Serological and genomic detection of bovine leukemia virus in human and cattle samples

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Key Words:
BLV infection, Human, Cattle, nested PCR, ELISA, Southern blot.

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Received: 4 November 2009
Accepted: 28 July 2010

Abstract

Bovine leukemia virus (BLV) is a retrovirus responsible for lymphoproliferative disorders in cattle. Although infections of BLV in animals are well known, little is known about its capacity to infect humans. This study investigated the presence of anti-BLV antibodies and BLV proviruses in human and cattle samples. An indirect enzyme-linked immunosorbent assay (ELISA) was used to detect anti-BLV antibodies while nested PCR was employed to identify BLV provirus sequences. The overall prevalence of anti-BLV antibodies in human and cattle samples were 12.50% and 16.73%, respectively. When using ELISA as a reference test, sensitivity and specificity for nested PCR were 0.625 and 0.970, respectively. The predictive value of a positive test was 0.862 and the predictive value of a negative test was 0.897. The percentage of cattle correctly classified by nested PCR assay was 89.1%. Nested PCR and Southern blot analysis, using primers specific for BLV gag sequences, revealed that BLV proviruses were detectable in cattle and human samples. Our results highlight the risk of human exposure to BLV and the need for further investigations to determine whether BLV infection poses a health hazard for humans.

Introduction

Bovine leukemia virus (BLV), a lymphotropic retrovirus structurally related to human T-cell leukemia virus type 1 (HTLV-1), leads to the development of enzootic lymphosarcoma-leukemia in cattle (Burny *et al*., 1984; Murphy, 1999; Oroszlan *et al*., 1984; Sagata *et al*., 1984). Infection with BLV can remain clinically dormant, with cattle in an a leukemic state, or it can emerge as a persistent lymphocytosis (PL), characterized by an increased number of B lymphocytes, and, more rarely, as B-cell lymphomas in various tissues after a lengthy latent period. A low percentage (0.6-5%) of BLV-infected animals develops tumors, while most (~95%) remain clinically healthy (Burny *et al*., 1988; Radostits *et al*., 2007). Under natural conditions, the disease occurs only in cattle, but under experimental conditions, the same virus can easily infect sheep, which develop B-cell lymphosarcomas at higher frequencies and after a shorter latent period than cattle (Aida *et al*., 1989; Jensen *et al*., 1990). Although the molecular biology of BLV and its infection in animals is well established, little is known about the possibility of BLV infections occurring in humans (Buehring *et al*., 2003; Burridge, 1981; Deschamps *et al*., 1981).

It is known that infected cows are allowed to mix with non-infected cows. Infected cows possess BLV-infected cells that are present in marketed beef and dairy products. It has been demonstrated that viruses present in bovine edible products may not be entirely inactivated by pasteurization or cooking (Buehring *et al*., 2003). Oral transmission of the closely related HTLV has been described in humans (Takahashi *et al*., 1991), suggesting the potential for oral transmission of BLV in humans.

Some serological surveys were capable of detecting human antibodies to BLV structural proteins. These studies used complement fixation (CF) and agar gel immunodiffusion (AGID) (Burny *et al*., 1984; Burridge, 1981; Donham *et al*., 1977). Nevertheless, the use of serological techniques more sensitive than CF or AGID, such as enzyme-linked immunosorbent assay (ELISA) and immunoblotting, have confirmed that humans might also be exposed to BLV (Buehring *et al*., 2003).

Antibodies in human sera that are reactive with BLV antigens might merely indicate an exposure to the virus. However, consumption of meat, milk or dairy products from infected animals may also lead to an immune reaction against viral antigens. Human exposure to BLV still raises important questions

concerning the potential for the presence of BLV in human blood or its integration into the human genome. There is some evidence for the capability of BLV to integrate into the human genome *in vitro*. Slavikova *et al*. (1978) have demonstrated that B-human myeloma cells could be infected with BLV. Altaner et al. (1989) have also shown that BLV propagated in a cell clone of fetal lamb kidney (FLK) origin could be transmitted by cell contact to different mammalian cells including human cells. In the cells containing the transmitted BLV, the viral genome was expressed to its protein products. Since we found human sera to be reactive to BLV, we further examined the possibilities of the presence of BLV in the human samples using a nested PCR technique. To determine the accuracy of this test, the concordance between ELISA and nested PCR was assessed.

It is still unclear whether exposure to the virus under natural conditions leads to its integration into the human genome. Scientists have investigated whether BLV is transmissible to humans. BLV has been reported to infect human cells *in vitro* and to cause tumors and erythroleukemia in primates (McClure *et al*., 1974; Slavikova *et al*., 1987). Therefore, it seems essential to investigate BLV proviruses in individuals with seropositive reactions. The present study was undertaken to determine the prevalence of anti-BLV antibodies in humans and cattle in Iran and whether BLV proviruses could be detected in the seropositive human or cattle samples.

Materials and Methods

Cattle serum and blood specimens

A total of 1,619 cattle serum samples from central, eastern and western parts of Iran were examined. Samples were collected from Holstein dairy cattle, ranging from three to eight years old, based on a random cluster sampling program. A total of 175 cattle blood samples were also collected for the detection of BLV from central and eastern parts of Iran, from where human samples had been received as well.

Human serum specimens

Stored human blood and serum samples were received from different regional diagnostic laboratories of central and eastern parts of Iran. This study used a total of 454 human samples without any particular illness recorded. The presence of anti-BLV antibodies in serum was confirmed by ELISA.

ELISA

Indirect ELISA for the detection of antibodies against BLV in human and cattle sera were performed according to standard methods with some modifications (Ausubel, 1999; Hay *et al*., 2002). ELISA polystyrene microplates (BioX BLV ELISA kit, Brussels, Belgium) were used that were coated in even-numbered columns with viral antigen (BLV) and in odd-numbered columns with control antigen. For cattle samples all procedures were carried out according to the instruction manual provided by the manufacturer. For human samples the procedure was the same as cattle samples with some modifications. Briefly, the primary antibody (incubated for 60 min) was whole human serum diluted 1:20 in dilution buffer. The secondary antibody (incubated for 60 min) was horseradish peroxidase-labeled goat anti-human IgG (Serotec, Oxford, UK) diluted 1:500 in washing buffer. Primary and secondary antibodies were added in duplicates to both even and odd columns. In order to check the direct reaction of secondary antibodies with coated antigens, goat anti-human IgG was directly added in duplicates to columns with no primary antibodies (human serum). After the primary and secondary antibody steps, the plates were rinsed five times. The 100 µl substrate solution containing tetramethylbenzidine and H_2O_2 (TMB) was added and reacted for 30 min. The plates were read on an ELISA reader at a wavelength of 450 nm. The corrected OD value for each serum sample was calculated by subtracting the OD value of the control well from the OD value of the coated viral antigen well. The OD cut-off values for the assay were calculated as the mean negative value plus 2 (standard deviation (S.D.), obtained from data of control BLV-seronegative humans included in the test. The BioX BLV ELISA positive and negative bovine serum controls (diluted 1:20 in dilution buffer) were run with each assay.

Extraction of DNA

FLK-BLV cells (Razi Vaccine & Serum Research Institute, Tehran, Iran) served as a positive control for extraction (Van der Maaten *et al*., 1975). DNA was prepared from cells and whole blood as described by Sambrook & Russell (2001). In cases where whole blood was unavailable DNA was prepared from serum samples instead. Aliquots (300 µl) of whole blood were added to 500 µl red blood cell lysis buffer [20 mM Tris–HCl (pH 7.6)] mixed and incubated at room temperature. After 10 min the tube was centrifuged at 12,000 g for 20 s and the supernatant discarded. The pellet was resuspended in 600 µl cell lysis buffer [10 mM Tris–HCl (pH 8); 1 mM EDTA (pH 8); 0.1% SDS] and homogenized. Then 200 µl potassium acetate (3 M potassium and 5 M acetate) was added and vortexed for 30 s. After centrifugation for 3 min at 12,000 g, the supernatant was transferred to a fresh tube containing 600 µl isopropanol to precipitate the DNA. The pellet was washed with 70% ethanol and resuspended in 50 µl TE (pH 7.6). DNA extraction from serum followed the same protocol as described above, except that the red blood cell lysis step was omitted.

Polymerase chain reaction

To detect BLV DNA, nested PCRs were performed using the DNAs extracted from blood and serum samples. Two sets of oligonucleotide pairs were used. For the first amplification round a pair of the outer primers specific to the BLV *gag* region was used as reported by Wang *et al*. (2002) (nt 628-648: 5'-ATGGGAAATTCCCCCTCCTAT-3', and nt 1803-1783: 5'-GTTTTTTGATTTGAGGGTTGG-3'). The second amplification round was performed to amplify a 385 basepair (bp) fragment, using a pair of inner primers of the gag region (nt 1068- 1087: 5'-AACACTACGACTTGCAATCC-3', and nt 1452-1434: 5'- GTTCCTTAGGACTCCGTCG-3') in the BLV genome (GenBank accession number K02120). The FLK-BLV DNA served as a positive control for amplification. A total of 466 bp bovine GAPDH and 265 bp bovine IFN-γ gene fragments were amplified by PCR as a control for human DNA contamination with bovine genomic DNA or FLK-BLV, respectively. Primers were as follows:

GAPDH F (5′-TGGCAAAGTGGACATCGTCG-3′) and GAPDH R (5′-TTGCGTGGACAGTGGTCATAAGTC-3′); IFN-γ F (5′ ACATCAACCTCTCTTTGTGCTC-3′) and IFN-γ R (5′-GTAAGAGCCTCTGCAATGATAC-3′)

The final reaction mixture contained the DNA sample: 100 ng of each primer, 200 µM of each dNTP, 50 mM KCl, 20 mM Tris–HCl, 1.5 mM $MgCl_2$ and 1 U/µl Taq DNA polymerase (Biotools, Madrid, Spain). In the first round each sample was amplified in a final volume of 50 µl for 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 60 s, 55°C for 60 s and 72°C for 120 s, with a final step of 10 min at 72°C. The second round of PCR was performed with 2 µl of the first-round amplification product, using the same concentration of reagents and the corresponding primers. The protocol for the second round was as follows: 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 60 s, with a final step of 10 min at 72°C. The conditions and amplification programs for GAPDH and IFN-γ were similar to the first round of BLV nested PCR. After amplification, 10 µl of the reaction mixture was electrophoresed on a 1% agarose gel and visualized on a UV transilluminator. Great care was taken to minimize sample carry-over contamination: laboratory activities were isolated in different rooms before and after PCR; fresh gloves and pipette tips were used; and blank and negative controls (human and bovine DNA) were included for each set and step of the PCR reactions.

Sequencing and sequence analysis

PCR products generated from human samples were sequenced on both directions. Sequencing was performed by Macrogen Inc. (Seoul, Korea) in an ABI 3730 XL automatic DNA sequencer. Sequence identification was completed utilizing BLAST (Altschul *et al*., 1997) via the NCBI website (http://www.ncbi.nlm.nih.gov/). The sequenced 385-bp PCR products were compared to the published sequence for the BLV reference sequence in the GenBank database (Accession number K02120). Sequence alignments were made using Clustal software, available in the Bioedit version 7.0.5.3 software package (http://jwbrown.mbio.ncsu.edu/Bioedit/bioedit.html). Sequences were submitted to the GenBank database (Benson *et al*., 2000) and assigned the accession number EF190192.

Southern blot hybridization

The specificity of the PCR products for all positive and negative samples was confirmed by Southern blot analysis. Ten µl of the PCR products were electrophoresed on a 1% agarose gel in 1x TAE buffer. After electrophoresis, the gel was denatured in 0.5 M NaOH and 1.5 M NaCl for 30 min, and then neutralized in 1.0 M Tris–HCl (pH 7.5), with 1.5 M NaCl for 30 min. The DNA samples were transferred onto a Hybond-N nylon membrane (Amersham, Bucks, UK) by the high-salt buffer method (Ausubel, 1999) and immobilized by heating at 80°C for 2 h. The oligonucleotide between the BLV inner primers (nt 1068–1452) was used as a probe, and labeled with a Gen Image random prime fluorescein labeling module (Amersham) according to the manufacturer's instructions. Membranes were pre-hybridized for 2 h at 65°C in hybridization buffer containing 5x SSC, 0.1% SDS, 5% dextran sulphate and liquid block (provided with the kit). Hybridizations were carried out for 16 h at 65°C in the same solution in the presence of the labeled probe. Membranes were washed in 2x SSC, 0.1% SDS for 15 min and in 0.2x SSC, 0.1% SDS for 15 min, each wash at 65°C. The hybridization signal was revealed by a Gen Image CDP-Star detection module (Amersham) and fluorography using Hyperfilm MP (Amersham).

Statistical analysis

The validity of the nested PCR technique for the detection of BLV in cattle was evaluated using ELISA as gold standard. ELISA and nested PCR results for 175 cattle samples were constructed in a 2-by-2 table in which the final nested PCR result was cross-tabulated with the ELISA results, thus defining true-positive, false-positive, false-negative, and true-negative values. They were used to calculate the standard diagnostic accuracy indices of sensitivity, specificity, negative predictive values, and positive predictive values. To determine the level of inter-rater agreement between ELISA and nested PCR in cattle samples, kappa values were calculated, and the strength of agreement was interpreted using a criteria in which a value of 0 to 0.20 is slight, 0.21 to 0.40 is fair, 0.41 to 0.60 is moderate, 0.61 to 0.80 is

substantial, and 0.81 to 1 is almost perfect, with a significant difference between observers at a P value of <0.05.

Results

The ELISA cut-off was determined according to the manual instructions by using a control serum supplied with the kit (positive control serum). The number of positive samples from different parts of Iran was 271, and the number of negative samples was 1348. The overall prevalence of anti-BLV in the cattle samples was 16.73%. ELISA was positive for 22.8% of samples collected from Tehran province (40/175). Nested PCR was positive for 16.75% (29/175) of blood samples. Within this group ELISA was positive for 22.85% (40/175) of serum samples. ELISA was positive for 86.20% (25/29) of samples with a final diagnosis of BLV infection. Nested PCR was positive for 62.50% (25/40) of samples with a final diagnosis of anti BLV antibodies. When using ELISA as a reference test, sensitivity and specificity were 0.625 and 0.970, respectively. Predictive value of a positive test was 0.862, and predictive value of a negative test was 0.897. The percentage of cows correctly classified by nested PCR assay was 89.1%. Interpretation of kappa scores for two methods was substantial (0.66).

Some individuals have been shown to possess antibodies that are reactive with BLV antigens. In some human sera samples high positive signals in ELISA were reproducibly observed. The absorbance of each test well was compared with the mean absorbance of all low-color test samples (mean = 0.113). Those wells with an absorbance of more than 2 S.D. (S.D. = 0.14992) above the mean were regarded as positive. The number of positive samples was 57, and the number of negative samples was 397. The overall prevalence of anti-BLV in the human samples was 12.5% (57/454). ELISA was positive for 10.41% of samples collected from Tehran province (10/96). Nested PCR was also carried out for 77 human samples (57 BLV-positive and 20 BLV-negative samples, determined by ELISA) (Figure 1). Only 12.3 % (7/57) of ELISA positive samples yielded a positive PCR product in the second round of nested PCR. As a control for DNA contamination with bovine genomic DNA, no fragments were observed when the bovine GAPDH and ovine IFN-γ amplification was performed with related primers. The alignment of the partial nucleotide sequence of 385 bp amplicons, resulting from nested PCR of human samples, is shown in Figure 2. There was 96-98% homology between these sequences and reference sequences, indicating a certain identity of the amplicons obtained from humans using BLV-specific primers. Figure 3 shows Southern blots after PCR amplifications of representative genomic DNA from blood and serum, using primers specific for the *gag*

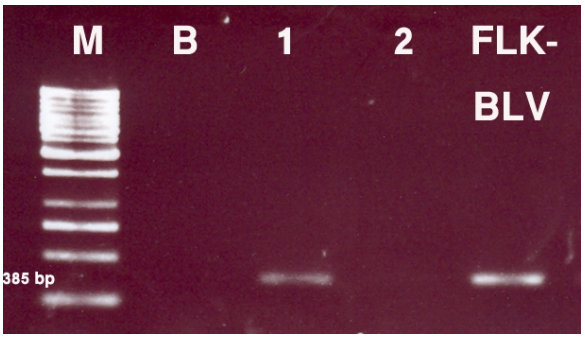

Figure 1: Ethidium bromide-stained agarose gel of PCR products after nested PCR amplification. Genomic DNA from seropositive and seronegative human samples together with FLK-BLV DNA was amplified with the primers described in the text (target size, 385 bp). M: 1 kb ladder (Fermentas); B: blank; lane 1: representative PCR products of seropositive human or cattle samples; lane 2: representative PCR products of seronegative human samples; FLK-BLV: PCR products of BLV-infected FLK.

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BLV, CG      AACACTACGA CTTGCAATCC TGCAGGCCGA CCCTACTCCG GCTGACCTAG
This study   .......... .......... .......... .......... ..........

BLV, CG      AACAACTTTG CCAATATATT GCTTCCCCGG TCGACCAAAC GGCCCATATG
This study   .......... .......... .......... .......... ..........

BLV, CG      ACCAGCCTAA CGGCAGCAAT AGCCGCCGCT GAAGCGGC-A ACACCCTCCA
This study   .......... .......... .......... .......... ..........

BLV, CG      GGGTTTTAAC CCCCAAAACG GGTACCCTAA CCCAACAATC AGCTCAGCCC
This study   .......... .......... ..-....... .......... ..........

BLV, CG      AACGCCGGGG ATCTTAGAAG TCAATATCAA AACCTCTGGC TTCAGGCC-G
This study   .......... .......... .......... .......... ..........

BLV, CG      GAAAAATCTC CCTACTCGTC CTTCAGCTAC AACCTTGGTC CACCATCGTC
This study   .......... .......... ......-... .......... ..........

BLV, CG      CAAGGCCCCG CCGAAAGCTC TGTAGAGTTT GTCAACCGGT TACAAATTTC
This study   .......... .........A .......... .......... ..........

BLV, CG      ATTAGCTGAC AACCTTCCCG ACGGAGTCCT AAGGAAC
This study   .......... .......... .......... .......
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Figure 2: Alignment of the partial nucleotide sequence of 385 bp amplicons obtained from a human with the reported sequence of the BLV reference (K02120; nt position 1068-1452 is shown). Primer positions are shown in bold letters.

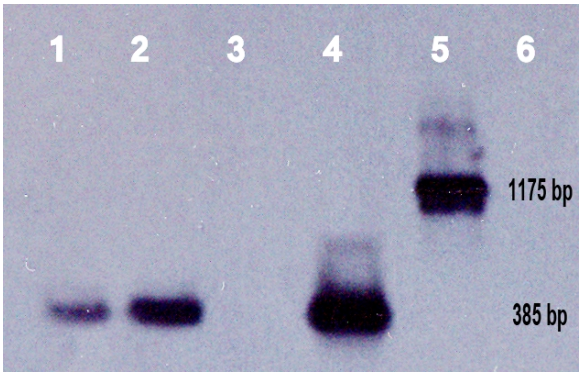

Figure 3. Southern blot analysis for PCR products. After nested PCR, the samples were analyzed by Southern blot hybridization with the *gag*-related probe as described in the text. Lanes 1-4: results of Southern blot hybridization against products of second round of amplification; lanes 1 and 2: PCR positive humans; lane 3: PCR negative human; lane 4: FLK-BLV; lane 5: BLV *gag* gene (PCR products of first round of amplification); and lane 6: blank PCR products.

gene of BLV. Southern blot hybridization against PCR products confirmed negative and positive results and was in accord with the first and second round of nested PCR. PCR products of first round (1176 bp) were also found in some human cases.

Discussion

BLV is the etiological agent of a chronic lymphatic leukemia in different species, such as naturally infected cows, but also experimentally infected sheep, goats and rabbits (Burny *et al*., 1985; Burny *et al*., 1988; Wyatt *et al*., 1989). Previous research has investigated whether BLV might be transmissible to humans. As demonstrated by a number of experiments, BLV infection does not appear to be the cause of any distinctive disease in humans (Burridge, 1981). Nevertheless, there is some evidence for human exposure to BLV infections. Serological studies have been performed to test serum samples from humans with cancer and from individuals with potential occupational exposure to BLV for evidence of BLV antibodies (Buehring *et al*., 2003; Onuma *et al*., 1987; Slavikova *et al*., 1987); antibodies to the virus in the sera of leukemia patients have been found (Cereda *et al*., 1984). Buehring *et al*. (2003) also stated that antibodies reactive with the BLV capsid antigen may serve as a biomarker for exposure to BLV and that this exposure may be widespread.

The detection of the BLV provirus DNA sequence by PCR is a sensitive method for a direct diagnosis of a BLV infection (Sherman *et al*., 1992). The majority of the PCR assays are based on a single assay. However, it has been reported that less than eight genome copies of the provirus could be detected in the background of 2 million negative lymphocytes using nested PCR (Ballagi-Pordany *et al*., 1992). Lee *et al*. (2005) have demonstrated that human cases were negative for a PCR based on the BLV envelope gene. For this reason we have utilized a simple gene amplification technique for detection of sequences from the major BLV gag gene. This study reports for the first time the use of the gag region for the detection of BLV in humans. The procedure resulted in good sensitivity and specificity, and viral copies were detected by nested PCR using primers specific for the BLV *gag* gene. Specificity of PCR products was further confirmed by sequencing and Southern blot hybridization assays. The present investigation revealed that the PCR amplicons obtained from human samples originate from the gag region of the BLV provirus DNA. Since proviruses may exist and circulate in body fluids as non-integrated elements, these findings do not necessarily prove that humans are actively infected with BLV. Nevertheless, in human cases more studies should be carried out to investigate the active BLV infection. This is not the first assay of human sera for the presence of antibodies to BLV, but to the best of our knowledge, this is the first report using detection of the BLV gag sequence in human samples. Our results underline the risk of human exposure to BLV, and the importance of further investigations to determine whether BLV infection is a human health hazard.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Mehdi Ghaffari, Iraj Ashrafi, and Mahmood Khormali. This research was supported by funds from the Veterinary Research Council of the University of Tehran

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