

Molecular Detection of *Bovine leukemia virus* (BLV) in the Semen Samples of Bulls

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Abstract: *Bovine leukemia virus* (BLV) is a causative agent of bovine leukosis and responsible for lymphoproliferative disorders in cattle. This virus identified in reproductive tissues and fluids such as fresh and frozen semen samples, embryos and uterine fluids. The objective of the present study was to detect BLV DNA in semen samples of bulls used for artificial insemination in Iran. Semen samples were obtained from 172 bulls and DNA was extracted and *gag* region of virus were amplified by two sets of oligonucleotide primers using nested-PCR technique and amplified products were visualized in a 1% agarose gel electrophoresis. Viral DNA was detected in 36 of the 172 (20.93%) semen samples of bulls that used for artificial insemination in Iran. The results of this study demonstrate the high presence of BLV DNA in bull's semen samples. According to these findings it is clear that test, segregate and cull programs can be effective to control and prevention of bovine leukosis disease and furthermore, examination of bull's semen samples that used for artificial insemination in cattle it seems to be necessary.

Key words: *Bovine leukemia virus* (BLV) • Bulls • Semen • Artificial insemination • Nested-PCR • Iran

Abbreviations: BLV • *Bovine leukemia virus*

INTRODUCTION

Bovine leukosis is a disease caused by the *bovine leukemia virus* (BLV). This virus is an oncogenic *retrovirus* of the BLV- HTLV group that causes lymphosarcoma in a proportion of infected individuals and can kill cattle and reduce overall productivity of a beef herd, even when there are no obvious signs of infection [1]. The genome structure, nucleotide sequence of the *provirus* and amino acid sequence of the structural and nonstructural proteins are very similar to that of human T cell lymphotropic virus (HTLV-1 and HTLV-2) [2]. Both BLV and HTLV-1 have a stable genome, distinctly unlike the more notable human *retrovirus*, HIV. Both viruses lack a chronic viremia, have a long latent period and lack preferred sites of proviral integration [3, 4]. Both BLV and HTLV-1 escape the immune response by low levels of viral replication. In BLV it appears that replication is blocked at the transcriptional level, but the mechanism has not been completely elucidated [5-7]. There are 4 main genes that encode for the virion proteins present in all *retroviruses*. They are 5'- *gag*,

pro, *pol*, *env*-3'. Some families may have additional genes encoding additional proteins required for viral function [1].

The infection with BLV can result in 3 different clinical states, with many animals remaining asymptomatic in an aleukemic state (AL) and acting as carriers. However, 20-30% of the cases develop a persistent lymphocytosis (PL) 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 [8]. PL is considered to be a benign condition associated with BLV infection. For this reason it is often overlooked. However, these cows may serve as a greater reservoir of infection on a farm. The increased lymphocyte count is attributed to a 45-fold increase of infected CD5⁺ and a 99-fold increase in infected CD5⁺ B cells [9]. In addition it has been suggested that cows with PL may be a greater risk for passing BLV infection on to their calves' in utero and may suffer from decreased milk production and alteration of milk components [10]. The development of lymphosarcoma occurs in 1-5% of

BLV-infected cattle. Clinical signs associated with the development of lymphosarcoma are highly variable, as the affected organ will dictate the predominant clinical signs. Animals with BLV associated lymphosarcoma will commonly show lesions in the central or peripheral lymph nodes leading to lymphadenopathy [10].

Cattle are infected with BLV through the transfer of blood and blood products that contain infected lymphocytes. Reproductive tissues and fluids such as semen, embryos and uterine fluids also have been evaluated for the presence of BLV *provirus* and their ability to transmit the virus [5]. This oncogenic virus is transmitted from infected cattle to other cattle via blood-to-blood transfer. In cattle infected with BLV only 1 of every 25,000 - 50,000 peripheral blood lymphocytes expresses viral proteins or has viral mRNA present in the cell cytoplasm [9]. Many common farm practices have been implicated in viral transmission including tattooing, rectal palpation, dehorning, injections and blood collection. Furthermore, blood sucking insects have been considered a risk for the spread of BLV infection in cattle. Vertical transmission may occur transplacentally from an infected dam to her fetus, intrapartum by contact with infected blood, or postpartum from the dam to the calf through the ingestion of infected colostrums [8].

Serologic methods for diagnosis of BLV infection, such as agar gel immunodiffusion test (AGID) and ELISA are commonly used to identify infected animals. These tests are sensitive (ELISA, 0.98; AGID, 0.946 to 0.985) and specific (ELISA, 1.0; AGID, 0.964 to 0.998) for detection of infection [3]. Despite the availability of tests that are both sensitive and specific for BLV, diagnosing infections in neonates and periparturient cows' remains problematic [11]. Passive transfer of colostral immunoglobulin from BLV-positive cows to their offspring may cause false-positive serologic test results, which persist for the first 6 months of life [12]. Consequently, testing by serologic methods is not reliable in post-colostral neonatal calves, severely limiting our ability to cull infected calves prior to substantial financial investment in those animals. Furthermore, relying on serologic tests to determine BLV status of recently purchased cattle is also problematic. Seroconversion may not develop for as long as 90 days following experimental viral exposure [11]. The inability to detect BLV in recently exposed cattle forces prolonged isolation of recently purchased cattle to assure that BLV-positive animals are not introduced to the herd. Most farms lack the facilities and the commitment to impose on-farm quarantine periods of long duration [3].

Molecular study such as polymerase chain reaction (PCR) assays may eliminate these concerns, because PCR can detect BLV provirus despite the presence of maternal antibody. PCR assays detect small quantities of virus, potentially leading to fewer false negative tests in recently exposed animals [13].

Previous studies by numerous investigators suggested that semen from BLV-infected bulls was noninfectious for recipient cows. But, in recent years BLV was demonstrated in fresh and frozen semen samples by researchers and artificial insemination is an important ways for transmission of this virus in cattle [14]. The purpose of the present study was to detection of *bovine leukemia virus* (BLV) DNA in bull's semen samples used for artificial insemination in Iran.

MATERIALS AND METHODS

Semen Sampling and DNA Isolation: A total of 172 bull's semen samples were obtained during July to December 2010, being careful of avoiding contamination with bacteria present in the prepuce. Prior to taking the samples, the prepuce was washed with a 1% benzalkonium chloride solution drying with sterile cotton. Semen samples were diluted according to standard procedures and sent to the laboratory in refrigerated boxes. Each of semen specimens was stored at -70°C for further use.

Genomic DNA was extracted from semen specimens using DNA extraction kit (Bioneer, Daejeon, Korea) according to the manufacturer's recommendations. The total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell [15]. The extracted DNA of each sample was kept frozen at -20°C until used.

Gene Amplification: To detect BLV DNA, nested-PCR was performed using the DNAs extracted from semen samples. Two sets of oligonucleotide primer pairs were used for amplification. The external primers specific to the BLV region was used as described by Wang *et al.* [16]. The sequence of primers for first round was BLV-F1: 5'-ATGGGAAATTCCCCCTCCTAT-3' and BLV-R1: 5'-GTTTTTTTGATTTGAGGGTTGG-3' [16]. The second amplification round was performed to amplify a 385 base pair (bp) fragment, using a pair of inner primers of the *gag* region (BLV-F2: 5'-AACACTACGACTTGCAATCC-3' and BLV-R2: 5'-GTTCCCTTAGGACTCCGTCG-3') in the BLV genome

(GenBank accession number K02120). The FLK-BLV DNA served as a positive control for amplification (FLK-BLV stands for fetal lamb kidney cells infected with *bovine leukemia virus*).

Two sets of nested-PCR program was carried out in 25 µl total reaction volumes, each containing 100 ng of template DNA, 0.2 pM of each primer, 2.5 µl of 10X PCR buffer, 1.5 mM MgCl₂, 200 mM dNTPs and 1 unit of *Taq* DNA polymerase (Fermentas, Germany). The amplification reaction consisted of 5 min of pre-denaturing at 94°C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min extension 72°C and then by a final extension at 72°C for 5 min. Two µl from the first round amplicon was used as a template for the second round PCR. The specimens were amplified in a Gradient Palm Cycler (Corbett Research, Australia). The second round PCR was performed with inner oligonucleotide primers for 25 cycles with the same concentration of reagents and temperatures condition.

Analysis of PCR Products: The amplified products were detected in 1% agarose gel electrophoresis. The electrode buffer was TBE (Tris-base 10.8 g 89 mM Boric acid 5.5 g 2 mM EDTA (pH 8.0) 4 ml of 0.5 M EDTA (pH 8.0), combine all components in sufficient H₂O and stir to dissolve). Gels were stained with ethidium bromide.

Aliquots of 10 µl of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. After electrophoresis, images were obtained in UVIdoc gel documentation systems (UK).

RESULTS

In present study, 172 bull's semen samples were examined for the presence of *bovine leukemia virus* (BLV) DNA. Nested-PCR specimens producing a band of the expected size (385 bp) were considered positive (Fig. 1). Viral DNA was detected in 36 of the 172 (20.93%) semen samples of bulls that used for artificial insemination in Iran. The positive control (FLK-BLV DNA) showed the expected amplification product specific for BLV (385 bp). The size of the bands matched the positive control.

The results showed a high presence of BLV DNA in bull's semen specimens that were used for artificial insemination. These results indicated that this viral infection is an important agent to decreased milk production, alteration of milk components, increased veterinary costs and mortality of calves and increased culling rate of infected adults in cattle of our studied region. These findings suggested that control and eradication programs for BLV infection it seems to be necessary in Iranian cattle.

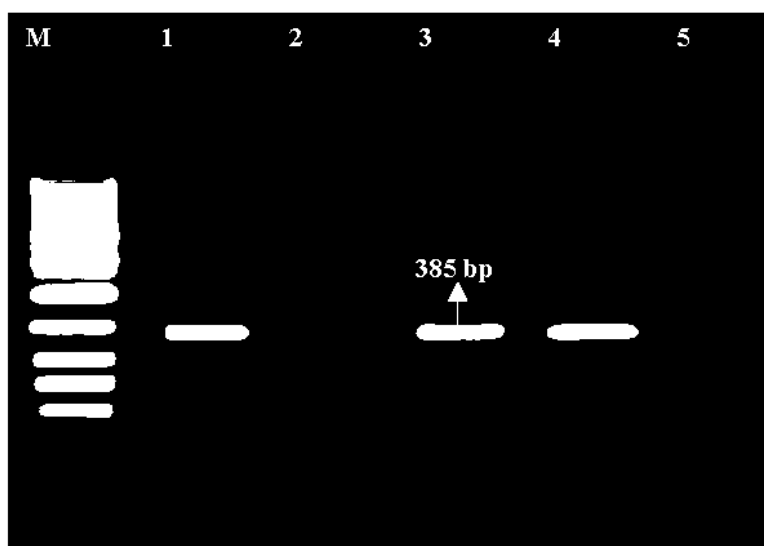


Fig. 1: Ethidium bromide-stained agarose gel electrophoresis of PCR products (385 bp) for detection of BLV DNA in bull's semen samples after nested-PCR amplification (Line M is 100 bp DNA ladder (Fermentas, Germany), line 1 is positive control (FLK-BLV DNA), line 2 is negative control (Blank), lines 3-4 are positive samples for BLV DNA and line 5 is negative sample)

DISCUSSION

BLV is a member of the family *Retroviridae*, genus *Deltaretrovirus*, together with human T-lymphotropic virus (HTLV) types 1 and 2 and a causative agent of bovine leukosis. This virus is the causative agent of bovine leukosis and a highly fatal form of cancer in cattle [10]. BLV is transmitted through white blood cells and very small amounts of blood can transfer the virus [17]. Nasal secretions, bronchoalveolar lavage fluid, semen and saliva have been evaluated for the presence of *provirus* and their ability to transmit BLV [12]. Several routine management practices such as dehorning, giving injections without changing needles, ear tagging and performing multiple rectal examinations with the same obstetrical sleeve can transmit BLV. The majority of BLV transmission is horizontal. Close contact between BLV negative and BLV positive cattle has been thought to be a risk factor [16].

Economic losses associated with BLV included decreased production, increased veterinary costs and increased culling rate of infected adults. More significant losses are experienced by purebred herds which the loss of domestic and foreign seedstock sales may constitute an important economic burden. Many countries, particularly those in the European Economic Community, will not accept cattle, semen, or embryos from positive individuals or herds [14].

The detection of the BLV in bull's semen is important and PCR is a sensitive method for a direct diagnosis of a BLV infection. The majority of the PCR assay is based on sensitivity and highly quantitative of this technique for detection of viral DNA such as BLV against other diagnosis method [18]. In current study the procedure resulted in good sensitivity and specificity and viral copies were detected by nested-PCR using primers specific for the BLV *gag* gene. The numbers of positive samples for BLV DNA in present study were 36 of the 172 (20.93%) bull's semen samples that used for artificial insemination.

Many studies were performed about BLV infection in semen of bulls and described its correlation with decreased production and increased veterinary costs in cattle. One epidemiologic study found natural service using BLV infected bulls to be a risk factor for transmission [19]. Another demonstrated the infectivity of semen from one BLV infected bull [20]. In another study BLV status of the sire was found to have no effect

on the BLV status of the progeny using either natural service or artificial insemination [21]. This is supported by a study that evaluated the risk of artificial insemination on a dairy herd. No increase in transmission could be attributed to the use of artificial insemination [22]. In 1975 a survey involving 4,394 dairy cattle in 100 herds and 2,794 beef cattle in 50 herds found BLV infection present in 66% of the dairy and 14% of beef herds. Overall, 10.2% of the dairy cattle and 1.2% of the beef cattle tested were positive [23]. The United States is endemic with BLV. A 1996 survey estimated that 89% of US dairy herds were endemically infected with BLV and greater than 40% of all US dairy cattle were infected. Although BLV is less prevalent in beef cattle, a 1997 survey of US beef cattle estimated that 38% of US beef herds and 10% of US beef cattle are infected with BLV [24]. Many states have voluntary control programs in place, but no mandatory or federal programs currently exist [25]. This is dramatically different from many other industrialized, cattle producing countries that have government controlled, mandatory control and eradication programs in place. Many of these countries in Europe, Australia and New Zealand have dropped the prevalence of viral infection to negligible levels [12].

The seroprevalence of BLV in Argentinean dairy cattle in year 2001 was 32.85% of infected individuals while the percentage of infected herds, harboring one or more infected animals, was of 84%. These results indicated a medium level of seropositive animals when taken individually, but a high prevalence of infected farms, which has been notoriously increased in the last 15 years as shown when compared with previous data from particular geographic areas, indicating that BLV constitutes a serious sanitary problem for dairy producers in Argentina [26]. The study of Nikbakht *et al.* in Iran showed that prevalence of anti-BLV antibodies in human and cattle samples were 12.50% and 16.73%, respectively [25]. Another research by Jafari and Asadpour on evidence of presence of part of *gag* gene in seminal plasma of naturally infected bulls in Iran showed that proviral DNA in 5 out of 45 (11.11%) seminal plasma samples [13].

Based on this information, examination of bull's semen samples that used for artificial insemination to control and prevention of bovine leukosis infection in cattle it seems to be necessary. Furthermore, it is clear that test, segregate and cull programs can be effective, even in high prevalence of BLV in bulls.

However, the lack of indemnity, the high prevalence of nation wide infection and the lack of mandatory control programs, cripples BLV control at the level of the farm. Disease control and eradication requires accurate, definitive tests for detection of BLV in Iranian bull's semen samples that are able to identify infected cattle early in the course of infection.

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