Seroprevalence and Field Isolation of Bovine Immunodeficiency Virus

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ABSTRACT. A seroprevalence study of bovine lentivirus, known as bovine immunodeficiency virus (BIV), was conducted in 12 different dairy herds in Hokkaido, where some herds were a high prevalence of bovine leukemia virus (BLV) infection. Amongst 611 cattle, 28.6% of cattle were BLV-seropositive, and 11.7% of cattle were seropositive for BIV, while 4.2% of cattle were seropositive for both BIV and BLV. For the isolation of BIV, 19 samples of peripheral blood mononuclear cells (PBMC) and one sample of milk-derived leukocytes were prepared from BIV-seropositive cows. These PBMC and leukocyte preparations were then co-cultivated with cc81 cells, a cat cell line transformed by mouse sarcoma virus. BIV was isolated from 17 PBMC and one milk-derived leukocyte samples. The isolated viruses showed slow replication and syncytia formation. Major core antigen, p26 from these isolates were reacted with anti-BIV serum. In addition, proviral DNA was detected in blood and milk samples by nested polymerase chain reaction (PCR) amplification of the gag gene products showed its 99.0 to 99.7% homology to that of BIV R-29. These results indicate that the Japanese BIV isolates appear to be antigenically and genetically similar to the American isolate R-29. Since BIV was isolated from milk samples, BIV could possibly be transmitted through milk. This is the first report of BIV isolation in Japan. — key words: bovine immunodeficiency virus, isolation, seroprevalence.

Bovine immunodeficiency virus (BIV) known as bovine lentivirus, R-29, was originally isolated in the United States, from a cow with persistent lymphocytosis, lymphadenopathy, central nervous system lesions, progressive weakness, emaciation and suspected of having bovine lymphosarcoma [28]. Ultrastructural studies found that this virus is morphologically similar to visna virus [2]. Further characterization of BIV was not undertaken until the interest in AIDS revitalized the study of all lentiviruses and their relationships. This virus was subsequently identified as a lentivirus having molecular, antigenic, genetic and structural similarities to HIV-1 [10, 11] and this finding raised questions about the relevance of BIV for veterinary and human medicine. Since the first report of BIV in 1972, prevalence of BIV infection has been reported in the United States [1, 7, 24], New Zealand [13], Switzerland [14], Australia [9], the United Kingdom [6], Canada [15], Germany [16], France [22] and Japan [12].

One report suggested that BIV infection may associate with decreased milk production in dairy cattle [15]. Calves experimentally inoculated with BIV developed a mild lymphocytosis and a moderate lymphoproliferation in small subcutaneous lymphatic nodules [28]. Although BIV infections induce dysfunction of monocytes [19] and neutrophils [8], BIV-inoculated calves did not exhibit severe clinical symptoms [5, 8, 19], and pathogenesis of BIV in cattle remain unclear. Unlike classical immunodeficiency virus such as HIV and feline immunodeficiency virus (FIV), there was no remarkable depletion of CD4+ cells although B-cell proliferation was observed in the calves inoculated with BIV [31]. The inoculated calves also did not develop severe clinical symptoms, similar to the calves inoculated with the R-29 strain [31].

Because of limited epidemiological data, difficulty in culturing new isolates in vitro and questionable diagnostic tests, whether BIV is associated with diseases in cattle remains unclear. The present study was designed to obtain additional information on the seroprevalence of BIV infection, isolation, identification and characterization of the Japanese BIV field isolates.

MATERIALS AND METHODS

Blood, serum and milk samples: Aliquots of 342 serum samples (primarily collected for brucellosis testing in Sorachi area) from 9 dairy herds and 193 serum samples (primarily collected for BLV testing in Tokachi area) from 2 dairy herds were collected in Hokkaido area. Seventy three blood samples were collected from another dairy herd and serum was separated. For BIV isolation, 19 blood samples were collected from BIV seropositive cows from 3 different dairy herds. One milk sample was collected and fat was removed by centrifugation.

Virus isolation: Peripheral blood mononuclear cells (PBMCs) were separated from blood samples by the Ficoll-Conray gradient centrifugation method. Leukocytes from
milk were separated by mixing milk with an equal volume of 0.15 M EDTA (pH 7.5), and subsequent centrifugation.

Purified leukocytes or PBMCs from BIV seropositive cows were then co-cultured with cc81 cells (cat cell line transformed by mouse sarcoma virus) in RPMI 1640 medium supplemented with 10% fetal calf serum, 200 U/ml penicillin and 200 µg/ml streptomycin. Cell cultures were incubated for 10 to 12 days at 37°C in 5% CO2 and passed up to 20 times. Supernatant of these cultures were collected at different passages and virus growth was monitored by syncytium assay, immunofluorescence assay (IFA) and polymerase chain reaction (PCR).

Western blotting assay: To detect antibodies against BIV protein, Western blotting was carried out by using culture supernatant fluid of bovine embryonic spleen cells infected with BIV-R29 (BESP-R29) as an antigen as described previously [12, 30]. Viral antigen was prepared by the same procedure as above, but culture supernatant from cc81 cells infected with BIV field isolates, were concentrated by ultracentrifugation. The virion suspension was mixed with 2 x SDS sample buffer, boiled briefly, and subjected to electrophoresis on 12% polyacrylamide slab gels. After electrophoresis, the size-fractionated proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, U.S.A.). The filters were incubated with 3% skim milk in phosphate buffered saline (PBS, pH 7.2) containing 0.05% Tween 20 (PBST) to block nonspecific reaction and were then incubated with fluorescein-isothiocyanate (FITC)-labeled rabbit anti-bovine IgG for 1 hr at room temperature, and then washed again with PBS. The filters were incubated with 3% skim milk in phosphate buffered saline (PBS, pH 7.2) containing 0.05% Tween 20 (PBST) to block nonspecific reaction and were then incubated with: 1: 50 dilution of anti-BIV serum 1141 (anti-BIV R-29) [30] with PBST for 1 hr at room temperature. Then, the membranes were washed, and incubated with peroxidase-conjugated anti-bovine IgG diluted with PBST for 1 hr at room temperature. After extensive washing, protein bands were visualized by the addition of substrate solution containing cobalt chloride hexahydrate, 3,3’ Diaminobenzidin and 0.2% H2O2.

Immunodiffusion test (IDT): For the detection of anti-BLV antibody, the immunodiffusion test using the glycoprotein antigen was performed as described previously [21].

Syncytium assay: Milk-derived leukocyte or PBMCs (2 x 10^6) from BIV-seropositive cows were co-cultivated with 1 x 10^6 of cc81 cells as an indicator [20]. After 5–6 days of incubation, the cells were stained by 4% Giemsa solution, and syncytia were counted under a light microscope.

Immunofluorescence assay (IFA): For the detection of virus antigens, co-cultured cells, as described above, were seeded onto chamber glass tissue culture slides, grown for 5–6 days at 37°C, washed with PBST and fixed with 70% cold acetone/30% methanol for 20 min at room temperature. These fixed cells on the slides were incubated for 1 hr with the 100 x dilution of anti-BIV R-29, 1141 serum [30] or 50 x dilution of anti-BIV serum from a cow naturally infected with BIV but BLV-seronegative. After several washing with PBS, these fixed cells were incubated with fluorescein-isothiocyanate (FITC)-labeled rabbit anti-bovine IgG for 1 hr at room temperature, and then washed again with PBS and examined for positive cells under fluorescence microscope.

Polymerase Chain Reaction (PCR): DNA samples were prepared from PBMCs, milk-derived leukocytes, and BIV-infected cc81 cells. These cells were lysed with a lysis buffer (0.1% SDS, 0.1 mg/ml proteinase K, 150 mM NaCl, 10 mM Tris, pH 8.0). The lysates were then extracted with phenol-chloroform, and cellular DNA was precipitated with ethanol in the presence of 3 M sodium acetate. The concentration of DNA was determined by OD at 260 nm. To detect the BIV proviral DNA, nested PCR was performed. The first amplification was done by using a pair of the outer primers specific to the BIV pol region (nt 2129–2148: 5’-GTATCAGGCCTCTTAAGGAAA-3’, and nt 2554–2522 : 5’-TAATCTTTCTGGTGTTAGTC-3’). The second amplification was performed to amplify a 298 bp fragment, using a pair of inner primers of pol region (nt 2181–2220 : 5’-TCCGAAAGCTCTTGGGATAA-3’, and nt 2479–2460 5’-TTCCACTGGAACCTCTCTAT-3’) in the BIV genome [3, 10]. The amplified products were fractionated on a 1.5% agarose gel, and visualized by the staining with ethidium bromide. The specificity of the PCR products were confirmed by Southern blot analysis. Ten µl of the PCR products was electrophoresed on an agarose gel, and transferred onto a nylon membrane filters (Hybond-N, Amersham, UK). Prehybridization was carried out at 42°C overnight in a mixture of 5 x SSPE, 50% formamide, 5 x Denhardt solution, 0.5% SDS, 250 µg/ml of denatured salmon sperm DNA. Then the filters were incubated with the 32P-labeled -BIV R-29 probe (1 x 10^6 to 6 x 10^6 cpm/µl). After hybridization, the filters were washed four times and were then exposed to X-ray films for 4 or 12 hr at -80°C.

DNA cloning and sequencing: The 298-bp DNA fragment of PCR products was purified and cloned into the pGEM-T vector (Promega, Madison, WI). The nucleotide sequences of the cloned inserts were determined by using the Taq DyeDeoxy™ Terminator Cycle Sequencing System (Applied Biosystems, Foster City, CA, U.S.A.) with an automated DNA sequencer (Applied Biosystems model 373A).

RESULTS

Seroprevalence of BIV and BLV in dairy herds: Antibodies against BIV-specific p26 protein were detected by Western blot analysis of sera collected from dairy cattle with purified BIV antigen (Fig. 1). The result of the seroprevalence of BIV and BLV in 12 different dairy herds is shown in Table 1. In all dairy herds, at least one cow was BIV-seropositive.

The same serum samples were also tested for BLV antibody by immunodiffusion test. As shown in Table 1, some dairy herds (Farms 4, 5, 10 and 12) showed higher prevalence while some farms did not have any BLV-seropositive cow. However, two dairy herds (11 and 12) were found to have higher prevalence not only of BLV, but
also BIV infection. Overall, among 611 of cattle tested, 175 (28.6%) were BLV-seropositive, 72 (11.7%) were BIV-seropositive and 26 (4.2%) were positive for both BIV and BLV.

Characterization of BIV field isolates: BIV was isolated from PBMCs and milk-derived leukocyte prepared from BIV-seropositive cows by co-cultivating them with cc81 cells, and the syncytium formation was observed during the passages. Half of the cultured cells were used for the identification of BIV as shown in Table 2, and remaining cells were continued to culture up to 20 passages.

Evidence of BIV replication was obtained by syncytium assay (Fig. 2, Table 2) in cc81 cells co-cultured with PBMC as well as milk-derived leukocytes prepared from BIV-seropositive cow. The number of syncytia formation was decreased during passages and the time to induce syncytia form was also prolonged. In contrast, co-cultured cells prepared from cows co-infected both BIV and BLV showed much more syncytium formations which might be mainly caused by BLV.

The expression of BIV proteins in cultured cells was detected by IFA. Structural proteins of BIV field isolates in co-cultured cc81 cells with PBMCs from BIV-seropositive cows within 5–6 days were recognized by anti-BIV serum, 1141 and about 70% of the cells were positive for BIV antigens (Fig. 3, Table 2). BIV-infected cc81 cells were also reacted with anti-BIV serum from a Japanese cow naturally infected with BIV but not BLV (data not shown).

Fig. 1. Detection of anti-BIV p26 antibody in sera from cattle of dairy herds in Hokkaido by Western blotting. Only 17 serum samples were shown in this figure. An arrow indicates the location of p26. P: serum from a cow experimentally inoculated with BIV (R-29), N: negative control.

Table 1. Seroprevalence of BIV and BLV in 12 dairy herds in Hokkaido

<table>
<thead>
<tr>
<th>Farm No.</th>
<th>No. of tested cattle</th>
<th>No. of seropositive cattle (%)</th>
<th>No. of cattle co-infected with BIV and BLV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>6 (20.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>1 (4.0)</td>
<td>5 (20.0)</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>4 (13.3)</td>
<td>7 (23.3)</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>1 (3.0)</td>
<td>16 (53.3)</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>2 (6.0)</td>
<td>14 (46.6)</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>2 (6.0)</td>
<td>4 (13.3)</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>1 (3.0)</td>
<td>6 (20.0)</td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td>4 (13.3)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>9</td>
<td>107</td>
<td>11 (10.2)</td>
<td>8 (7.5)</td>
</tr>
<tr>
<td>10</td>
<td>75</td>
<td>5 (6.0)</td>
<td>27 (36.0)</td>
</tr>
<tr>
<td>11</td>
<td>121</td>
<td>24 (19.8)</td>
<td>67 (55.3)</td>
</tr>
<tr>
<td>12</td>
<td>73</td>
<td>11 (15.0)</td>
<td>21 (28.7)</td>
</tr>
</tbody>
</table>

Total 611 72 (11.7) 175 (28.6) 26 (4.2)

a) Seroprevalence of BIV and BLV was tested by Western blotting and immunodiffusion test, respectively.
By Western blot analysis, the BIV isolate was found to have a virus-specific protein, p26, similar to American BIV, R-29 (Fig. 4, Table 2). A stretch of 10 amino acids within this protein (strain R-29) had been observed to have a high degree of similarity to HIV-1 p24 and several other lentiviruses [10] and was speculated that p26 is the major core, or capsid, protein of BIV [29].

By using nested PCR, a 298-bp fragment specific to BIV proviral DNA was amplified from BIV-infected cc81 cells, blood and milk samples (Fig. 5, Table 2). However, no fragment was amplified from 2 blood samples from seropositive cows although probed with $^{32}$P-labeled inner fragments. Moreover, the nucleotide sequence of the 298-bp PCR product corresponding to the pol gene amplified from three field isolates from three different dairy herds, including uncultured isolates, cultured isolate and milk isolate were analyzed, and compared with the sequence of BIV R-29 [10] (Fig. 6). Nucleotide sequences of the amplified fragments from Japanese BIV field isolates had 99.0 to 99.7% homology with R-29. Sequences of the fragments amplified from all field isolates were compared with each other and were 99.0 to 99.3% homologous. Comparison of sequences of DNA fragments amplified from cultured (after 15 passages) and uncultured PBMC isolated from a cow (No. 234) showed almost 100% identity.

Proviral sequences amplified from PBMCs and milk-derived leukocytes obtained from the same host at the same time of collection, showed 99.7% homology. Homology in the predicted amino acid sequences between BIV field isolates and R-29 were 97.0 to 99.0%. The BIV R-29 isolate had 99.6% nucleotide sequence homology with the published sequence of molecular clone R-29, with a single, silent nucleotide substitution [10]. Based on genetic characteristics of the pol region, it appeared that Japanese BIV isolates are very similar to American BIV R-29.

**DISCUSSION**

This is the first report of BIV isolation in Japan. These BIV isolates were obtained by co-culture of PBMC from...
Fig. 3. Immunofluorescence test of BIV-infected cc81 cells. Infected cells were reacted with serum (1141) from a cow experimentally inoculated with BIV R-29 (American isolate).

Fig. 4. Comparison of BIV p26 between BIV R-29 and a Japanese BIV isolate. Viral antigens purified from R-29 (lane 1) or Japanese BIV isolate (lane 2) were incubated with BIV R-29 serum (1141). Location of BIV p26 was indicated by an arrow.

clinically healthy cows but seropositive for BIV with cc81 cells. Antigenic and genetic characteristics of these BIV field isolates appeared to be similar to those of American R-29. These BIV isolates are difficult to culture *in vitro* to produce a maximum amount of viral proteins, which is in agreement with previous reports [25, 30]. The nucleotide sequences of 298-bp sequence of the amplified *pol* gene products were compared and found that the Japanese BIV isolates are very similar to strain R-29. The *pol* sequences are highly conserved among lentiviruses. The sequence analysis of the *env* gene of nine different isolates from cattle in the United States suggested that R-29 may not be representative of BIV currently prevalent in cattle population in the United States [26, 27]. Comparison of nucleotide sequences of the viral genes, particularly in the *env* region of Japanese isolates to BIV R-29 is now under investigation.

Seroepidemiological studies of BIV infection in cattle have been reported in many countries around the world. The prevalence rate varied in different dairy herds. In the present study seroepidemiological survey of BIV and BLV in 12 different dairy herds from Hokkaido was performed to know the relationship between BIV and BLV infections. It was noted that BIV prevalence rate was higher than previously reported [12], however there was no correlation between BIV and BLV infections. This observation is similar to previous reports [12, 15]. Nevertheless, we found a cow co-infected with BIV and BLV and showing persistent lymphocytosis, weight lose, wasting and enlarged lymph node. Proviral BIV was detected in brain, liver, kidney,
mammary gland, spleen and bone marrow of this cow at the time of necropsy by nested PCR (data not shown). It is interesting to know whether the mixed infection of BIV and BLV can enhance any clinical symptoms when compared to single infection with BLV.

Although BIV infection has worldwide distribution, the mode of BIV transmission is unknown. The facts that BIV has been detected in semen [18], and that seroprevalence of BIV infection increases according to aging of animals in the same dairy herd suggests that horizontal transmission of the virus is possible through natural or artificial inseminations and through iatrogenic transmission with blood contaminated instruments or blood-sucking insects [24]. Moreover, BIV has been detected in milk-derived leukocytes [17]. In this study, proviral DNA and BIV were found in milk from a sero-positive cow. These findings

| Fig. 5. Detection of BIV proviral DNA in BIV-infected cc81 cells, blood and milk samples from BIV-seropositive cows (Nos. 234, 212 and 16). Nested PCR was performed to amplify a part (298 bp, nt.2181-2479) of the pol gene of BIV. |
| A. Nucleotide sequences |
| BIV R29-127 pol | >2181 | GCAATGATA | TAAAGATGC | CTAAGTTACT | ATCCCTTAC | AGAAGGCTT | TAGACCGCTT |
| 212 BIV pol Milk | >2181 | .......... | .......... | .......... | .......... | .......... | ...........

| B. Amino acid sequences |
| BIV R29-127 pol | >61 | AIDIKDAYPT | IPLHEDRPFF | TAPSVPVPVR | ESLPDRPFW |
| 212 BIV pol Milk | >61 | .......... | .......... | .......... | .......... |

Fig. 6. Comparison of proviral (A) and predicted amino acid (B) sequences of a portion of the nested PCR-amplified DNA fragments derived from blood (BIV pol blood), BIV-infected cc81 cells (BIV pol culture), and milk (BIV pol milk). These sequences were compared with the published sequence of BIV R-29 (nt2181-nt2299, aa61-aa99). Dots indicate identical bases (A) or amino acid residues (B), and letters indicate substitutions.
suggest that BIV-infected dams can transfer not only antibodies against BIV but also BIV-infected cells to their offsprings in uterus or via colostrum or milk. In fact, this route of transmission has been reported in other lentiviruses [4].

In conclusion, this study provides additional informations on seroprevalence of BIV infection, and biological and genetic characteristics of Japanese BIV field isolates. Because isolation of BIV from milk samples it suggests viral transmission through milk. Further studies are required to clarify the pathogenesis of BIV on Japanese cattle population and its possible involvement in other disease(s).

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REFERENCES


