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SHORT COMMUNICATIONS

Molecular detection of bovine immunodeficiency virus in water buffaloes (*Bubalus bubalis*) from the Amazon region, Brazil

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Abstract Bovine immunodeficiency is a chronic progressive disease caused by a lentivirus that affects cattle and buffaloes. Although the infection has been described in cattle in some countries, including in Brazil, there are only two reports of infection in buffaloes: one in Pakistan and one in Cambodia. The aim of the present study was to survey the occurrence of bovine immunodeficiency virus (BIV) in water buffaloes from the Amazon region, Pará state, Brazil. BIV proviral DNA was surveyed in 607 whole blood samples of water buffaloes from 10 farms located in the state of Pará using semi-nested polymerase chain reaction (PCR) (PCR-SN) to amplify the *pol* region of the viral genome. Of the 607 samples tested, 27 (4.4 %) were positive for BIV proviral DNA. The amplified fragments were confirmed by sequence analysis after cloning and nucleotide sequencing. The sequence obtained had 99 % similarity to the reference strain (R-29). The present study provides important epidemiological data because BIV was detected for the first time in water buffaloes in Brazil. Further, the results suggest the possibility of the virus being a risk factor for herd health because it may be a potential causal agent of chronic disease and, also may be associated to other infectious diseases.

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Introduction

Buffalo rearing has been increasing globally. This species plays an important role in food production, especially in developing countries, which are mostly located in tropical regions (Borghese 2005).

Over the last 30 years, the Brazilian buffalo herd grew by 129.48 %. In 2013, according to official data from the Brazilian Institute of Geography and Statistics (Instituto Brasileiro de Geografia e Estatística- IBGE 2013), the national herd comprised approximately 1.1 million animals, distributed among the five Brazilian regions. The Amazon region has the largest number of water buffaloes, with 64 % of the total. Within the Amazon region, the state of Pará has the largest buffalo herd, with 461.275 animals, which represents 39 % of the national total.

Despite the importance of buffaloes for food production, several aspects of the diseases that affect these animals are still unknown. Because of their significant hardiness, it has been assumed that buffaloes are resistant to many diseases that affect bovines. However, it is known that buffaloes are susceptible to most of the infectious agents that affect bovines (Adlakha et al. 1992).

Bovine immunodeficiency virus (BIV) causes chronic progressive disease and induces signs of persistent leukocytosis, lymphadenopathy, weight loss, and damage to the central nervous system (Van Der Maaten et al. 1972; Gonda and Oberste 1992). Furthermore, there is evidence that BIV may cause immunosuppression, triggering secondary bacterial infections and reducing the immune response to vaccines (Brujeni et al.

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2010). BIV is also associated with weight loss and reduced milk production (Corredor and St-louis 2010).

Some serological and molecular studies on BIV infection in cattle have been conducted around the world, showing a non-uniform distribution of the infection, with incidence rates ranging from 1.4 to 80 % (Meas et al. 2002; Andrews et al. 2008).

However, there are only two reports of BIV infection in buffaloes, one in Pakistan and the other in Cambodia, with incidences of 10.3 and 16.7 %, respectively (Meas et al. 2000a, b).

Due to the few works about the worldwide distribution of BIV and the absence of studies on BIV occurrence in buffaloes from South America, this study aimed to survey the occurrence of bovine immunodeficiency virus in buffaloes from the Amazon region, Pará state, Brazil.

Materials and methods

Samples

The occurrence study was conducted in 607 buffaloes from Brazil. The samples were collected during technical visit in many rural properties on Pará state by jugular venipuncture in EDTA tube. Whole blood samples were obtained from Murrah, Mediterranean, Jafarabadi, and crossbred water buffaloes (*Bubalus bubalis*) of both sexes, over the age of four, that had been raised on extensive grazing.

Sample collection was performed at 10 farms, each located in a different municipality of the state of Pará, northern Brazil: A Abaetetuba, B Cachoeira do Arari, C Castanhal, D Ipixuna, E Nova Timboteua, F Ponta de Pedra, G Porto de Moz, H Salvaterra, I Soure, and J Vigia.

During the visits to the farms, epidemiological data were collected, such as the rearing system, management practices, and sanitation.

DNA extraction from leukocytes

Whole blood samples (4 mL) were centrifuged for 10 min at 4000x*g*; the buffy coat was collected. Subsequently, the total genomic DNA of the buffy coat leukocytes was extracted using the commercial kit QIAamp® DNA Blood Mini Kit (Qiagen®, Hilden, Germany) according to the manufacturer's instructions. The DNA concentrations were determined by the absorbance at 260 nm.

Polymerase chain reaction

To test the quality and accessibility of the DNA samples for polymerase chain reaction (PCR), a fragment of the normalizing gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified (Oliveira et al. 2013).

The BIV proviral DNA was searched in the DNA samples using the semi-nested PCR technique (PCR-SN), which amplifies a portion of the conserved region of the BIV *pol* gene. Primers BIV F (5'CCCTCCAGGAATTAAGGAATG3') and BIV R (5'TCACTTTCTCTTCCTGGACCTT3') were used in the outer reaction to amplify a 385-bp fragment. The primers BIV Rand BIV semi-nested F(5'AGCCACCCAGACATCATGTT 3') were used in the inner reaction to amplify a 154-bp fragment.

The reaction was composed of 1 U GoTaq® Flexi DNA Polymerase (Promega, USA), 1× of buffer 5× Green GoTaq® Flexi, 1.5 mM MgCl2, 0.2 mM dNTP mix (Invitrogen, USA), 20 uM of each primer, 300 ng of DNA for outer reaction or 1 uL from outer reaction as template for inner reaction, and ultrapure water 25 uL qsp. The amplification cycles consisted of initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 54 °C for 40 s and extension at 72 °C for 40 s, and final extension at 72 ° C for 5 min, for outer reaction. For the inner reaction, the annealing was at 58 °C.

The amplified products were observed under UV light after electrophoresis on a 1.5 % agarose gel stained with ethidium bromide.

Cloning and sequencing

The fragments amplified in the outer reaction (385 bp) were extracted from the gel and purified using the Kit Wizard® Plus Minipreps DNA Purification System (Promega, USA) following the manufacturer's instructions.

The products were cloned into pGEM®-T Easy Vector System II (Promega, USA) and were transformed and grown in DH5alpha bacteria. Plasmids were extracted from three clones using the Wizard® Plus SV Minipreps DNA Purification System (Promega, USA). The insert region (385 bp) in the plasmid was sequenced using the outer reaction primers on an ABI 3130 genetic analyzer using BigDye and POP7polymer (Applied Biosystems, USA). The sequences of the BIV *pol* fragment were analyzed in the software MEGA version 5.0 (Tamura et al. 2011). Next, the sequences were subjected to similarity searches in BLAST (NCBI, http://www.ncbi.nlm.nih.gov/BLAST) and aligned with American BIV strain R-29 available at GenBank database (Access number M32690.1).

Results and discussion

Of the 607 DNA samples obtained from buffalo peripheral blood and tested for BIV by PCR-SN, 27 (4.4 %) amplified the 154-bp target fragment (Fig. 1). This occurrence (4.4 %) was lower than the results observed in buffaloes by other authors in Pakistan (10.3 %) (Meas et al. 2000a) and

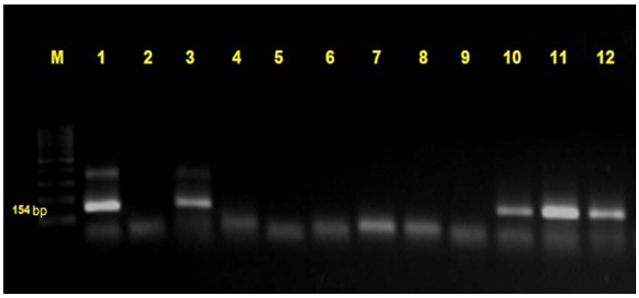

Fig. 1 Amplification of the BIV *pol* gene from DNA samples extracted from the buffy coat of water buffalo blood. The amplified fragments were visualized in a 1.5 % agarose gel stained with ethidium bromide. *M* molecular weight marker of 100 bp. *1* positive control. *2* negative control. *3*, *10*, *11*, and *12* positive samples. *4* and *9* negative samples

Cambodia (16.7 %) (Meas et al. 2000b) who used Western blotting as the serological diagnostic method.

Despite the existence of two BIV infection occurrence reported in cattle in Brazil, one in the South, with the occurrence of 11.7 % (Meas et al. 2002) and the other in the Southeast, with an incidence of 12.5 % (manuscript in preparation), this is the first report of the detection of BIV in buffaloes in the country. However, the present study is the first to report the detection of BIV in buffaloes from Brazil.

In most studies about BIV occurrence, antibodies against the virus were detected using serological tests. However, the development of direct detection tests that are properly standardized ensures greater accuracy of BIV detection (Tajbakhsh et al. 2010) since the BIV RNA or proviral DNA can be detected even in seronegative animals. Thus, the false negative animals from serological tests may be potential transmitters of the disease (Takiuchi et al. 2003).

When using PCR, the gene regions most commonly used for BIV detection are the genes *env* and *pol* (Meas et al. 1998, 2000a, b; Gradil et al. 1999; Orr et al. 2003; Momtaz et al. 2010). In our study, we decided to use the gene *pol* because it is highly conserved among lentiviruses, therefore making it suitable for diagnosis (Meas et al. 1998).

When comparing the BIV occurrence among the farms (Table 1), it was observed that farms E, I, and J had the highest number of BIV-positive animals, with an average of 12.3 %. This rate may be a result of the management practices adopted in those farms, where despite the fact that the animals are raised extensively, they are often taken to the pen for vaccination, deworming, pregnancy diagnosis, blood collection for brucellosis and tuberculin tests, and other procedures, causing the animals to crowd together. The contact between animals through the exchange of body fluids can favor the horizontal transmission of the virus. In addition, BIV transmission may occur via the iatrogenic route because needles and other fomites are commonly shared among animals.

These same observations have also been made by other authors (St Cyr Coats et al. 1994), who additionally argued that environmental factors such as the presence of hematophagous insects may transmit BIV mechanically. Snider et al. (1997) reported that the time during which the animals remain crowded is the primary means to increasing virus transmission among the herd.

Farms C and F did not have any BIV-positive animals, and farm G had the lowest occurrence of the disease (0.5 %). The absence of positive animals in farms C and F and the low occurrence observed in farm G can also be explained by the management practices adopted in these farms. Farm C is located in the municipality of Castanhal; however, its animals came from a farm located on Marajó Island, where the rearing systems is similar to the one adopted in farms F and G. In these farms, the animals are reared in an ultra-extensive system in which the buffaloes are reared freely with no fences and are rarely taken to the pen for sanitary procedures, which

Table 1 Occurrence of bovine immunodeficiency virus in water buffaloes from Brazil, Pará, state detected by semi-nested PCR (PCR-SN)

Farm/municipality	Management system	Number of samples	Number of BIV-positive animals	Percentage
A Abaetetuba	Extensive	28	1	3.6
B Cachoeira do Arari	Extensive	29	1	3.4
C Castanhal	Ultra-extensive	28	0	0.0
D Ipixuna	Extensive	95	4	4.2
E Nova Timboteua	Extensive[a]	35	5	14.3
F Ponta de Pedra	Ultra-extensive	40	0	0.0
G Porto de Moz	Ultra-extensive	183	1	0.5
H Salvaterra	Extensive	95	5	5.3
I Soure	Extensive[a]	61	9	14.8
J Vigia	Extensive[a]	13	1	7.7
Total	607	27	4.4	

[a] Animals are often taken to the pen for vaccination, deworming, pregnancy diagnosis, blood collection for brucellosis and tuberculin tests, and other procedures, causing the animals to crowd together

reduces the risk of horizontal transmission of the virus through iatrogenic route.

Although differences were observed in the molecular detection of BIV between buffalo herds in Brazil, unfortunately we could not associate this virus to illness in these animals. Other studies for this purpose comparing positive and negative animals and herds should be carried out considering the possibility of persistent leukocytosis, lymphadenopathy, weight loss, and damage to the central nervous system as observed in cattle. Additionally, cattle and buffalo are commonly raised together in farms from Brazil, and the epidemiological importance of buffalo for BIV infection in bovine could be analyzed.

The nucleotide sequencing confirmed the BIV detection in buffaloes by semi-nested PCR. A high similarity (99 %) was observed between the nucleotide sequences obtained in the present study and the sequence of the American strain R-29 previously deposited in GenBank (Access number M32690.1). Meas et al. (2002) reported that the BIV *pol* region amplified from cattle samples from Brazil also showed high similarity to the R-29 strain.

In conclusion, BIV occurs in water buffaloes from Brazil. Our study provides epidemiological data on the occurrence of BIV in the Amazon region, state of Pará, and draws attention to the fact that this virus may be a risk factor for buffalo health, possibly acting as a causal agent of chronic disease or being associated to the other infectious diseases. Thus, a more detailed study on the prevalence, epidemiology, and pathogenesis of BIV in buffaloes is needed.

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Conflicts of interest The authors declare no conflicts of interest.

Statement of animal rights All procedures and animal handling followed the ethical principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation of UFMG/CEUA, under Protocol n° 133/2012.

Informed consent Informed consent was obtained from all individual participants included in the study.

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