomic regions most frequently used to characterize the genus and species of new strains using phylogenetic
methods (Becher et al., 1999, 2003; Vilcek et al., 2001, 2005; Schirrmeier et al., 2004; Cortez et al., 2006; Kirkland et al., 2007; Liu et al., 2009a,b; Decaro et al., 2011; Xia et al., 2011, 2012; Peletto et al., 2012). However, the envelope glycoprotein 2 (E2) gene has been used in some works for phylogeny (Becher et al., 1999, 2003; Schirrmeier et al., 2004; Liu et al., 2009a,b; Xia et al., 2011, 2012; Peletto et al., 2012).

Bovine viral diarrhea virus and BDV may cause acute or persistent infection in cattle. Acute infections generally are subclinical but when present, signs include diarrhea, fever, leukopenia, ocular and nasal discharge. Most acute infections, in animals with non-compromised immune systems, are cleared within 14–21 days. Persistent infection is established when a virus, belonging to the non-cytopathic biotype, crosses the placenta and infects a non-immunocompetent foetus. The persistently infected (PI) calves resulting from this infection may have retarded growth and congenital defects or appear normal. Regardless of appearance PI calves excrete virus throughout their lives, spreading the infection in the herd. Many PI calves die in the first 2 years of its life from mucosal disease (MD) or secondary infections, probably as a consequence of virus-induced immune suppression (MacLachlan and Dubovi, 2011). MD occurs when cattle are infected with both non-cytopathic and cytopathic viral biotypes that are antigenically homologous (Pocock et al., 1987; Brownlie, 1990; Baker, 1995).

In 2004, an atypical bovine pestivirus, strain D32/00_Hobi, was isolated from a contaminated batch of foetal calf serum (FCS) originating from Brazil (Schirrmeier et al., 2004). This strain was proposed as the prototype of an emerging pestivirus species variously known as atypical bovine pestivirus, ‘HoBi’-like pestiviruses and BVDV-3 (Liu et al., 2009a). Thereafter, other ‘HoBi’-like strains were detected in other bovine materials including FCSs from different regions of the world (Stalder et al., 2005; Liu et al., 2009b; Xia et al., 2011, 2012; Peletto et al., 2012), in a Brazilian buffalo (Stalder et al., 2005), in aborted foetuses in Brazil (Cortez et al., 2006; Bianchi et al., 2011) and Italy (Decaro et al., 2012a), in a naturally infected calf from Thailand (Stähl et al., 2007), associated with a severe respiratory disease in Italy (Decaro et al., 2011, 2012b, 2013a) and causing persistent infection (Decaro et al., 2013b). However, little information is available about the clinical signs and pathology caused by acute ‘HoBi’-like natural infections and MD like clinical presentation has not been reported. Here, we describe an outbreak of bovine viral diarrhoea-like disease that included signs resembling mucosal disease, associated with the presence of different strains of ‘HoBi’-like viruses in one cattle herd.

Materials and Methods

Clinical history and sample collection

Clinical symptoms of disease were observed in a Brown Swiss cattle herd of 123 animals in Pombal, in the semi-arid region of Paraíba state, north-eastern Brazil, from November 2011 to March 2012. The epidemiologic data and clinical signs were collected and observed during four visits (November, December and twice in March) to the farm during this period and in the affected bovines that were sent to the Veterinary Hospital. Serum samples and biopsies of the skin of the ear were collected from live animals. Six calves with retarded growth from four to seven months of age and four cows with reproductive disorders were observed. Data about signs in the other animals were not available or were taken from non-veterinary staff of the farm and were not further described.

Necropsies were performed on four calves identified as positive by RT-PCR (Deregt et al., 2006), between the ages of 5 and 7 months, that either died (LV01/12, LV02/12 and LV03/12) or was euthanized in extremis (LV04/12). Samples from organs of the thoracic (oesophagus, trachea, lungs and heart) and abdominal cavities (rumen, omasum, reticulum, abomasum, duodenum, jejunum, ileum, colon, rectum, kidneys, spleen, liver, pancreas and adrenal), the skin and the entire central nervous system were fixed in 10% buffered formalin. Samples of these organs were embedded in paraffin, cut into 4–5 μm sections and stained by haematoxylin and eosin for histologic studies.

Immunohistochemistry

Initially, for a first screening in the herd, skin biopsies of the extremity of the ear (ear notches) were collected from two calves (LV01/12 and LV02/12) and submitted for immunohistochemistry (IHC) in November 2011. Later, ear notches were collected, from four cows with a history of infertility or abortion and from four 5–7-month-old calves with retarded growth or respiratory signs and submitted for IHC analysis. The IHC was performed using the monoclonal antibody #15c-5 (Syracuse Bioanalytical Inc., Ithaca, NY, USA), which specifically binds the E<sup>rm</sup> protein of pestiviruses (Santos et al., 2011).

ELISA for antibody detection (ELISA-Ab)

The 123 bovine serum samples, collected from animals in this herd in December 2011, were tested for antibodies against BVDV using a commercial indirect ELISA kit (SVA-NOVIR<sup>TM</sup> BVDV-Ab kit, SVANOVA Biotech, Uppsala, Sweden), following the manufacturer’s recommendations. Serum from seven animals, identified as being negative by the test, were collected a second time and tested for the presence of antibodies in March 2012.
RT-PCR

Total RNA was extracted from the 123 serum samples using TRIzol™ LS Reagent (Life Technologies, Carlsbad, CA, USA) and suspended in 50 μl of ultrapure water, according to the manufacturer’s instructions. The cDNAs were synthesized using the SuperScript™ III Reverse Transcriptase Kit (Life Technologies), in a total volume of 20 μl, following the manufacturer’s recommendations. The PCRs were prepared in a total volume of 25 μl that contained 2 μl of cDNA, using two protocols to amplify fragments of the 5′NCR: the first used primers 324 5′- ATGCCCWGTAGGACTAGCA-3′ (position in BVDV-1 strain NADL: 108-128) and 326 5′- TCAACTCCATGTGCCATG- TAC-3′ (position in strain NADL: 395-375) that amplify a 288 bp described by Vilcek et al. (1994) and the second using the primers forward 5′-CATGCCCRYAGTAGGACTAGC-3′ (position in strain NADL: 107-127) and reverse 5′-ATGTGCCCAGTAGCAGAG-3′ (position in strain NADL: 387-368) that amplify a 280 bp described by Deregt et al. (2006). The amplification products were stained with Blue Green Loading Dye I (LGC Biotecnologia, Cotia, Brazil) and electrophoresed in 2% agarose gels. The BVDV-1 NADL strain was used as positive control. Serum from the RT-PCR positive animals were collected again in March 2012 and retested to verify persistent infection.

For DNA sequencing, partial sequences of 5′NCR (Deregt et al.,) Npro (position in HoBi-like virus strain Th_04/KhonKaen: 384-405 and 878-868; fragment size: 494 bp; Liu et al., 2009c) and glycoprotein E2 (position in strain NADL: 395-375) that amplify a 288 bp described by Deregt et al. (2006). The amplification products were stained with Blue Green Loading Dye I (LGC Biotecnologia, Cotia, Brazil) and electrophoresed in 2% agarose gels. The BVDV-1 NADL strain was used as positive control. Serum from the RT-PCR positive animals were collected again in March 2012 and retested to verify persistent infection.

Virus isolation

The serum from the RT-PCR positive calves was used to inoculate 25 cm² flasks containing Madin-Darby bovine kidney (MDBK) cells in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% FCS tested free for BVDV and anti-BVDV antibodies, antibiotics (200 IU/ml of penicillin and 200 mg/ml of streptomycin) and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 4 days. The presence of non-cytopathic pestivirus was monitored by immunoperoxidase monolayer assay using an anti-NS2/3 monoclonal after the third passage.

Purification, sequencing and phylogenetic analysis of amplification products

The RT-PCR amplified fragments of the 5′NCR (Liu et al., 2006), Npro (Liu et al., 2009c) and glycoprotein E2 genes (Liu et al., 2009b) were purified using a NucleoSpin Extract II kit (Macherey–Nagel, Düren, Germany). Both DNA strands were sequenced from three independent RT-PCRs with an ABI PRISM 3100 Genetic Analyzer using a BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

The partial sequences of the present study were assembled using SeqMan (DNASTAR Lasergene® 11, Madison, WI, USA). Sequence alignment was performed with Molecular Evolutionary Genetics Analysis version 5 (MEGA 5) (Tamura et al., 2011) using CLUSTAL W. The sequences of the primers were excised and the resulting sequences submitted to GenBank nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for confirmation of their identity.

For phylogenetic analysis, three independent alignments were performed using 189 (5′NCR), 430 (Npro) and 317 (E2) nucleotides, including gaps, of the RT-PCR products and 18 reference and representative strains within the species (GenBank accession numbers are reported in Table S1). MEGA 5 was used for phylogeny inference according to the neighbor-joining criterion (Saitou and Nei, 1987) and Kimura 2-parameter model (Kimura, 1980). The robustness of the hypothesis was tested in 1000 non-parametric bootstrap analyses.

Results

Clinical signs and gross pathology

The disease occurred in a Brown Swiss cattle herd of 123 animals that were monitored by veterinarians from November 2011 to March 2012. Three animals had been introduced into the herd in the second semester of 2010; two cows came from a farm in the same region, and one bull came from south Brazil. The animals were not tested for pestivirus before their introduction into the herd, which was raised in a semi-intensive system and was not vaccinated for BVDV. The farm also had 140 sheep. The farmer reported that four cows aborted or returned to oestrus. Six of the 62 calves that were less than 1-year-old died after clinical manifestations of respiratory signs, diarrhoea that was sometimes haemorrhagic, thickening of the skin, crust- ing around the nose and eyes, corneal opacity, and nasal and ocular discharge over a period of 15 to 20 days. The affected animals had been treated with antibiotics unsuccessfully. Two out of these six calves were tested and found RT-PCR positive for pestiviruses (LV01/12 and LV02/12). Two additional calves tested RT-PCR positive for pestivirus. These calves were subsequently euthanized in extremis (LV04/12) or died (LV03/12). The clinical signs and gross pathology observed with the 4 RT-PCR-positive calves are as follows.

LV01/12, a 7-month-old calf, died in December 2011 and showed mixed dyspnoea, nasal discharge, emaciation, soft faeces and erosions in the lips and hard palate. The skin was thickened with crusts, mainly in the head, neck and
inguinal region. The skin of the coronary band was ulcerated. A clear exudate covered the periorbital skin and both eyes showed corneal opacity, which was more severe in the left eye. The animal died 2 weeks after arriving at the Veterinary Hospital. At necropsy, several rounded or linear ulcerations were observed in the esophagus.

LV02/12, a 5-month-old calf, died in December 2011 and showed marked retarded growth and severe mixed dyspnoea in an orthopneic position. After being treated with antibiotics and anti-inflammatory drugs, the animal improved and returned to the farm, but the clinical signs recurred and the animal died. A necropsy was not performed. On immunohistochemistry, the ear biopsy from this calf displayed pestivirus-positive keratinocytes.

LV03/12, a 7-month-old calf, died in March 2012 and showed severe respiratory signs. Two days after the first clinical signs this calf was found recumbent and subsequently died. At necropsy, the animal was emaciated, with a rough coat and congested ocular mucosa. An abscess was observed on the right caudal lobe of the lung. Adherences were observed between the cranial lobe, the pleura and the pericardium.

LV04/12, a 7-month-old calf, showed depression, emaciation, soft faeces and nasal seromucosal discharge. The farmer reported that the first clinical signs were observed 15 days before. The right parotideal and left retropharyngeal lymph nodes were enlarged, and in some areas, the keratinocytes and basal cells showed hyperplasia, vacuolization or single-cell necrosis. Severe infiltration by neutrophils was observed in the submucosa, mainly around blood vessels. A large area of necrosis with severe infiltration by neutrophils was observed in the muscle of the tongue of LV04/12. Various histological lesions were observed in the abomasum: in LV01/12, multifocal lymphoplasmocytic infiltrates in the submucosa; in LV04/12, a focal area of necrosis that affected the mucosa and the submucosa. In the epithelium of the forestomachs of LV01/12, LV03/12 and LV04/12, cell vacuolization, focal necrosis or single cell necrosis, acanthosis, and pustules were observed. Oedema of the lamina propria was also present. All of the intestinal segments of LV01/12 and LV04/12 showed mild to severe depletion of the lymphoid tissue, and the crypts had herniated into the Payer patches. In LV04/12, a moderate to severe lymphoplasmocytic infiltrate was observed in the lamina propria of the jejunum and colon.

In the respiratory tract, LV01/12, LV03/12 and LV04/12 showed similar lesions: oedema, hyperaemia and interstitial pneumonia characterized by thickening of the alveolar septa. Perivascular infiltrate of lymphocytes and plasma cells were observed in the submucosa of the trachea. The more frequent lesions of the lungs were oedema, hyperaemia and mild to severe histiocytic, interstitial, acute pneumonia characterized by multifocal areas of thickening of the alveolar septum by histiocytic infiltration. A focally extensive abscess was also observed in LV03/12.

The lymph nodes showed two different patterns of lesions: diffuse hyperplasia of either the cortex and or the paracortex (LV01/12 and LV04/12). These two patterns were also observed in LV03/12 as well as necrosis of the lymphoid tissue. In both patterns of lesions, foamy macrophages and necrotic lymphocytes in the cortical and paracortical regions, thickening of the medullary cords by plasma cells and plasmoblasts, and histiocytic infiltration of the medullary sinus were also present. In the spleen of LV03/12, lymphoid depletion with necrosis and absent lymphocytes was observed. Thickening of the periarteriolar sheets with 15-20 layers of cells was observed in LV03/12. In the thymus of LV01/12, there was severe depletion of the follicular lymphocytes and lymphoid necrosis with infiltrated macrophages. The follicles of the tonsils of LV01/12 and LV03/12 were necrotic and surrounded by lymphocytes.

In the skin of LV01/12, multifocal areas of necrosis with microabscesses and exocytosis of neutrophils were observed in the epidermis and in the epithelium of the hair follicles. Coagulative necrosis of the sebaceous glands was also observed. Lymphoplasmocytic perivascular infiltrate and oedema were observed in the skin of LV01/12 and LV04/12.

ELISA-Ab, immunohistochemistry, RT-PCR and virus isolation

Table 1 summarizes the results obtained in the four distinct assays. From the two ear biopsies submitted first to IHC for pestivirus, LV01/12 was negative and LV02/12 was positive.
The ear notch biopsies of the eight animals submitted later, including LV04/12 and three others calves with retarded growth and four cows with a history of infertility or abortion, had negative results. LV02/12 had antigen marking in the keratinocytes (Figure S1A) and the epithelium of the hair follicles (Figure S1B) and was positive by RT-PCR. Two calves that were negative by IHC (LV01/12 and LV04/12) were positive by RT-PCR. LV03/12 that was also positive in RT-PCR but was not tested in IHC.

RT-PCR (Deregt et al., 2006) revealed that four calves (LV01/12, LV02/12, LV03/12 and LV04/12) out of the 123 cattle in the herd contained pestivirus RNA in their serum. All the serum yielded negative results when tested by the other RT-PCR protocol (Vilcek et al., 1994). LV03/12 and LV04/12 were collected again after 30 days (March 2012) and tested positive again. LV01/12 and LV02/12 died before they could be retested.

Viral isolation was performed with the four positive RT-PCR serum collected in December 2011 (LV01/12, LV02/12, LV03/12 and LV04/12) and in two collected in March 2013 (LV03/12 and LV04/12). LV01/12 and LV02/12 could not be tested a second time because the animals died in December 2011. Cytopathic effect characteristic for pestivirus (cytoplasmic vacuolization, cell rounding and detachment from the monolayer) effect could be observed in LV04/12 collected in March 2012. In the other five samples, no cytopathic effect was observed until the third passage. The immunoperoxidase monolayer assay that was performed in serum samples, had negative results. LV02/12 had antigen marking in the keratinocytes (Figure S1A) and the epithelium of the hair follicles (Figure S1B) and was positive by RT-PCR.

Sequencing and phylogenetic analysis

Comparison of sequences from the 5’NCR, N\textsuperscript{pro} and glycoprotein E2 gene regions of the four RT-PCR-positive samples showed that they are more related to ‘HoBi’-like virus than to the other pestivirus species, although the four sequences are unique because they had at least three nucleotides of difference in N\textsuperscript{pro} segment (Table S2). The name and accession number for each genetic region are summarized in Table S1.

The obtained phylogenetic trees presenting partial segments of 5’NCR (Fig. 1a), N\textsuperscript{pro} (Fig. 1b) and glycoprotein E2 (Fig. 1c) showed six well-separated clusters corresponding to the known BVDV-1, BVDV-2, CSFV, BDV, the giraffe pestivirus, Pronghorn antelope virus and ‘HoBi’-like pestivirus. Each monophyletic clade was supported by a bootstrap value of 98 to 100%, thus confirming the robustness of the tree topology. The four viral samples detected in the present study clustered with the ‘HoBi’-like strains in each of the phylogenetic trees with LV01/12 and LV03/12 positioned in a different terminal node compared with LV02/12 and LV04/12. Moreover, these two separate terminal nodes that are closer to the other ‘HoBi’-like strains than to one another. Figure 1a shows LV01/12 and LV03/12 grouping with the Italian isolates (Italy-1/10-1, Italy-280/11-A) and one virus detected in a FCS in Italy with Brazilian origin (IZPLV_To), while LV02/12 and LV04/12 grouped with a Brazilian FCS detected in Sweden (SVA/cont-08). Figure 1b shows LV01/12 and LV03/12 in a separated terminal node while LV02/12 and LV04/12 grouped with the Chinese isolate (JS12/01). Figure 1c presents LV01/12 and LV03/12 grouping with SVA/cont-08 and LV02/12 and LV04/12 in a separate terminal node.

Discussion

Bovine infections caused by pestiviruses are generally not apparent clinically but can present with respiratory, digestive and reproductive signs (Baker, 1995; MacLachlan and Dubovi, 2011). In the outbreak reported herein, the main
Clinical signs and lesions were observed in the respiratory and digestive systems, although skin lesions and corneal opacity were also observed. Abortions and other reproductive problems were not observed by the veterinarians during the period of this study, although the farmer reported previous abortions and returns to oestrus which may have been due to infection with HoBi-like viruses based on the time frame. Little information is available about the clinical and pathological features of natural infections of ‘HoBi’-like pestivirus. Respiratory syndrome has been reported in both natural infections in Italy (Decaro et al., 2011, 2012b) and experimental infections (Decaro et al., 2012c; Larska et al., 2012). Other reports of ‘HoBi’-like pestiviruses described abortions in natural infections (Cortez et al., 2006; Bianchi et al., 2011; Decaro et al., 2012a) and pyrexia without other clinical manifestation under controlled conditions (Ridpath et al., 2013). Altogether, ‘HoBi’-like pestivirus infections seem to be clinically indistinguishable from those caused by BVDV-1 and BVDV-2.

MD is triggered when a PI calf is superinfected with a cytopathic strain and briefly develops erosions and ulcers in the epithelium of oral and nasal cavities, oesophagus and foregut and in the small intestinal mucosa overlying Peyer’s patches (Brownlie, 1990; Baker, 1995; MacLachlan and Dubovi, 2011). A pestivirus with cytopathic biotype could be isolated from calf 04/12 and postmortem and histopathological findings similar from those observed with MD were detected. The other two calves necropsied showed similar lesions but the cytopathic virus was not observed. The isolation of a cytopathic ‘HoBi’-like virus and similarity of some of the observed lesions to mucosal disease suggests that a mucosal disease like syndrome may also occur in cattle infected with ‘HoBi’-like viruses.

The combination of IHC, viral isolation, RT-PCR and an ELISA-Ab were used to assay the presence of pestivirus and the status of infections (acute or persistent) of the animals in the herd. IHC of the ear biopsies showed that only one calf (LV02/12) was positive by that test. The RT-PCR test also showed that this animal was positive, whereas two other calves (LV01/12 and LV04/12) were found to be positive by RT-PCR but negative by IHC, a test that is known to detect persistent infections (Grooms and Keilen, 2002). Because PI cattle spread virus throughout their lives while transient infections are spread only during 5-7 days (MacLachlan and Dubovi, 2011), PI animals could be identified by performing RT-PCR with samples collected at a 15-day interval (Helal et al., 2012). Accordingly, LV03/12 and LV04/12 were tested again by RT-PCR and shown to be positive, suggesting that they were PI animals. Because LV01/12 was negative by IHC but was tested only once by RT-PCR and identified as positive, it was not possible to confirm that the animal was a PI or acutely infected animal. In the present study, more samples tested positive by RT-PCR than by IHC.

The herd studied had not been vaccinated for BVDV; therefore, the ELISA for antibody detection was used to examine exposure to circulating pestiviruses based on the presence of antibodies. The ELISA-Ab performed with samples from the first blood collection (December 2011) showed that 12 of the 123 animals in the herd were antibody-negative, including the four calves that were shown to be BVDV-positive by the first RT-PCR test. Tests of the serum from the second blood collection (March 2012) revealed that three of the animals had seroconverted, demonstrating the presence of an active pestivirus infection in the herd. The four other animals remained seronegative.
two of them (LV03/12 and LV04/12) were positive in the two RT-PCR assays, while the other two were RT-PCR-negative. BVDV seronegative animals in herds with active infection could be PI animals, could be animals that have not yet become infected or could be animals that are currently infected and have not yet seroconverted (MacLachlan and Dubovi, 2011). Previous reports showed that the ELISA kit used is able to detect not only anti-BVDV-1 and BVDV-2 antibodies, but also anti-'HoBi'-like pestivirus antibodies (Decaro et al., 2011; Bauermann et al., 2012; Larska et al., 2013), and can be used for the early detection of the seroconversion (Larska et al., 2013).

Initially, all of the 123 samples were assayed using the conventional RT-PCR protocol with the pan-pestivirus primers 324/326 (Vilcek et al., 1994), and no positive samples were identified. However, the positive results in the IHC test led to the use of additional RT-PCR primers (Deregt et al., 2006) that identified the four positive samples. Moreover, the failure of primers 324/326 for 'HoBi'-like pestivirus detection has been reported (Schirrmeier et al., 2004; Peletto et al., 2012) to be due to the sequence diversity in the primer-binding site. To prevent false-negative results, it is necessary to exercise care in the choice of the RT-PCR protocol for pestivirus detection. The RT-PCR used in the present study (Deregt et al., 2006) was able to detect the atypical pestiviruses and may be an option for use in diagnostic laboratories. Furthermore, the optimization of commonly diagnostic procedures for BVDV-1 and BVDV-2 detection to also detect 'HoBi'-like viruses is necessary due to the crescent number of reports worldwide (Cortez et al., 2006; Decaro et al., 2011; Mao et al., 2012; Xia et al., 2011, 2012).

The amplification products from the four RT-PCR-positive samples were submitted to DNA sequencing to characterize the pestivirus species. All of the four samples shared a higher degree of nucleotide sequence similarity with 'HoBi'-like virus than with other pestivirus species (Fig. 1). The relationships attributed to the different pestiviruses species appears to vary according to the genome region analysed. Moreover, the sequence divergence between 'HoBi'-like pestivirus and the recognized members of the genus Pestivirus as well as the antigenic differences reported previously (Schirrmeier et al., 2004; Stähl et al., 2007; Bauermann et al., 2012, 2013) reinforce the hypothesis that this atypical pestivirus is a new species of the genus.

The sequence analysis of the 5'NCR, Npro and glycoprotein E2 gene fragments of the four positive samples showed that they are four different 'HoBi'-like strains (Table S2). In the three phylogenetic trees generated (Fig. 1) the four 'HoBi'-like isolates clustered in two separate terminal nodes that are closer to the other 'HoBi'-like strains than to one another suggesting at least two independent viral introductions into that herd. Therefore, most likely, at least two 'HoBi'-like pestivirus-infected animals were introduced into the herd. The source of the viral samples could be the bull from south Brazil or the cattle bought from the same state in which the herd resides (Paraíba) that were all introduced in 2010. Another possibility is the sheep herd that was reared on the farm or indirectly through contaminated semen or fomites. These two independent introductions could lead to the four strains through mutations during replication in the susceptible cattle. The hypothesis that there were at least two independent introductions is supported by previous reports that showed that the descendent BVDV retained approximately 99% of its nucleotide identity with the original strain in all ORF extension after a single passage in PI cattle (Neill et al., 2011), as noted for the sequencing results in the fragments of the Npro and E2 genes of the present study (Table S2). Furthermore, another study revealed that sequence identity remained approximately 99% during at least 2 years in an endemic situation (Ridpath et al., 2006). It has been shown that genetic changes are established more rapidly during persistent infections and during acute infections of pregnant cows than during acute infections of non-pregnant cattle (Neill et al., 2011, 2012).

Our study described the genetic and pathologic characterization of the disease associated with natural infection with 'HoBi'-like pestivirus, in which respiratory and digestive signs predominated, and reports for the first time a syndrome resembling MD associated with isolations of cytopathic and non-cytopathic atypical pestivirus from one animal. Moreover, four different 'HoBi'-like strains from apparently two different genetic origins were identified, suggesting at least two independent introductions of the virus in this herd. This report reinforces the presence of 'HoBi'-like pestivirus in Brazilian herds and suggests that this virus can induce a syndrome with similarities to MD. Further these studies concur with previous studies that demonstrated genetic variability, between HoBi-like pestivirus and other pestiviruses can interfere with commonly used 'pan pestivirus' detection protocols. This may lead to false-negative diagnoses of pestiviruses in bovines using currently accepted protocols. These findings indicate that epidemiologic surveillance, using tests specific for HoBi-like virus, is necessary assess the extent to which these atypical pestivirus strains are present in cattle populations worldwide and their impact on animal health and production.

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Positive immunohistochemistry (IHC) from the ear biopsies of bovine LV02/12 using the monoclonal antibody 15c-5, stained with hematoxylin and eosin. Arrows indicate positive IHC on keratinocytes (A) and in the epithelium of hair follicles (B).

**Table S1.** Pestiviruses names and accession numbers of the sequences included in the phylogenetic tree.

**Table S2.** Nucleotide identity of different genomic regions of the ‘HoBi’-like strains identified in the present study.