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## Seroprevalence and molecular evidence for the presence of bovine immunodeficiency virus in Brazilian cattle

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### Abstract

Data on the worldwide distribution of bovine immunodeficiency virus (BIV) and bovine leukemia virus (BLV) is limited. A prevalence study of antibodies to BIV and BLV was conducted in six different cattle herds in Brazil. Out of a total of 238 sera analyzed, 11.7% were found positive for anti-BIV p26 antibodies as determined by Western blot analysis, 2.1% were positive for anti-BLV gp51 antibodies as detected by immunodiffusion test. Peripheral blood mononuclear cells from BIV seropositive cattle were found to have BIV-provirus DNA, as detected by nested polymerase chain reaction. A nucleotide sequence corresponding to a 298 bp fragment of the BIV *pol* gene was also analyzed. Amino acid sequences of these Brazilian *pol* gene products showed 98.0 to 100% homology to the American strain BIV R29, 97.0 to 99.0% to Japanese BIV isolates, and divergence ranged from 0 to 4.0% among Brazilian BIV isolates. This evidence of the presence of BIV and BLV infections in Brazil should be considered a health risk to Brazilian cattle populations and a potential causative agent of chronic disease in cattle.

Key words : bovine immunodeficiency virus ; bovine leukemia virus ; Brazil ; seroprevalence

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## Introduction

Bovine immunodeficiency virus (BIV) is a member of the family *Retroviridae*, genus *Lentivirus*. BIV R29 was originally isolated from an 8-year-old dairy cow in Louisiana in the United States of America (USA). The cow demonstrated persistent lymphocytosis, progressive weakness, and emaciation. At necropsy, lymph node hyperplasia and cerebral lymphocytic perivascular cuffing were noted<sup>28</sup>. Studies have shown that BIV resembles human immunodeficiency virus (HIV) type 1 and simian immunodeficiency virus in its structural, genetic, antigenic, and biological properties<sup>8,9</sup>. In the years between the original description of BIV and the recognition of its relationship to HIV, there was little research related to BIV. Therefore, much remains to be learned regarding the epidemiology, molecular biology, pathogenesis, host range and emerging potential of the virus.

Although several pathological changes have been reported in BIV infected cattle, including monocyte dysfunction<sup>21</sup>, encephalopathy, lymphadenopathy, and immunodeficiency<sup>8,28</sup>, the detailed pathogenesis of BIV in infected cattle still remains unclear. However, BIV has been detected in dairy and beef cattle in the USA<sup>1,7</sup>, New Zealand<sup>13</sup>, Switzerland<sup>12</sup>, Australia<sup>7</sup>, the United Kingdom<sup>6</sup>, Canada<sup>14</sup>, Germany<sup>20</sup>, France<sup>24</sup>, Japan<sup>11,15</sup>, Costa Rica<sup>10</sup>, Italy<sup>4</sup>, Korea<sup>5</sup>, in buffaloes in Pakistan<sup>18</sup>, in draught animals in Cambodia<sup>16</sup> and Indonesia<sup>2</sup>. All these studies reveal rates of BIV detection ranging from 1.4 to 80% depending on the method of detection and source of samples. Nonetheless, there is little knowledge regarding BIV and bovine leukemia virus (BLV) infection in cattle in Brazil. BIV seropositivity has been associated with milk production in dairy cattle<sup>14</sup>, but has not been directly linked with clinical disease

in naturally infected cattle. In many cases, such a demonstration is complicated by the presence of confounding factors including coinfection with BLV, an oncogenic retrovirus that can cause lymphoid tumors and persistent lymphocytosis in its host with most infected cattle remaining clinically and hematologically normal<sup>3</sup>. BLV is closely related to the human T cell leukemia virus type I and II<sup>25</sup>. Many molecular aspects of BIV have been examined<sup>8,9</sup>, but relatively little is known about the *in vivo* pathogenicity.

The purpose of this study was to gather initial data on the seroprevalence of BIV and BLV in Brazilian cattle; in addition the nucleotide sequence of the *pol* gene of BIV proviral DNA from BIV seropositive cattle was also analyzed.

## Materials and Methods

### *Blood, Plasma and DNA samples*

Blood samples were collected with EDTA or heparin from a total of 208 cattle in five different herds (beef cattle in farms I to IV and farm V was dairy cattle, Table 1) in the Pelotas area and from 30 cattle in a beef herd from the Campo Grande area in Brazil. All animals

Table 1. Seroprevalences of BIV and BLV in six cattle herds in Brazil

| Farm No. <sup>a)</sup> | No. of tested cattle | No. of seropositive cattle (%) <sup>b)</sup> |        |
|------------------------|----------------------|--|--------|
|                        |                      | BIV  | BLV    |
| I                      | 42                   | 0(0.0)                                       | 0(0.0) |
| II                     | 40                   | 5(12.5)                                      | 0(0.0) |
| III                    | 42                   | 2(4.7)                                       | 1(2.4) |
| IV                     | 42                   | 9(21.4)                                      | 0(0.0) |
| V                      | 42                   | 4(9.5)                                       | 4(9.5) |
| VI                     | 30                   | 8(26.6)                                      | 0(0.0) |
| Total                  | 238                  | 28(11.7)                                     | 5(2.1) |

a) Farms I to IV (beef cattle) and farm V (dairy cattle), were located in the Pelotas area and farm VI (beef cattle) was in the Campo Grande area in Brazil.

b) Seroprevalences of BIV and BLV were tested by WBA and IDT, respectively.

were clinically normal and older than one year at the start of this study. Plasma, peripheral blood mononuclear cells (PBMCs) and red blood cells were separated by centrifugation. All plasma samples were mixed with  $\beta$ -propiolacton (final conc., 0.4%) to inactivate live pathogens. DNA was extracted from PBMCs by *SepaGene* kit according to the manufacturer's instruction (Sanko Junyaku Co., Ltd, Tokyo, Japan) and was then transported to our laboratory. All plasma and DNA samples were stored at -20 and -80°C, respectively, until further use.

#### *Western blot analysis (WBA)*

In order to detect antibodies against BIV protein, WBA was performed using culture supernatant fluid of bovine embryonic spleen cells infected with BIV R29 as an antigen. This BIV p26 *gag* antigen was prepared as described previously<sup>11, 15, 18, 19</sup>.

#### *Immunodiffusion test (IDT)*

For the detection of anti-BLV antibodies, the IDT using the glycoprotein antigen prepared from culture supernatant fluid of fetal lamb kidney cells infected with BLV. This BLV gp51 antigen was prepared as described by Onuma et al.<sup>23</sup>

#### *Polymerase chain reaction (PCR)*

To detect the BIV proviral DNA, nested PCR was performed to detect a portion of the *pol* gene. A BIV-specific band with the predicted size of 298 bp was detected in each of the DNA samples tested with primer sets as described earlier<sup>15</sup>. Each PCR was done in a buffer containing 45 mM Tris-HCl (pH 8.8), 11 mM ammonium sulfate, 4.5 mM MgCl<sub>2</sub>, 6.7 mM 2-mercaptoethanol, 4.4 mM EDTA (pH 8.0), 113 µg/ml BSA, 1 mM dNTPs, 20 pM each of the oligonucleotide primers and 2.5 U of *Taq* DNA polymerase (TaKaRa Shuzo

Co., Ltd., Kyoto, Japan). Cycling conditions for the first step as well as second step PCR were 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 57°C for 45 sec and 72°C for 30 sec, with a final extension step at 72°C for 7 min. The PCR products were separated in a 1.5% agarose gel with ethidium bromide and electrophoresed at 100V for 35 min. The reaction was determined to be positive or negative based on the product of the appropriate size being visible on UV exposure.

#### *DNA cloning and sequencing*

PCR products of *pol* gene of five Brazilian, and two Japanese BIV<sup>19</sup> field isolates were excised from the gel and purified with the GeneClean II kit (Bio101, La Jolla, USA), and cloned into pGEM-T easy vector (Promega, Madison, USA). The pGEM-T plasmids containing the inserts corresponding to these *pol* regions of BIV field isolates were purified by standard mini-prep method, and sequenced using the BigDye terminator sequencing kit (Applied Biosystems, Foster City, USA). At least 4 individual plasmid clones per each BIV isolate were used for sequencing. DNA sequence analysis was done using the GENETYXMAC 10.1.2 package (Software Development Co., Ltd, Tokyo, Japan) in combination with the BLAST program at the GenBank for homology search and comparison with known BIV gene sequences.

## **Results**

### *Seroprevalence of BIV and BLV in Brazilian cattle*

Antibodies against BIV p26 protein were detected in Brazilian plasma samples by WBA with purified BIV *gag* antigen and the same samples were also tested for anti-BLV antibodies by IDT. Plasma samples were considered BIV seropositive if they showed specific band in WBA. As summarized in Table 1, cat-

Table 2. Comparison of nucleotide and amino acid sequences of *pol* gene of Brazilian and Japanese BIV field isolates<sup>a)</sup>

|           | BIV R29 | JpnD164 | JpnOff164 | Brazil-P 2 | Brazil-P 3 | Brazil-P 4 | Brazil-P 5 | Brazil-C 6 |
|-----------|---------|---------|-----------|------------|------------|------------|------------|------------|
| BIV R29   |         | 98.7    | 99.7      | 99.3       | 99.3       | 99.0       | 99.7       | 99.7       |
| JpnD164   | 99.0    |         | 99.0      | 98.7       | 98.0       | 98.3       | 99.0       | 99.0       |
| JpnOff164 | 100.0   | 99.0    |           | 99.7       | 98.0       | 99.3       | 100.0      | 100.0      |
| Brazil-P2 | 100.0   | 99.0    | 100.0     |            | 99.3       | 99.0       | 99.7       | 99.7       |
| Brazil-P3 | 98.0    | 97.0    | 98.0      | 98.0       |            | 98.0       | 99.0       | 99.0       |
| Brazil-P4 | 98.0    | 97.0    | 98.0      | 98.0       | 96.0       |            | 99.3       | 99.3       |
| Brazil-P5 | 100.0   | 99.0    | 100.0     | 100.0      | 98.0       | 97.0       |            | 100.0      |
| Brazil-C6 | 100.0   | 99.0    | 100.0     | 100.0      | 98.0       | 98.0       | 100.0      |            |

a) Percentage nucleotide (upper half) and amino acid (lower half) sequences similarities of the *pol* gene (nt 2181-2479) of the predicted size of 298bp-99aa amongst Brazilian (P 2 -P 5 were obtained from farm II-V in the Pelotas area, C 6 was obtained from a single herd in the Campo Grande), Japanese and American R29 BIV isolates. Nucleotide sequence of BIV strain R29 (molecular clone R29-127) was obtained from the GenBank. Japanese BIV isolates from cow No. 164 (JpnD164) and her offspring (JpnOff164) at 1 day post-parturition before colostrum feeding, were obtained from previous work<sup>19)</sup>. Because this *pol* gene sequence was highly conserved, the nucleotide sequences of difference of BIV field isolates reported here were not submitted to the database.

tle from farm No. I was shown to be free of both BIV and BLV antibodies. For the other four farms (II to V) in the Pelotas area, the prevalence of BIV antibodies ranged from 4.7 to 21.4%. In the case of BLV, three farms of them were antibody free, while the other two farm's prevalence ranged from 2.4 to 9.5% in the Pelotas area; the farm in the Campo Grande area had 26.6% cattle positive for BIV and no BLV-infected cattle. Overall, 11.7 and 2.1%, of tested cattle were BIV- and BLV- seropositive, respectively, and no co-infection for both BIV and BLV was determined in this study, suggesting that the rate of dual infection is very low in cattle in Brazil as in agreement with our previous observations in cattle and buffaloes in Pakistan<sup>18)</sup> and draught animals in Cambodia<sup>16)</sup>.

#### *Molecular characterization of Brazilian BIV isolates within pol genes*

In order to further confirm BIV seropositivity in Brazilian cattle, PBMCs from BIV seropositive animals were found to have BIV-

provirus DNA using nested PCR to detect a 298 bp fragment of the *pol* gene (nt2181-2479). The nucleotide sequences were also analyzed. By using the BLAST program, all nucleotide sequences were shown to be highly homologous to the respective sequences of a molecular clone, BIV R29 and other known BIV sequences in the database. As shown in Table 2, nucleotide sequence of Brazilian BIV isolates (P2, P3, P4, P5 and C6) showed 98.0 to 100% homology with the American BIV strain R29, 98.0 to 100.0% with Japanese (JpnD164 and JpnOff164) BIV isolates and 99.0 to 100% similarities to each other. Deduced amino acid sequences were 97.0 to 100.0% homologous to Japanese (JpnD164 and JpnOff164) BIV isolates and there was 96.0 to 100.0% similarities to among Brazilian BIV isolates (Table 2). There is high homology between the nucleotide sequences of the *pol* gene of BIV isolated from a Japanese cow (JpnD164) and her offspring (JpnOff164) on the day after parturition (99.0% homology) (Table 2). Whether size variation

occurs within the surface envelop (*env*) gene of BIV *in utero* infected cattle is under investigation.

### Discussion

Two methods, WBA and nested PCR were used to confirm BIV infection in cattle. Data of this study demonstrated that BIV was more common than BLV in Brazilian cattle populations and the prevalence rate of BIV was not so high as compared to other countries in our previous studies<sup>16,18</sup>. These results also support the conclusion that infection with BIV and BLV can occur independently of one another (Table 1) which is in agreement with other studies<sup>5,16,18</sup>. Indeed, BIV seropositivity had no correlation with BLV infection in this study. However, BIV infection in cattle has been associated with secondary bacterial infections, stresses of parturition and early lactation and/or with unusual environmental stress in a Louisiana dairy cattle herd<sup>26</sup>. BIV infection was associated with a wide-range of clinical spectrum that including weight loss, nerve degeneration, mouth ulcers and respiratory infections as the first case of bovine AIDS in the United Kingdom<sup>6</sup>. However, the R29 isolate of BIV originated from cattle co-infected with bovine viral diarrhea virus<sup>28</sup> and these observations were not confirmed in Brazilian cattle. Limited studies of the pathogenesis of BIV in infected cattle have been reported. Macrophages are important target cells for the most lentiviruses. Onuma et al.<sup>21</sup> reported that some macrophage functions including superoxide anion release, phagocytic activity, and chemotactic responsiveness were depressed, and a slight delay in antibody responses against mouse serum proteins were observed in calves inoculated with BIV R29.

Additionally, new Florida BIV isolates (F L112) demonstrated no remarkable depletion of CD4<sup>+</sup> cells, unlike classical immunodeficiency

viruses such as HIV and feline immunodeficiency virus. However, B-cell proliferation was observed in the calves inoculated with BIV FL112 in short-term studies<sup>29</sup> and further investigation of effects of BIV infection on acquired immune responses in cattle is warranted.

Lentiviruses in general are transmitted both horizontally and vertically. Although the route and detailed mechanism of BIV natural transmission in cattle is largely unknown, our previous work has found that BIV can be transmitted to offspring *in utero* or transplacentally (Table 2) and BLV can be transmitted through colostrum or milk if dams are co-infected with both BIV and BLV under natural conditions<sup>19,22</sup>. The reason for the reported difference in seroprevalence of BIV in two Mississippi herds is not readily apparent, but considering the age related distribution of BIV in these animals and the separate geographic location of the two production herds, it appears that BIV transmission is predominantly horizontal, rather than vertical, and may be environmentally influenced, perhaps by blood sucking insects<sup>27</sup>. Pelotas is located in the southern region of Brazil with a landscape of rice plantations and cattle raising. Neither vertical nor horizontal transmission of BIV/BLV occurred in farm I in the Pelotas area, and BIV and BLV infection may have occurred either vertical or horizontal transmission in farms II to V in the Pelotas or in a beef cattle herd (farm VI) in the Campo Grande (Table 1). The nucleotide sequences of the 298 bp DNA fragment corresponding to a part of the *pol* gene of Brazilian BIV isolates are very similar to strain R29 and Japanese BIV isolates (Table 2). It has been shown that several distinct strains of BIV may exist worldwide<sup>13,17,24</sup>. More recently, Meas et al.<sup>17</sup> reported that the nucleotide sequences of the surface envelope genes (*env*) of all Japanese,

Pakistan and Cambodian BIV field isolates were shorter, and several base substitutions were observed in the V1 region, and deletions were also found in the V2 region when compared to the American BIV isolates (R29, Florida112, and Oklahoma40). Since these genotypes of Asian BIV isolates were different from those of the American BIV isolates, the nucleotide sequence analysis of the V2 region in the *env* gene of Brazilian BIV isolates are in progress and will be compared with those other BIV field isolates. A larger serological study with detailed long term epidemiological observation will be necessary to confirm these preliminary findings and the role of BIV in diseases progression in cattle has to be elucidated.

In summary, this epidemiological survey provides additional evidence that BIV and BLV infections are widespread in some cattle herds in Brazil and primary BIV infection in cattle may cause problems in animal health as reported in other countries around the world.

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