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Evidence of bovine immunodeficiency virus (BIV) infection: Serological survey in Argentina

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Abstract

This is the first report of serological evidence for bovine immunodeficiency virus (BIV) infection in Argentina. The analysis was performed in 589 dairy bovine sera samples, applying indirect enzyme-linked immunosorbent assay (I-ELISA) using a synthetic antigen (transmembrane peptide, TM) and Immunofluorescent assay (IFA). In this study, 9 dairy herds from 4 Argentinian provinces were evaluated and 12% of the animals tested positive for BIV. Seven of the 9 herds tested were BIV seropositive and the percentage of BIV seropositive animals in the herds ranged from 2% to 42%. Direct detection of BIV provirus applying nested PCR was not conclusive. Antibody detection against bovine leukemia virus (BLV) in all sera was also performed applying immunodiffusion (ID) assay and 59% resulted seropositive. Statistical analysis of the results was carried out and possible evidence of association between BIV and BLV infection was considered. Future studies should be performed including local field isolates strains of BIV.

Keywords: BIV; BLV; I-ELISA; IFA; Seroprevalence

1. Introduction

A Bovine Visna-like virus, because of similarities to the ovine lentivirus, was isolated by Van Der Maaten and colleagues (1972) from an 8-year old dairy cow from Louisiana that had a persistent lymphocytosis, lymphadenopathy, central nervous system lesions and wasting. New interest was shown in Bovine Visna-like virus (named bovine immunodeficiency virus, BIV) after the isolation of human immunodeficiency virus (HIV), because of the urgent need for developing animal models for the acquired immunodeficiency syndrome (AIDS) (Gonda et al., 1987; Straub and Levy, 1999).

BIV is structurally, genetically and biochemically similar to HIV but unlike it, BIV has not been associated with severe acquired immunodeficiency syndrome. The early pathogenic effects of BIV were studied by Carpenter and colleagues (1992), in experimentally inoculated calves. Levels of BIV replication *in vivo* appeared to be low, since during peak periods of viral replication *in vivo*, less than 0.03% of peripheral blood mononuclear cells (PBMC) expressed detectable levels of viral RNA. Later efforts were directed toward understanding the virus-host interactions which restrict virus replication *in vivo* and contribute to long-term clinical quiescence in virus infected cattle (Gonda et al., 1994; Snider et al., 1996, 2002).

Although several pathological changes have been reported in BIV-infected cattle, including monocyte dysfunction (Onuma et al., 1992), encephalophathy and lymphadenophathy (Van Der Maaten et al., 1972; Snider et al., 2002), the detailed pathogenesis in infected cattle still remains unclear. BIV seropositivity has been shown to be variably associated with decrease in animal production, weight loss, secondary diseases, and diminished milk

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production (McNab et al., 1994). Serologic evidence for BIV infection has been reported in many countries around the world: Netherlands, (Horzinek et al., 1991), France (Polack et al., 1996), Japan (Usui et al., 2003), Canada (McNab et al., 1994), Australia (Burkala et al., 1999), Brazil (Meas et al., 2002), Costa Rica (Gonda et al., 1994), Venezuela (Walder et al., 1995) and Turkey (Meas et al., 2003). Cyrcoats and colleagues (1994) revealed that greater than 50% of dairy cattle in Lousiana, USA were seropositives to BIV. The impact of BIV is controversial due to the difficulty in culturing new isolates *in vitro* and the complexity in identifying BIV-infected animals (Evermann and Jackson, 1997; Gradil et al., 1999; Lew et al., 2004). Beef herds appears to have a lower prevalences (<5%) compared to dairy herds (3–50%), (Amborski et al., 1989), which may be the result of herd management practices and of the extended productive life of dairy relative to beef cattle.

Even though a correlation between BIV and bovine leukemia virus (BLV) has been reported, the information on this issue is still not clear (Meas et al., 2002; Jacobs et al., 1995).

In this study we applied both an Immunofluorescent assay (IFA) and an indirect enzyme-linked immunosorbent assay (I-ELISA) for the detection of antibodies against BIV, and PCR as a direct method of BIV provirus detection. The immunodiffussion (ID) assay for the detection of BLV antibodies was also included in the analysis.

2. Materials and methods

2.1. Bovine samples

Serum samples were obtained during the period of 2003–2004. A total of 589 sera were collected from 4 provinces (9 dairy herds). The sera were stored at −20 °C until further use. All serum samples were analyzed to detect antibodies against BIV applying I-ELISA and IFA. In order to determine infection with BLV, all serum samples were analyzed to detect antibodies against the virus by ID.

For PCR assay, blood samples with heparin were obtained from 50 seropositives and seronegatives dairy cows and DNA was extracted from peripheral PBMC within 48 h.

2.2. Immunofluorescent assay (IFA)

The IFA was carried out using 10-well commercial slides (VMRD, Inc., Pulman, WA, USA) according to the manufacturer's instructions. Slides are virus infected Madin-Darby bovine kidney epithelial cell cultures (MDBK) grown on the surface of Teflon masked slides. All wells contain both positive and negative cells. The serum samples were diluted 1:10 in serum dilution buffer, pH 7.2. Substrate slide were equilibrated to room temperature and 50 μl of the diluted test serum was placed in one well; seven animals were tested on each slide. Each slide was incubated with a positive and a negative control serum diluted 1:10 (provided by VMRD). The slides were incubated in a humidified chamber at 37 °C for 30 min, flushed with PBS, then soaked for 10 min in PBS and blotted. Fifty microliter of a 1:200 dilution of fluorescein isothiocyanate-labeled goat anti-bovine IgG (VMRD) was added to each well and the slides were incubated at 37 °C for 30 min and then washed as before. The slides were dried and a coverslip mounted with one drop of mounting fluid (VMRD). Slides were examined for fluorescence immediately using a Zeiss fluorescent microscope at 400×. Seventy dairy serum samples were analyzed by this technique.

2.3. Indirect enzyme-linked immunosorbent assay (I-ELISA)

Serological analysis was performed on 589 serum samples using a synthetic peptide derived from the available sequence of the transmembrane (TM) glycoprotein of BIV-R29, produced at the Veterinary Laboratories Agency, Surrey, UK (Scobie et al., 1999).

The detection of antibodies against this TM peptide was performed under the following conditions: a volume of 100 μl peptide (12 μg/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6) was absorbed to each well of a microtitre plate (Nunc-Immunoplate. MaxiSorp™ Surface) overnight at 4 °C. The wells were washed three times with 200 μl TBS-T (138 mM NaCl, 2.6 mM of Calf Intestinal Phosphatase (CIP), 24.8 mM Tris–Cl, 1% Tween-20, pH 7.5) and blocked with dried milk powder (2%) and goat serum (20%) in TBS-T for 1 h at room temperature. Following three washes with TBS-T, 100 μl aliquots of bovine sera diluted 1:10 in TBS-T were incubated for 1 h at room temperature. After three additional washes, antibovine immunoglobulin conjugated with horseradish peroxidase diluted 1:5000 in TBS-T with 1% of non-fat milk, were added to each well and incubated 1 h at room temperature. After three washes with TBS-T, the peroxidase reaction was visualized with ABTS: 2.2-azino-bis-3ethylbenz-thiazoline-6-sulfonic acid (Sigma–Aldrich Chemical Co., St. Louis, MO, USA) and the results were expressed as the absorbance at 405 nm. A sample to positive ratio was calculated based on the positive and negative control sera included in each plate.

2.4. Immunodiffussion (ID)

For the detection of BLV antibody, the ID test using the glycoprotein antigen was performed as described previously (Gonzalez et al., 2001b).

2.5. PCR assay

In order to confirm BIV infection on BIV-I ELISA seropositive cattle, DNA was extracted from PBMC using the Dneasy™ tissue kit (QIAGEN GmbH, Hilden, Germany) and its concentration was determined by optical density

(OD) at 260 nm. To detect the BIV proviral DNA, nested PCR was performed (Meas et al., 1998). The first amplification was performed using a pair of outer primers specific to the BIV *pol* region (nt 2129–2148: 5′-GTATCAGGCTCTAAGGAAA-3′ and 2554–2522: 5′-TAATCTTCTGGGTGGTAGTC-3′). The second amplification was performed to amplify a 298 bp fragment, using a pair of inner primers from the *pol* region (nt 2181–2220: 5′-TCCGAAGCTGCTTGGATAA-3′ and nt 2479–2460 5′-TTCCACTGGAACCTCTCTAT-3′). Briefly, PCR was performed as following: final concentrations in the reaction mixes were 1 × Taq polymerase buffer (Promega, Corp., Madison, WI, USA), 1.5 mM $MgCl_2$, 200 M dNTPs, 10 pmol of each primer, 2U Taq polymerase and 1 μg of genomic DNA, in a total volume of 50 μg. The thermal cycling conditions for the first round of amplification were 1 cycle for 4 min at 94 °C, then 30 cycles of 45 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C, with a final extension step of 20 min at 72 °C. Five microliter of the first round reaction were used in the second reaction under identical cycling condition. Ten microliter of the amplified products were loaded on a 2% agarose gel, and visualized by staining with ethidium bromide. A positive control BIV-DNA, obtained from The National Institute of Animal Health, Tsukuba, Japan, was included in each analysis. DNA for negative control reactions was obtained from unrelated animals constantly assayed as BIV negative. Additionally, a water only negative control reaction was included in each reaction. Subsequent to negative results using the primers mentioned above, additional nested PCR reactions were performed with BIV *pol* degenerate primers as follows. The first amplification was performed using a pair of outer primers specific to the BIV *pol* region (Suarez et al., 1995) (P1: 5′-ATGCTAATGGATTTTAGGGA-3′ and P36: 5′-CATTTCTTGGGTGTGAGCTC-3′). The second amplification was performed using an internal pair of degenerate primers from the *pol* region (LV3-BIV: 5′-GAYRTARRRGATGCCTA-3′ and DDMY-BIV: 5′-AWRTCRTCCATRTAYTG-3′). PCR was performed as follows: final concentrations in the first round reaction mix were 1 × Taq polymerase buffer (Promega), 2.5 mM $MgCl_2$, 200 μM dNTPs, 4 μM of each primer, 2.5U Taq polymerase and 500 ng of genomic DNA, in a total volume of 50 μl. The thermal cycling conditions for the first round of amplification were a hot start with 10 min at 94 °C followed by addition of the Taq enzyme, then 2 min at 94 °C, 15 s at 51 °C, and 2 min at 72 °C, followed by 36 cycles of 45 s at 94 °C, 15 s at 51 °C, and 1 min at 72 °C, and a final extension step of 10 min at 72 °C. Two microliter of the first round reaction were used in the second reaction, where final concentrations in the reaction mix were 1 × Taq polymerase buffer (Promega), 2.5 mM $MgCl_2$, 40 μM dNTPs, 1 μM of each primer, and 1U Taq polymerase, in a total volume of 50 μl. The thermal cycling conditions for the second round of amplification were 35 cycles of 30 s at 92.5 °C, 30 s at 46 °C, and 45 s at 72 °C, and a final extension step of 10 min at 72 °C. Amplified products were visualized as before, and negative control reactions (DNA and water) were included as before. Positive control material consisted of DNA extracted from PBMC of a calf experimentally infected with the FL-112 strain of BIV (Suarez et al., 1993).

2.6. Statistical analysis

The serological results were analyzed using chi square test and odds ratio (OR) from STATCALC, EpiInfo version 6.0.

3. Results

3.1. IFA

The immunofluorescent assay was able to detect BIV antibody-positive animals from 70 dairy cattle and established a prevalence of 2.8% (2 positive animals). Positive cells showed a diffuse cytoplasmic staining pattern (Fig. 1). In agreement with Orr et al. (1999) positive fluorescence brighter than the negative serum control and centralized around syncytial nuclei indicated the presence of

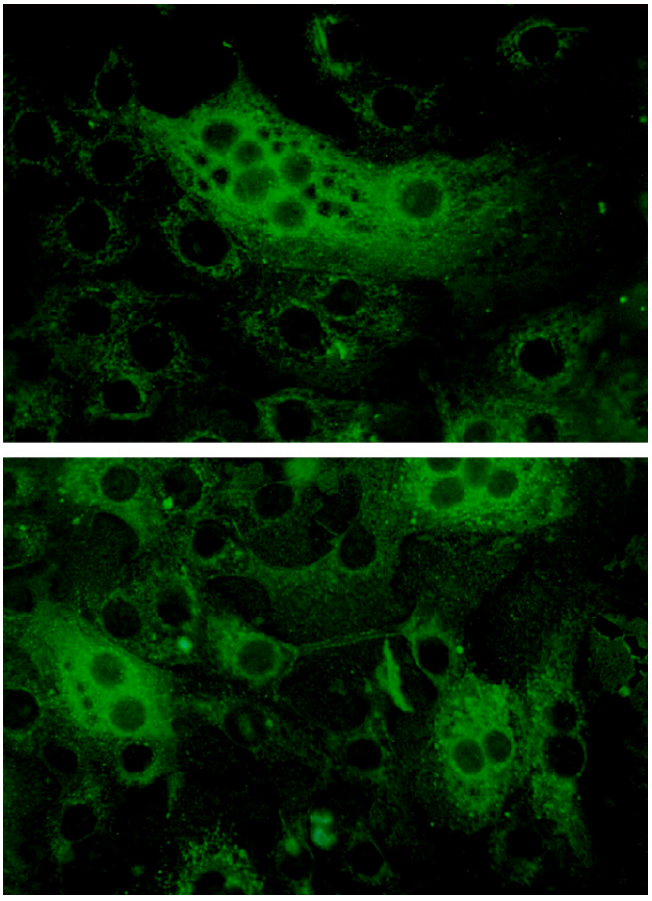

Fig. 1. IFA to detect BIV antibodies. The assay was performed as described in material and methods. Positive cells shows a diffuse cytoplasmic staining pattern, centralized around syncytial nuclei.

anti-BIV antibody in the test serum. Only one of these IFA positive sera was confirmed as positive by I-ELISA.

3.2. I-ELISA and ID assay

589 serum samples were analyzed to detect antibodies against BIV, and the results are shown in Table 1. Two dairy herds (farm 2 and 6) showed higher prevalence (>20%) while two farms (3 and 9) did not have any BIV-seropositive cow. Two dairy herds (2 and 6) were found to have higher prevalence not only of BIV, but also BLV infection. Overall, among 589 cattle tested, 70 (12%) were BIV positive, 348 (59%) were BLV positive and 51 (9%) animals were positive to both BIV and BLV. Interestingly, BLV seropositive farms ranged from 15% to 92%.

In order to explore the association between BIV and BLV, Table 2 shows the 589 serum samples, sorted by positive or negative results to BIV and BLV. The statistical analysis shows a p value <0.05 ($p = 0.012$) for the chi square test, and OR larger than 1.0 (OR: 2.1 ($1.12 < \text{OR} < 3.63$) demonstrated the association between BIV and BLV.

3.3. PCR

The PBMC DNAs from bovine samples were tested by nested PCR, with the primers mentioned in materials and methods. No BIV-specific amplification products were obtained from any of the bovine samples tested positive to serological assays. However, a BIV-specific band with the predicted size of 298 bp was detected in DNA positive control sample, from experimentally infected animals – The National Institute of Animal Health, Tsukuba, Japan – using nested *pol* gene primers (data not shown).

4. Discussion

Serological survey is an important way to determine the distribution of BIV on livestock and data on BIV seropositive animals in a South American country, may contribute to the awareness of the worldwide prevalence of the disease. This is the first report of serological evidence for BIV infection in Argentina. In this study, which was limited to the oldest animals from 9 dairy herds from different Argentinian provinces, we found BIV seropositivity in 12% of the total tested animals (78% of the herds). An overall low prevalence of BIV has been confirmed in studies undertaken in other countries (Horzinek et al., 1991; Gonzalez et al., 2001a).

Currently, there is no a gold standard (a completely accurate test) to detect BIV infection (Nash et al., 1995; Orr et al., 2003) and one of the difficulties in assessing the role of BIV in bovine disease is inconsistency with the methods used to detect infected cattle. Variations in infection prevalence might be influenced by the disparity of assays used for BIV detection. Substantial misclassification of infection would be expected in epidemiological studies of BIV regardless of which assay was used (Orr et al., 2003). Concerning IFA, there are some disadvantages in the use of this technique for BIV. Antibodies to BIV antigen used in IFA tests can be undetectable when antibody levels are diminished, as in long-term infection. Currently there is no commercially available IFA test for BIV. A previously available commercial test was used in this work to detect anti-BIV antibodies in serum, but IFA is time consuming and interpretation is subjective because of irregularly occurring background fluorescence. Horzinek and colleagues (1991) have reported that IFA may give some false positive results. In our study 1 sera positive by IFA were not confirmed by I-ELISA. This could be attributed to the fact that IFA and I-ELISA may not detect the same antigen. Additional, both test IFA and ELISA may detect different antibodies and/or have a different sensitivity level.

Table 2
Concordance between bovine immunodeficiency virus (BIV) and bovine leukemia virus (BLV) serological results

	BLV (+)	BLV (−)	Total
BIV (+)	51	19	70
BIV (−)	297	222	519
Total	348	241	589

$p < 0.05$ ($p = 0.012$) OR: 2.1 ($1.12 < \text{OR} < 3.63$).

Table 1
Seroprevalence of antibodies against bovine immunodeficiency virus (BIV) by I-ELISA and bovine leukemia virus (BLV) by immunodiffusion assay, in 9 dairy herds from 4 Argentinian provinces

Farm no.	Tested cattle	Positive cattle		BIV–BLV negative cattle	Co-infected cattle (BIV and BLV)
		BIV	BLV		
1	70	2 (3%)	49 (70%)	21 (30%)	2 (3%)
2	76	19 (25%)	50 (66%)	23 (30%)	16 (21%)
3	26	0 (0%)	4 (15%)	22 (85%)	0 (0%)
4	25	3 (12%)	0 (0%)	22 (88%)	0 (0%)
5	25	4 (16%)	23 (92%)	2 (8%)	4 (16%)
6	76	32 (42%)	61 (80%)	7 (9%)	24 (32%)
7	153	9 (6%)	102 (67%)	46 (30%)	4 (3%)
8	46	1 (2%)	33 (72%)	13 (28%)	1 (2%)
9	92	0 (0%)	26 (28%)	66 (72%)	0 (0%)
Total	589	70 (12%)	348 (59%)	222 (38%)	51 (9%)

Culture of the virus is difficult and as expected, virus could be isolated only within a short time frame. Although the use of PCR for detection of BIV proved to be more sensitive than either serologic testing or virus isolation, the genetic variation (7–8% nucleotide divergence in the conserved *pol* segment) of field isolates, probably plays a negative role in the results of these diagnostic tests. In our studies, as has been reported by Lew and colleagues (2004), the purification of DNA from PBMC appears to be critical for the PCR detection of BIV. In addition, prolonged difficulties, during the development of PCR assay carried on locally in Argentina as well as in UK, were experienced in amplifying Argentinian BIV sequences and no conclusive results were obtained using the BIV *pol* primers based on the same region utilized for Meas and colleagues (1998) or degenerate primers. Our data showed that PCR reactions of DNA positive control from experimentally infected animal were successful. Although in naturally infected animals, it seems much more difficult to obtain positive results. This issue may be explained by low levels of virus integration in the lymphocytes during natural infection. In an experimental infection, Baron and colleagues (1998) obtained positive PCR results using *pol* primers at 4 and 8 weeks post infection (p.i.) with DNA extracted from 2.5×10^5 cells, but not from lower amounts, and PCR products were no longer detected 12 months p.i. If in fact, nested PCR is only 80% sensitive and 85% specific (Orr et al., 2003), then misclassification will likely occur even if PCR is the sole diagnostic assay applied. This is supported by our negative PCR analysis and previous results (Gonzalez et al., 2001; Lew et al., 2004). On the other hand, Suarez and Whettone (1997) demonstrated variability (from 0 to 10 out of 12) in the classification of infection status of samples from experimentally infected bovines using the PCR assay.

Antigenic variation between European and American strains of BIV has been reported (Polack et al., 1996), since European sera react weakly with the American isolate antigen R-29. Consequently, wild-type isolates are also needed in order to develop specific detection assays based on antibodies or nucleic acids (Belloc et al., 1996). For this purpose, an assay that could detect all BIV strains should be essential too.

ELISA tests are, in general, relatively accurate. They are considered highly sensitive and specific and compare favorably with other methods used to detect specific antibodies. Therefore I-ELISA should also be presented as a confirmatory test for BIV. We propose here that the I-ELISA based on recombinant viral antigen or synthetic peptides, as used in this work, should decrease the number of false positive reactions occurred when the serum react with non-relevant proteins, and could be recommended as a diagnosis test to detect BIV seropositive animals.

Even though the serum samples were not randomly collected but chosen among the oldest of dairy cattle with previous or current BLV infection, the results presented here give an accurate estimate of BIV prevalence in Argentina. The prevalence rate varied in different dairy herds and the higher prevalence in some dairy cattle may be the result of herd management practices and of the extended productive life. Large scale serological and molecular studies with detailed long-term epidemiological observation of BIV-incidences will be necessary to confirm these preliminary findings.

In the present study, a seroepidemiological survey of BIV and BLV was performed to determine a correlation between BIV and BLV infections. The p value for chi square and the odds ratio value provide evidence of association between BIV and BLV. However, these data expressed a statistical correlation and the probability that BIV infection predispose the animals to infection with BLV needs to be confirmed. Meas and others (2002) have reported some evidence for the co-infection of BLV and BIV; however disagreements with these results were published before by Jacobs and colleagues (1995). Co-infection may simply represent prior exposure to both viruses and not in itself indicate any biological association and/or probably synergisms.

In conclusion, the seroprevalence of BIV in dairy cattle herds in Argentina ranged from 2% to 42%. Further studies should be designed to investigate pathogenic and biological properties of local field isolate strains of the virus, and these strains should be included in the assays chosen to detect BIV antibodies.

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