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Bovine Leukemia ProVirus: Evidence of Presence of Part of *Gag* Gene in Seminal Plasma of Naturally Infected Bulls

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Abstract

It is of critical importance to understand the modalities of BLV presence in semen, especially with regard to artificial insemination (AI). Presence of bovine leukemia provirus was demonstrated in fresh and frozen semen samples by researchers. In this study paired blood and semen samples from 45 bulls were assessed for the presence of part of *gag* gene and antibodies to BLV in blood, semen and cell-free fraction of the semen (seminal plasma). Proviral DNA was detected in 5 out of 45 seminal plasma samples. PCR products were sequenced and submitted to gene bank. This data strongly suggested that seminal plasma of seropositive bulls can be positive in PCR.
Keywords: Bovine leukosis provirus; Semen; Seminal plasma; *gag* gene; PCR.

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Introduction

Enzootic bovine leucosis (EBL) is a chronic lymph proliferative disorder in cattle caused by the exogenous retrovirus, bovine leukemia virus (BLV).[1]

The infection can result in three different clinical states, with many animals remaining asymptomatic in a leukemic state and acting as carriers. However, 20-30% of the cases develop a persistent lymphocytosis characterized by a polyclonal expansion of B-lymphocytes and a small percentage (1-5 %) of seropositive cattle develops malignant lymphoma, the fatal clinical form of BLV infection[2]. Transmission of BLV occurs essentially horizontally by direct exposure to biological fluid contaminated with infected lymphocytes, mainly blood. The major route of transmission is the iatrogenic through procedures permitting the transfer of blood between cattle. In some areas, where the density of hematophagous insect is important, tabanids play a considerable role in the spread of BLV under natural condition. Although viral antigens and proviral DNA has been identified in semen, milk and colostrums, natural transmission through these secretions has not been demonstrated.[3-6]

Cellular damages could force cells to shed free DNA into body fluids. Numerous studies have demonstrated tumor or viral specific sequences of DNA or RNA recovered from plasma or seminal fluid of patients with various diseases, a finding that has potential for molecular diagnosis and prognosis.[7-10]

Presence of bovine leukemia provirus was demonstrated in fresh and frozen semen samples by researchers. In first step they have tried to centrifuge and then extract genomic DNA from cell fraction of the semen samples.[11]

It is of critical importance to understand the modalities of BLV presence in semen, especially with regard to artificial insemination. In this study, paired blood and semen samples from 45 bulls were assessed for the presence of part of *gag* gene and antibodies to BLV in blood, semen and cell-free fraction of the semen. The objective of this study was to detection of BLV provirus in seminal plasma of bulls.

Materials and Methods

Samples.Semen samples (n = 45) were obtained from Iran's Artificial Insemination Centre, Tabriz, Iran. Semen samples were diluted according to standard procedures at the AI centre and sent to the laboratory in refrigerated boxes. Semen was collected from animals once a week for 10 weeks, and processed within 24 h of collection. Each of semen specimens were centrifuged at 12000 × *g* for 10 mins in a micro-centrifuge, and the pellets were harvested for further use. After semen collection, 45 blood samples with and without EDTA were taken from the caudal vein of the animals and immediately transported to the laboratory. Serum was removed after centrifugation at 1200 × *g* for 10 mins. Each serum was kept in micro tubes and stored at −20 °C until tested for antibody against BLV.

ELISA. All sera were tested for antibody to BLV (in Central Lab., Faculty of Vet. Med, University of Tabriz) using ELISA commercial kit (Pourquier Institute, France) according to the manufacturer's instructions.

DNA isolation. Genomic DNA from blood and semen samples was extracted according to Chomezynski extraction method. DNA concentration was measured in 260 and 280 nm (Biophotometer plus, eppendorf, Germany). Electrophoresis of each DNA sample on 2% agarose gel in 1X TBE buffer was undertaken to check the integrity of the DNA. An aliquot of total DNA was produced from each sample and stored at −20 °C until required for analysis.

PCR primers. Two different primes were used for the PCR as described by

Santos and colleagues. A BLV specific primer pair *gag 3* (aac act acg act tgc aat cc) and *gag 4* (ggt tcc tta gga ctc cgt cg) that anneals to *gag* region.[11] Furthermore, two primers *HL033* (5- CGA gtc ctt atg agc ttg att ctt -3) and *HL035* (5- gcc ttc cag aag tcg ttt gtt ttc -3) that target part of the bovine prolactin gene were considered as an internal control.

Polymerase chain reaction (PCR). All PCR reactions were performed in a 20 µl volume containing 1µl of sample containing 100 ng DNA, 0.4 µL of 0.2 mM dNTPs mix, 1.4 µl of 3.5 Mm MgSo4, 2 µl of PCR buffer, 0.12 µl of 0.6U of platinm Taq polymerase, 0.4 µl of 0.2 Mm of each primer. PCR was performed in a primus 96, MWGAG b701ECH thermocycler with the following conditions: initial denaturation at 95 ˚C for 5 mins, followed by 35 cycle at 94˚C for 1 min, 65 ˚C for 1 min and 72˚C for 2 min s, with a final extension of 72˚C for 10 min s. A not template control (water blank) and a positive control DNA from lymph node of cow with lymphosarcoma (were mixed with extracted DNA from semen) included in each reaction. Amplificated products were analyzed by electrophoresis through a 2% agarose gel for the specific BLV PCR.

Sequencing of the PCR products. Positive PCR amplicons were sequenced from both ends. DNA sequencing was carried out using the dideoxy chain termination procedure (Chemistry V3.1, Applied Biosystems) and the 3730XL DNA analyzer (Applied Biosystems) by MilleGen sequencing service Labège, France). The DNA sequence databases were searched using the nucleotide-nucleotide BLAST (BLASTn) at the National Center for Biotechnology Information, USA. Comparison of the different *gag* fragments with each other and with previously reported sequences were done using Algin two sequence (bl2seq)(http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) at the National Center for Biotechnology Information,USA (http/www.ncbi.nlm.nih.gov/BLAST/).

Results

Of 45 bulls, blood, semen and seminal fluid of six bulls were positive in PCR, the same animals had ELISA positive results except one of them. Products of ~ 370 bp were amplified from semen, seminal plasma and blood of five seropositive and one seronegative bulls, using primer pair *gag* 3 and *gag* 4 (Fig 1).

The size of the bands matched the positive control. The PCR products were sequenced from both ends by *gag* 3 and 4. When the sequence results were subjected to BLAST search, the best score of 99% homology was returned with bovine leukemia virus (EF600696, K02120, AY277948, FJ914764, AF257515) in listed order. The nucleotide sequences of all samples were identical when compared and some of them can be viewed in Gene Bank with gene accession numbers: GQ491115, GQ491116, GQ 491117, GQ491118, GQ491119.

Amplification of the *prolactin* gene with primer pair (*HL033* and *HL035*) gave product of ~ 156 bp in all blood, semen and seminal plasma samples.

Discussion

The introduction of AI has changed the epidemiology of animal virus diseases. The utilization of AI techniques prevents the natural transmission of virus by direct contact between males and females, allowing the control of many diseases. Nevertheless, semen contaminated with viral agents may cause an enormous spread of certain diseases, since it could infect numerous farms, areas, or even countries in a short period of time.[12] Besides, affecting production efficiency, the sanitary and economic impact of BLV infection is associated with the interference in the international movement

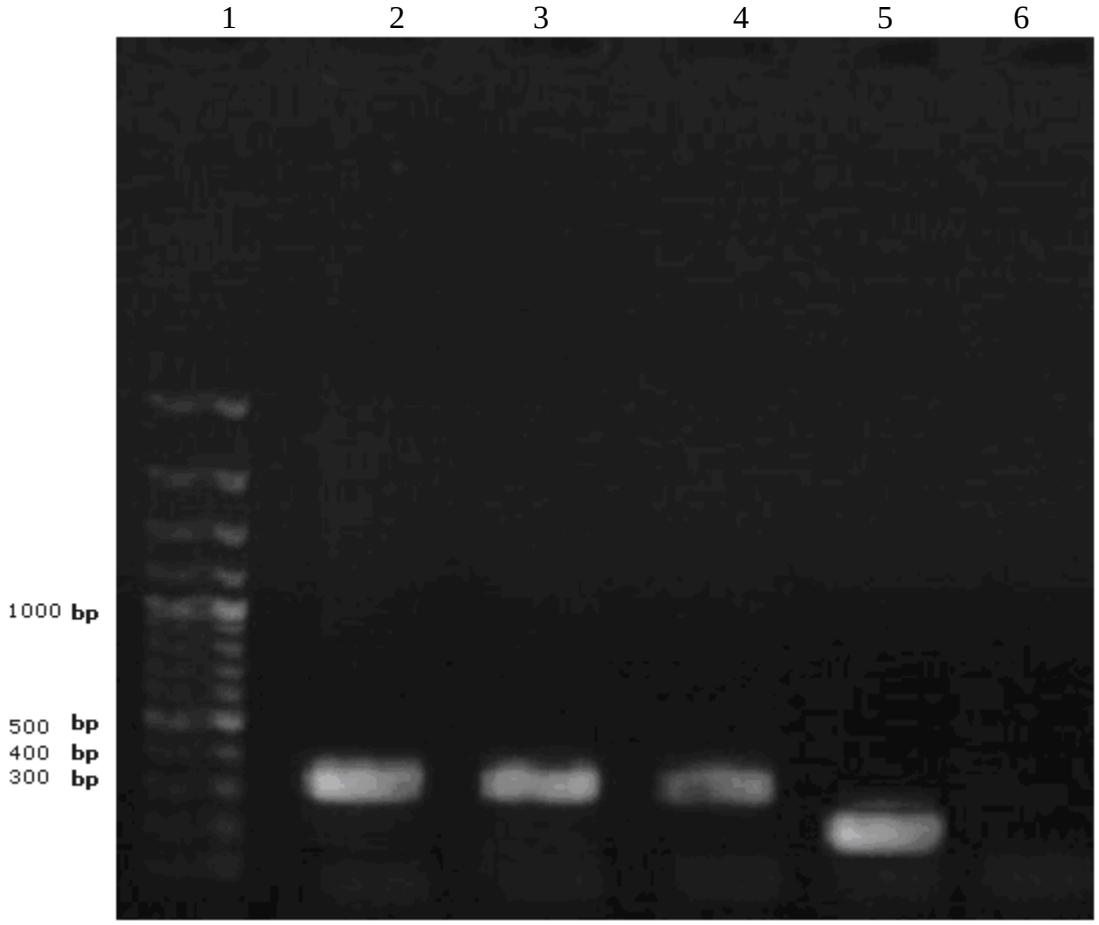

Fig 1. PCR products of *gag* and *prolactin* genes. Lane 1 kb+ ladder, Lane 2; 3 and 4: products of *gag* gene from positive control, semen and seminal plasma samples of a seropositive bull, Lane 5: products of *prolactin* gene from seminal plasma samples of the same bull, Lane 6: negative control.

of cattle and their germ plasm, due to sanitary restriction imposed by countries that have worked in control and eradication of EBL. Utilization of molecular biology techniques, specifically PCR, to detect the presence of BLV provirus in semen should facilitate certification of germ plasm as being free of contaminating virus.

This study was conducted to establish the use of PCR amplification in detecting BLV in semen plasma. All bulls in the study were serologically tested for the presence of BLV antibody using a commercial ELISA.

ELISA revealed that five out of 45 bulls had antibodies against BLV, in the meantime all of them had PCR positive reactions in blood, semen and seminal plasma samples. Although ELISA was not able to detect antibodies against BLV in one bull, the PCR showed the presence of intended sequence in blood, semen and seminal fluid of the same bull. We think this might be due to the low sensitivity of the ELISA as it was found as negative by this test indicating the possibility that this animal is seropositive but has a low antibody concentration and was misclassified as seronegative.

The recent discovery that cell-free DNA can be shed into the body fluids as a result of cell death has generated great interest. Presence of pathogens in cell-free fluid of bovine semen has been shown before, Rocha and colleagues could detect Bovine herpesvirus in cell-free seminal fluid by a nested PCR.[13]

But in case of bovine leukosis, previous studies have shown that in seropositives

bulls, the semen might contain Bovine Leukemia Provirus. As far as we found out all the studies were designed to investigate the presence of the provirus in cellular fraction of the samples.[6,11] In this regard the sperm and associated cells of fresh or extended semen samples have been concentrated by centrifugation. Accidentally we understood that seminal plasma of a positive sample was also contained the targeted part of the *gag* gene. Repeated tests showed that seminal plasma of the seropositive bulls contains the some part of the provirus.

In first step it was considered that some sperm cells might remain in the cell free fluid because of the carry over due to mistreatment of the samples or inadequate *g* force, but the cells never observed under light microscopy. Based on these observations it was concluded that the cell free fraction of PCR positive semen might contain provirus or part of that, by own. The presence of BLV in serum of infected animal was demonstrated by Poon and colleagues.[7] Although many papers reported that pathogens or specific sequences of the genome are track able in body fluids by PCR,[8-10] to our knowledge this is the first report that demonstrate the presence of part of *gag* gene in cell-free fraction of the semen of naturally infected bulls.

The results of other studies suggest that cell-free DNA originates from apoptotic or necrotic cells; and levels of them are elevated in acute disorders such as trauma, suggesting a marker of clinical severity.[14] Although at this moment we do not have any reason or hypothesis for the origin of the DNA in seminal plasma, we are planning to check it out with more primers for different house keeping genes and by amplification a large sequence of the Bovine Leukemia Provirus.

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