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Diagnosis and phylogenetic analysis of bovine viral diarrhea virus in cattle (*Bos taurus*) and buffaloes (*Bubalus bubalis*) from the Amazon region and Southeast Brazil[1]

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ABSTRACT.- Assunção S.F.V., Antos A., Barbosa J.D., Reis J.K.P., Larska M. & Oliveira C.H.S. 2022. **Diagnosis and phylogenetic analysis of bovine viral diarrhea virus in cattle (*Bos taurus*) and buffaloes (*Bubalus bubalis*) from the Amazon region and Southeast Brazil.** *Pesquisa Veterinária Brasileira 42:e06955, 2022*. Escola de Veterinária e Zootecnia, Universidade Federal de Goiás, Goiânia, Brazil. E-mail: m.larska@piwet.pulawy.pl, cairo.oliveira@agro.gov.br

Bovine viral diarrhea virus (BVDV) is a highly infectious pathogen that affects bovines worldwide leading to great economic impact. Although Brazil has the largest commercial cattle population throughout the world and an increasing buffalo breeding industry, the country has no control or eradication program for BVDV. In this perspective, the aim of this study was to evaluate the occurrence of BVDV in cattle and buffaloes from two Brazilian states. Four different ELISA tests were performed and confirmed by virus neutralization testing (VNT). The presence of BVDV antibodies in the serum or plasma from 77 cattle from six herds (ELISA-1 and ELISA-4) and from 89 buffaloes from three herds (ELISA-1 through ELISA-4) was detected. Extraction of viral RNA was performed from the serum or plasma samples for the detection of BVDV by RT-PCR analysis. Amplified nucleotide sequences were used to construct a phylogenetic tree. In cattle, ELISA-1 detected 49.4% of seropositive animals, while ELISA-4 detected 37.7%. In buffaloes, ELISA-1 failed to detect any seropositive animals, while ELISA-2 and ELISA-3 detected 20.2% of seropositive animals, and ELISA-4 detected 21.3%. Eight of the nine herds tested had seropositive animals. The rate of PCR positive animals was 6.5% in cattle and 9% in buffaloes. Subtype 1d was found in cattle, and subtypes 1d and 1f were found in buffaloes. This is the first-time subtype 1f has been reported in Brazil. The absence of a control and eradication program seems to be favoring the spread of BVDV in the Brazilian herds. In addition, the improvement of diagnostic strategies for BVDV in buffaloes are required.

INDEX TERMS: BVDV, ELISA, buffalo, bovine, epidemiology, phylogenetic, *Bos taurus*, *Bubalus bubalis*, Brazil.

RESUMO.- [**Diagnóstico e análise filogenética do vírus da diarreia viral bovina em bovinos (*Bos taurus*) e búfalos (*Bubalus bubalis*) da região amazônica e Sudeste do Brasil.**] Diarreia viral bovina (BVDV) é um patógeno altamente infeccioso que afeta bovinos em todo o mundo elevando o impacto econômico. Apesar de o Brasil possuir a maior população bovina comercial em todo o mundo e uma indústria de criação bubalina em ascendência, o país não

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tem programa de controle e erradicação para BVDV. Nessa perspectiva, o objetivo deste estudo é avaliar a ocorrência do BVDV em bovinos e bubalinos de dois estados brasileiros. Quatro testes de ELISA foram realizados e confirmados por teste de vírus neutralização (VNT). A presença de anticorpos contra BVDV no soro ou plasma de 77 bovinos de seis rebanhos (ELISA-1 e ELISA-4) e de 89 búfalos de três rebanhos (ELISA-1 ao ELISA-4) foi detectada. Extração de RNA viral foi realizada em amostras de soro ou plasma para detecção de BVDV por análise de RT-PCR. Sequências de nucleotídeos amplificadas foram utilizadas para construção de uma árvore filogenética. Em bovinos, o ELISA-1 detectou 49.4% dos animais soropositivos, enquanto ELISA-4 detectou 37.7%. Em búfalos, o ELISA-1 falhou em detectar animais soropositivos, enquanto o ELISA-2 e o ELISA-3 detectaram 20.2% dos animais soropositivos e o ELISA-4 detectou 21.3%. Oito de nove rebanhos continham animais soropositivos. A frequência de animais PCR positivos foi de 6.5% em bovinos e 9% em búfalos. Os subtipos 1d foi encontrado em bovinos e os subtipos 1d e 1f foram encontrados em búfalos. Este é o primeiro relato da presença do subtipo 1f no Brasil. A ausência de um programa de controle e erradicação parece favorecer a disseminação de BVDV nos rebanhos brasileiros. Além disso, a melhora de estratégias de diagnóstico para BVDV em búfalos é necessária.

TERMOS DE INDEXAÇÃO: BVDV, ELISA, búfalo, bovino, epidemiologia, filogenética, *Bos taurus, Bubalus bubalis,* Brasil.

INTRODUCTION

Large numbers of highly variable pestiviruses are encompassed by the term bovine viral diarrhea virus (BVDV), which causes bovine viral diarrhea (BVD) and mucosal disease (MD) among bovines worldwide (Goens 2002), leading to great economic impact USD 0.50-687.80 per animal (Richter et al. 2017). Furthermore, BVDV is a common contaminant of fetal bovine serum (FBS) that is used to supplement culture media for cell culture so it can result in impairment of biological assays and contamination of several immunobiological products (Polak et al. 2008); e.g., BVDV proteins and anti-BVDV-specific antibodies were found in 25% and 45% of FBS batches in a study performed by Uryvaev et al. (2012).

Although there are only two recognized species of BVDV (1 and 2), the emerging HoBi-like pestivirus is being considered a third species because it shares 75% and 80% of genomic identity with BVDV 1 and 2, respectively, and since it produces similar symptomatology in infected animals (Dias et al. 2017, Mósena et al. 2017). The two BVDV species are divided into subtypes and into cytopathic (CP) or non-cytopathic (NCP) biotypes according to their ability to injure cells *in vitro* (Goens 2002, Uryvaev et al. 2012).

CP and NCP strains can cause transient infection (TI) of BVD, which is usually an asymptomatic or mild disease that presents with respiratory, gastroenteric and/or reproductive signs such as erosions and ulcerations of mucosal tissues, pneumonia, hemorrhagic enteritis, decreased fertility in bulls, infertility and abortion in dams and fetal malformations (Goens 2002, Brodersen 2014, Gebauer et al. 2014). When a NCP strain infects cows between 40 and 120 days of pregnancy, it leads to the development of persistently infected (PI) animals that are immunocompromised and immunotolerant to the infecting strain. Moreover, in PI animals, NCP strains can mutate into CP leading to MD, which is an aggressive and lethal illness that cause death of the calf in the first two years of its life (Goens 2002, Brodersen 2014, Gebauer et al. 2014).

Currently, there are two vaccines available against BVDV: one based on modified live virus (MLV) and another based on killed or inactivated virus (KV) (Lokhandwala et al. 2017). Although vaccination is recommended for prophylaxis in Brazil, the identification of positive animals by serological tests and their removal from the herds is also essential to control bovine pestiviruses (Walz et al. 2010). However, they are not usually performed in Brazil. Virus neutralization testing (VNT) is the standard serological test, but it is laborious and more expensive than enzyme-linked immunosorbent assay (ELISA); thus, ELISA and reverse transcription polymerase chain reaction (RT-PCR) are the main method for BVDV diagnosis (Deregt et al. 1998, OIE 2018).

Brazil has the world's largest commercial cattle (*Bos taurus*) population, an increasing population of water buffaloes (*Bubalus bubalis*) and it is one of the main exporters of FBS (Brunner et al. 2010, IBGE 2019). Some reports in Brazil detected 22.2% to 66.32% rates of BVDV infection in cattle and 8.8% to 97.9% rates of infection in buffaloes (Fernandes et al. 2016, Soares et al. 2017), and subtypes 1a, 1b, 1c, 1d, 1e, 1i, 2a, 2b and HoBi-like viruses are found in Brazilian livestock (Dias et al. 2017, Yeşilbağ et al. 2017). Pará and Minas Gerais states have large herds of cattle and Pará has the main herd of buffalo in Brazil, the livestock of these states are exported to other states as well as to other countries, important to BVD epidemiology. The subtype 1d has been reported in buffalo from Pará (Paixão et al. 2018) but little is known about the subtypes amongst cattle from Pará and Minas Gerais.

Therefore, the aim of this study was to evaluate the efficacy of different ELISA kits for BVDV in cattle and buffaloes, the occurrence of the disease in samples from Pará (PA) and Minas Gerais (MG) states, Brazil, the sort of infection present in these states and to establish the subtypes of BVDV circulating in the animals.

MATERIALS AND METHODS

Samples

A total of 166 serum and plasma samples were collected from 89 buffaloes and 77 cattle. The buffaloes belong to three different herds located in three different counties of the state of Pará in Brazil and they had no history of disease outbreak. The cattle belong to six different herds, one of which had a poxvirus outbreak in the past, four herds were located in four different counties of the state of Pará, and two herds were located in two different counties of the state of Minas Gerais in Brazil. All the animals were males and females apparently healthy, aged between one and four years old vaccinated against foot and mouth disease and brucellosis only.

Blood samples were processed to collect serum and plasma and were stored at -70°C until testing was performed.

Serological tests

ELISA-1. The indirect ELISA kit (BVDV Total Ab Test, IDEXX) was used for the detection of BVDV antibodies in bovines. Samples with S/P values less than 0.20 were classified as negative; those with S/P values greater than or equal to 0.30 were positive; and those with S/P values greater than or equal to 0.20 but less than 0.30 were considered suspect. This test had a specificity and sensitivity of 99.5% and 96%, respectively.

ELISA-2. Samples were tested to detect antibodies against the highly conserved non-structural p125/p80 protein of pestiviruses using a commercial blocking ELISA (BVDV p80 Ab, IDEXX). Samples with a percentage inhibition less than or equal to 40%, 40%-50%, and greater than or equal to 50% were considered positive, doubtful and negative, respectively. Compared with the virus neutralization test, the relative sensitivity and specificity of this ELISA test was 97.6% and 97.2%, respectively.

ELISA-3. In a blocking ELISA that detects antibodies anti the non-structural protein p80/125 of BVDV (SERELISA BVD p80 Ab Mono Blocking, SYNBIOTICS, France) in ruminants, samples with optical density (OD) values compared with the positive control OD value that were greater than or equal to 50% were considered positive, and those with OD values less than 30% were considered negative. The specificity and sensitivity of this test relative to the virus neutralization test were 100% and 94% respectively, according to the manufacturer.

ELISA-4. An ID Screen BVD p80 antibody competition ELISA (ID. vet, Montpellier, France) that detects specific antibodies against the non-structural p80-p125 protein of BVDV in samples from ovine, bovine, caprine and all sensitive species was used. Samples with a competition percentage less than or equal to 40% were considered positive; those with a competition percentage greater than 50% were considered negative; and those with a competition percentage in the range of 40% to 50% were considered doubtful. The specificity and sensitivity of this competitive ELISA were 100% and 100%, respectively, according to the manufacturer.

All serological ELISA tests were performed on serum and plasma samples according to the instructions provided by the manufacturer.

Virus neutralization test (VNT). To confirm the positive results from the BVDV antibody ELISA tests, serial dilutions (from 1:5 to 1:320) of seven randomly selected serum samples from buffalo and nine cattle sample with doubtful result in ELISA-1 and ELISA-4 were tested with VNT as described previously (Larska et al. 2013). The Singer strain of CP BVDV-1a was used to determine neutralizing antibody titers in BT (bovine turbinate) cells. This strain produces a cytopathic effect (CPE) that can be evaluated in infected cells with an optical microscope. The antibody titers were determined as the reciprocal of the highest serum dilution that neutralized the virus in at least 50% of the cells in the well. Positive and negative control sera were included in each test.

Virus antigen detection by ELISA. A commercial ELISA (BVDV Ag/Serum Plus, IDEXX) kit was also used. Testing was performed on serum samples according to the manufacturer's instructions. All the samples with S/P values greater than or equal to 0.3 were considered positive. This test had a specificity of more than 99.7% and a sensitivity of nearly 100%, according to the manufacturer.

Molecular tests

Viral RNA detection. To determine current infection, RT-PCR was performed to detect BVDV RNA. First, extraction of the viral RNA was performed with the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. The samples were tested in pools of an average of 18 samples. The sera from the RT-PCR positive pools were tested individually. A standard one-step RT-PCR was performed using a Transcriptor One-Step RT-PCR Kit (Roche Diagnostics GmbH) according to the manufacturer's instructions, and a set of the panpestivirus primers 324F (5'-ATG CCC WTA GTA GGA CTA GCA-3') and 326R (5'-TCA ACT CCA TGT GCC ATG TAC-3') (Vilcek et al. 1994) were used. The primers amplified a 288 bp fragment of the 5'-UTR region of the BVDV genome. The final volume of the RT-PCR mixture was 25µl comprising: 15.5µl of RNase free water, 5µl of reaction buffer, 1µl of each primer (10µM), 0.5µl of the enzyme mix and 2µl of the RNA template. The reaction conditions were set at 50°C for 30 min and 94°C for 7 min, followed by 10 cycles at 94°C for 10 s, 53°C for 30 s, 68°C for 30 s, followed by 25 cycles at 94°C for 10 s, 53°C for 30 s, 68°C for 33 s with a final extension step at 68°C for 7 min. The RT-PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide with a weight standard.

Atypical bovine pestivirus detection. To evaluate the presence of HoBi-like viruses in-house real-time RT-qPCR was performed using AgPath-ID Kit Reagents (Life Technologies, USA) according to the manufacturer's protocols in the StepOnePlus real-time PCR system (Life Technologies, Republic of Singapore). The primers T134-F (5'-GACTAGTGGTGGCAGTGAGC-3'), T220-R (5'-GAGGCATTCCTTGATGCGTC-3') and the probe T155r-P (FAM-5'-ACTCGGGGCTTCGGTGATCCAGGG-3'-BHQ1), which are specific for atypical pestiviruses, were used (Liu et al. 2008). The probe was produced and labelled with the fluorescent reporter FAM at the 5' end and the quencher BHQ-1 at the 3' end (Metabion International, Germany). The assay was run using the following thermal program: 48°C for 10 min (RT-step), 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 45 s. The reaction was performed for 42 cycles with a C_t (threshold cycle) value of 40 as a cut-off.

Statistical analysis

The results were submitted to descriptive statistical analysis.

Phylogenetic analysis

All RT-PCR products using panpestivirus primers were purified and submitted to DNA sequencing as described previously (Kuta et al. 2013).

Multiple sequence alignment was performed using the CLUSTAL W software (Thompson et al. 1994) and compared with sequences available on GenBank using BLAST software (NCBI). The phylogenetic analysis was performed using the neighbor-joining method in MEGA software (version 5.05). The reliability of the constructed phylogenetic tree was evaluated by running 1000 replicates in the bootstrap test. Bootstrap figures ≥70% are presented. The sequences of the reference sequences of the known BVDV viruses were retrieved from the NCBI GenBank.

RESULTS

The serological results are summarized in Table 1. The ELISA-1, ELISA-2, ELISA-3 and ELISA-4 were performed in buffalo sample, but only ELISA-1 and ELISA-4 were performed in cattle sample. No BVDV antibodies were found in any of the 89 buffalo sera samples with ELISA-1 while ELISA-2, ELISA-3 and ELISA-4 results were comparable with each other showing similar results, 20.2%, 20.2% and 21.3%, respectively. For cattle, ELISA-4 detected 29 seropositive animals against 38 seropositive animals detected in ELISA-1.

Virus neutralization tests (VNT) were performed on 22 serum samples: nine bovines and six buffaloes to clarify the doubtful results from the ELISA tests and seven serum samples to confirm the positive results from randomly selected buffalo samples that tested positive with ELISA-4. Neutralization studies on seven randomly selected ELISA-4 buffalo positive samples with BVDV-1 showed that all these samples had BVDV-specific antibodies (titers for BVDV ranged from 5 to 80). All the doubtful results from cattle sample in

ELISA-1 were negative on VNT. Out of five doubtful results in buffalo sample on ELISA-3, one was found positive and three negatives on VNT, while one serum sample was cytotoxic. Two doubtful results from cattle sample in ELISA-4 were found to be positive. The mean neutralizing titer against the Singer strain of BVDV in all the sera samples that tested positive on VNT was 19.1 (range 5-80, 95% CI: 4.0-34.1), while the mean titer of the buffalo sera samples was 21.1 (range 5-80, 95% CI: 2.2-40.0)

In Table 2 we have the results among the nine different herds of buffalo or cattle in each state; the number of seropositive animals by the number of tested ones; the percentage of seropositive animal withing the tested ones; the number of virus positive animal in standard PCR by the number of tested animals; the number of tested animals by the size of the herd; the percentage of seropositive animals withing each heard; and the BVDV genotype find in each heard. None of the tested serum samples from cattle nor buffalo were positive on antigen ELISA testing. Four of nine pooled serum samples were positive on standard PCR with panpestivirus primers (Kuta et al. 2013). After repooling, 61 serum samples were tested individually, and 13 were positive on PCR (eight originated from buffaloes and five from cattle). All the pooled and individual serum samples were negative on PCR for atypical bovine pestivirus.

It was possible to obtain nucleotide sequences of sufficient length and quality from only three samples from buffaloes (including one pooled sample from one herd) and one individual sample from cattle. It was impossible to propagate BVDV in cell culture to increase the virus titer, because all serum samples needed to be heat inactivated in 56°C for 60 min. The four Brazilian BVDV isolates were deposited in the GenBank database with the following accession numbers: MF977719 (Castanhalpool_buf); MF977720 (Ipixuna179_buf); MF977722 (Ipixuna181_buf); MF977721 (Castanhal108_bov); the first three were from buffaloes, and the last one was from cattle. All isolates derived from the state of Pará and were from three different farms (Table 2).

The nucleotide sequence analysis of the fragment revealed that the Ipixuna179_buf isolate belonged to BVDV-1f and shared the highest identity of 92% with the 25-KP/10 isolate from Poland, 91% with Vostok from Ukraine, 90% with 0-1897/00-175 from Slovenia and 89% with TR-2006-AK171449 from Turkey, whereas the isolates Castanhalpool_buf, Castanhal108_bov and Ipixuna181_buf were typed as BVDV-1d. Analysis of the 5'UTR sequences of the these three isolates showed that the sequences were identical with a difference of only one base pair. They had the highest homology with isolate 34-DE/11 from Poland, 98% with HLB_nb_02 from Denmark, 97% with 10JJ-SKR from Korea and 96% with UEL7-BR/11 from Brazil. In one buffalo herd, coinfection with two different BVDV (subtypes 1d and 1f) only 83.8% sequence similarity

Table 1. The serological results for buffalo (N=89) and bovine (N=77) samples using four different ELISA tests

Results	No. of positive (%)		No. of doubtful (%)		No. of negative (%)	
	Buffalo	Cattle	Buffalo	Cattle	Buffalo	Cattle
ELISA-1	0 (0%)	38 (49.4%)	0 (0%)	7a (9.0%)	89 (100%)	32 (41.6%)
ELISA-2	18 (20.2%)	nt	0 (0%)	nt	71 (79.8%)	nt
ELISA-3	18 (20.2%)	nt	5b (5.6%)	nt	66 (74.2%)	nt
ELISA-4	19* (21.3%)	29 (37.7%)	1c (1.1%)	2d (2.2%)	69 (77.5%)	46 (59.7%)

nt = not tested; * Seven samples were randomly selected and tested positive on virus neutralization test (VNT); [a] All negative on VNT, [b] one sample positive, three negative and one cytotoxic on VNT, [c] negative on VNT, [d] both positive on VNT with neutralizing titers of 10.

Table 2. BVDV-1 ocurrence by serology and virus detection by herd, state and species

			ELISA results	Standard PCR		
Herd no.	Species	State	No. positive/ No. tested (%)[a]	No. positive/No. tested[b] - % positive[c] (% seronegative[d])	BVDV infection occurrence rate[e]	BVDV genotype[f]
1	Buffalo	Pará	1/19 (5.3%)	4/19 - 21.5% (22.2%)	5/19 (26.3%)	1d (pool)
2	Buffalo	Pará	12/34 (35.5%)	nt - 0% (0%)	12/34 (35.5%)	-
3	Buffalo	Pará	6/36 (16.7%)	4/16 - 11.1% (13.3%)	10/36 (27.8%)	1d, 1f (179,181)
TOTAL buffaloes			19/89 (21.3%)	8/35 - 9.0% (11.4%)	27/89 (30.3%)	1d, 1f
4	Cattle	Pará	8/11 (72.7%)	nt - 0% (0%)	8/11 (72.2%)	-
5	Cattle	Minas Gerais	1/10 (10.0%)	2/9 - 20.0% (22.2%)	3/10 (30.0%)	-
6	Cattle	Pará	3/7 (42.9%)	nt - 0% (0%)	3/7 (42.9%)	-
7	Cattle	Pará	2/9 (22.2%)	2/4 - 22.2% (28.6%)	4/9 (44.4%)	1d (108)
8	Cattle	Pará	18/25 (72.0%)	1/5 - 4.0% (14.3%)	19/25 (76.0%)	-
9	Cattle	Minas Gerais	6/15 (40.0%)	0/8 - 0% (0%)	6/15 (40.0%)	-
TOTAL cattle			38/77 (49.4%)	5/26 - 6.5% (12.8%)	43/77 (55.8%)	1d
TOTAL			57/166 (34.3%)	13/61 - 7.8% (11.9%)	70/166 (42.2%)	1d,1f

[a] Percentage of seropositive buffaloes according to ELISA-4 and cattle according to ELISA-1 withing the heard, [b] ratio of number of animals positive on PCR to number of animals tested individually, [c] percentage of animals positive on PCR in proportion to all animals tested in a herd, [d] percentage of animals positive on PCR in proportion to all seronegative animals tested in a herd or species including negatives tested in pools, [e] number of BVDV-infected cases (seropositive + PCR positive) out of the total number of animals tested, [f] identification of the virus used for phylogenetic analysis given in the brackets; nt = not tested.

was identified. The phylogenetic tree including the Brazilian isolates with the strains selected from the GenBank database is shown in Figure 1. The identity percentage of Brazilian strains based on the 5'UTR region ranged between 83.8% and 100%. In the nucleotide sequencing and phylogenetic analyses of the Brazilian strains, the emergence of a new subtype of BVDV-1f that has not been previously described among Brazilian BVDVs should be concluded.

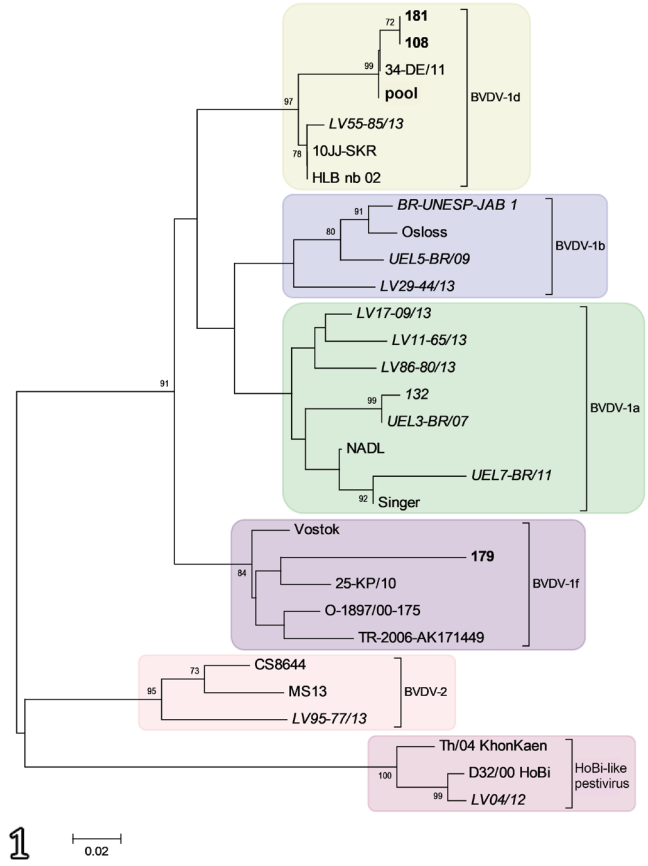

Fig.1. Phylogenetic analysis of the Brazilian isolates (in bold) and other geographical regions (in italics for Brazilian isolates) based on the partial 5'UTR region. The GenBank accession numbers of the reference strain sequences included in this analysis are: 34-DE/11 (JN715027); LV55-85/13 (KM007115); HLB_nb_02 (AY363072); 10JJ_SKR (KC757383); UEL7-BR/11 (KJ188147); NADL (AJ133738); Singer (L32875); UEL3- BR/07 (JQ513588); 132 (EF683540); LV11-65/13 (KM007106); LV86-80/13 (KM007118); LV17-09/13 (KM007108); Vostok (KJ131535); 25-KP/10 (JN715018); O-1897/00-175 (AY323895); TR-2006-AK171449 (EU716131); LV29-44/13 (KM007111); UEL5-BR/09 (JQ513585); Osloss (M96687); BR-UNESP-JAB 1 (FJ895327); LV95-77/13 (KM007130); CS8644 (Z79774); MS13 (GU395546); Th/04_KhonKaen (FJ040215); D32/00_HoBi (AB871953); and LV04/12 (KC465391). The sequences derived from this study included the following (accession numbers in brackets): Castanhalpool_buf (MF977719); Ipixuna179_buf (MF977720); Castanhal108_bov (MF977721); and Ipixuna181_buf (MF977722). The tree was constructed using the neighbor-joining algorithm with Kimura 2-parameters and the bootstrap test (n=1000) using MEGA version 5.05.

DISCUSSION AND CONCLUSION

All antibody ELISA tests used in the studies are dedicated to cattle samples, therefore the cattle samples were tested with only two ELISAs. ELISA-1 was chosen because it is the test which we use on daily basis, and the results for cattle samples were validated in an internal method verification process (data not shown), according to OIE recommendations. Second ELISA-4 we choose because the specificity and sensitivity of this competitive ELISA were the highest from all tests, 100% and 100%, respectively, according to the manufacturer.

ELISA-1 did not detect any seropositive buffalo but different from the other three ELISAs (ELISA-2, ELISA-3, ELISA-4), it is an indirect essay, which requires the recognition of the fragment crystallizable (Fc) region of the antibody by the conjugated antibody of the test; according to the manufacture, ELISA-1 kit was based on cattle, so possibly its conjugated antibody do not have reactivity to the Fc region of buffalo antibody. ELISA-2 and ELISA-3 had equal results in buffalo sample detecting 20.2% seropositive animals from all the buffalo tested, but ELISA-2 had no doubtful results and ELISA-3 had three doubtful results among which one was seropositive, so ELISA-3 was more reactive than ELISA-2. ELISA-4 detected 21.3% seropositive buffalo with one doubtful result that was confirmed negative. From all the four ELISAs performed in buffalo sample, we conclude that ELISA-4 was more reactive, followed by ELISA-3, while ELISA-1 is not efficient for diagnostic of BVDV seroprevalence in buffalo. The two ELISA performed in cattle had different results, ELISA-1 detected 49.4% positive animals with seven doubtful results that were all confirmed negative in VNT and ELISA-4 detected 37.7% positive animal with two doubtful results, both confirmed positive in VNT. We did not perform VNT in every sample to confirm ELISA-1 positive results were in fact all positive, but out of the two doubtful results in ELISA-4, the two were positive in VNT and in ELISA-1, suggesting that ELISA-1 was indeed more reactive than ELISA-4 for cattle sample. Ag ELISA did not detect any positive animals, it may be due to the fact that the herds were apparently healthy, none of the animals had symptomatology suggesting that it was an infection that happened a while ago or the infection is latent, when it is not spreading to the bloodstream, so it cannot be detected.

In this study, the seropositivity of 49.4% assessed in cattle matches the reported seroprevalence found in previous studies in Brazil (Fino et al. 2012). The seropositivity of 21.3% assessed in buffaloes is higher than the 14% reported in a previous study performed in Pará, Brazil (Silva et al. 2016), but it was the only epidemiologic one performed in Pará before ours. None of the animals that were virus positive at the PCR were seropositive; they could be at the beginning of a transient infection (TI) prior to seroconversion, they could be persistently infected (PI) animals, or have undetectable antibody titers due immunosuppression or illness, for example. The last hypothesis seems unlikely as they were apparently healthy animals at sampling.

Although HoBi-like pestivirus was first detected in a Brazilian batch of FBS and in buffaloes from Asia, South Europe and South America, none of the samples tested in this study were positive for this species (Schirrmeier et al. 2004, Bauermann et al. 2013, Pecora et al. 2017). BVDV-1d was isolated in cattle, and BVDV-1d and 1f were isolated in buffaloes. BVDV-1d has been reported with low occurrence

throughout the world, including in Brazil (Luzzago et al. 2014, Bianchi et al. 2017) with less than 10% occurrence rate in the country (Silveira et al. 2017, Flores et al. 2018), while BVDV-1f has been reported in restricted regions of Austria (Hornberg et al. 2009), Finland (Vilcek et al. 2004) and northern Italy (Luzzago et al. 2014). This is the first report of BVDV-1f in Brazil. As we import buffaloes and genetics from Italy, the emergence of subtype 1f may be associated with transboundary spread from infected animals and/or contaminated bioproducts.

The absence of an official control and eradication program for BVDV in Brazil (Weber et al. 2014) seems to be favoring the ocurrence and the spread of subtypes among cattle and buffaloes. While we are not assessing the health status of animals and the biosafety of semen, FBS batches, vaccines and other biological products being imported from and exported to other countries, BVDV RNA has been reported in FBS, cell cultures and human live viral vaccines (Studer et al. 2002, Giangaspero 2013, Silveira et al. 2015). Overall BVDV diagnostics developed for bovines should be performed carefully in buffaloes.

In conclusion, ELISA-1 was efficient in the detection of anti-BVDV antibodies in cattle sample but should not be performed to assess buffaloes' seropositivity to BVDV. ELISA-2, ELISA-3, ELISA-4 were able to detect anti-BVDV antibodies in buffaloes' sample. BVDV subtype 1d was found to be circulating in Pará and Minas Gerais, but as it was reported by other authors (Bianchi et al. 2017, Flores et al. 2018) with low occurrence, we were surprised to find it as the only infecting BVDV subtype amongst the nine herds in two different states. It is the first report of subtype 1f in Brazil. Although the herds assessed were overall healthy, BVDV infection was detected, which indicate that our surveillance program should be improved.

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