

Identification of bovine leukemia virus in raw milk samples in North-West of Iran

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Abstract

Bovine leukemia virus (BLV) is one of the most important carcinogenic viruses genetically related to the human T-cell lymphotropic viruses (HTLV-1 and HTLV-2). The virus infects type B lymphocytes and creates lymph glands tumors. Recently, the association between the presence of this virus and breast cancer has been addressed in humans. Here, we studied the prevalence of BLV in the samples of raw milk of native Iranian and Iranian-foreign cows in traditional, semi-industrial and industrial dairy farms in rural and urban areas of Zanjan province. Raw milk samples of cows were collected manually in sterile tubes. The samples were tested by nested-PCR method. Forty samples (9.93%) out of 403 samples showed BLV contamination. In this study, nested-PCR was successfully applied to determine the level of contamination in raw milk samples from cows infected with BLV. Furthermore, a relatively high rate of BLV infection was found in dairy cows in Zanjan province, northwestern of Iran.

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Introduction

Retroviridae, a member of the large family of enveloped viruses with 80 to 100 nm in diameter, bears a complex structure and an unusual enzyme called reverse transcriptase. The family has a unique diploid genome consisting of two strands of a single-stranded positive-sense RNA of 7 to 10 kb. BLV is a member of genus *Delta retrovirus*, belonging to Retroviridae family. The virus is one of the most important tumor causing virus that infects type B lymphocytes of cattle ¹⁻³ and causes lymph glands tumors.⁴⁻⁶ BLV is genetically associated with human T-cell lymphotropic viruses (HTLV-1 and HTLV-2).^{7,8}

The BLV is highly dependent on the cell and remains within a group of B-lymphocytes that are reproduced as a result of the infection. When an animal is infected, the infection remains in the infected host's chromosomes for a lifetime.⁹ The virus can be transmitted experimentally to different species of animals such as sheep, goats, pigs, rabbits, rhesus macaque monkeys, chimpanzees, and buffalo. A few weeks after the infection, the livestock's

body begins to secrete an antibody against it.^{4, 10} BLV genome has 8714 bp. The ends of the two sides of the genome have multiple repetitive sequences and three consecutive zones called U3, R, and U5.¹¹

Milk and colostrum of infected animals contain BLV-infected cells which are one of the main ways of transmitting the animal retroviruses to the next generation. Early detection and isolation of infected livestock from healthy livestock in the herd is the only way to prevent it from being transmitted to other animals.^{10,12} It is estimated that swallowing only 2,000 BLV-infected cells causes infection in the new host which finally leads to death in 1.00-5.00% of the infected animals via causing severe lymphocytosis as well as the lymphatic cancer.¹³

Most people drink cow's milk a lot during their lives. Raw milk of healthy cows contains some microorganisms. According to the Food and Drug Administration (FDA), the milk of the cows may have some pathogenic microorganisms such as enterotoxygenic *Staphylococcus aureus*, *Salmonella*, *Campylobacter jejuni*, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Brucella* species and some

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viruses such as BLV, and may be transmitted to humans by consumption of contaminated milk.¹⁴ According to a study, it was found that 74.00% of the human subjects had antibodies against BLV virus in their blood. Therefore, there are concerns about the probable relationship between the use of non-pasteurized milk and the development of cancer in the human body, especially in relation to BLV.^{14,15}

Cancer is a major public health problem in the world and the second leading cause of death in the United States.¹⁶ Breast cancer has had the highest rate among women's cancers worldwide and the highest mortality rate in 2012.^{17,18} About 1.70 million new cases, with 521,900 deaths have been reported in 2012 all around the world. Breast cancer alone accounts for 25.00% of all women's cancers and 15.00% of women's deaths.¹⁹ In Asia, 27.20% of new cancers and 19.00% of deaths associated with various cancers are due to women's breast cancer. Reportedly, the incidence of the disease in Iranian women is rising and they are at risk of the disease at least 10 years earlier than developed countries.¹⁹

In Australia, one of the countries with a high incidence of breast cancer, a significant relationship was found between the presence of BLV and breast cancer in women. Also, in the United States, DNA of BLV virus was found in the breast tissue of women that was correlated with breast cancer.²⁰ On the other hand, global reports show a significant geographical correlation between the incidence of breast cancer and milk consumption, so raw milk consumption may be one of the transmission routes of the virus to humans.²⁰

Previous studies in Iran have often been conducted on blood samples from cattle. Considering the importance of the issue in terms of food safety as well as public health, in the present study, we studied the prevalence of BLV virus using nested-PCR method in raw milk samples in the northwest of the country.

Materials and Methods

Sampling and Sample processing. In this study, 419 raw milk samples were collected from different breeds of dairy cows at various geographical locations of Zanjan province during July and Aug, 2017. The farms were selected from north, south, east, west, north-east, and north-west parts of the province. The sampling was done from different types of husbandry including traditional, semi-industrial, and industrial farms. The breed of the animals was determined by a veterinarian. The samples were taken manually and collected in 50 mL sterile tubes. Milk sampling from collection tanks as well as milking machines was avoided due to the possibility of cross-contamination.

The collected samples were immediately placed at 4.00 to 8.00 °C and transferred to the laboratory of the Department of Microbiology, Zanjan School of Medical. Then, the samples were centrifuged using a refrigerated centrifuge

(3,200 *g* per 30 min).⁵ Among the three phases formed, the upper phase contained milk fat, the middle phase had water or the supernatant of milk, and the lower phase had somatic cells deposition. The upper and middle phases were discarded, and 1.00 mL of somatic cell deposition was collected in 1.50 mL sterile micro-tubes and stored for further evaluation at - 20.00 °C.⁵

DNA extraction. After melting the samples at room temperature, using DNA extraction kit (GeneAll exgene, Seoul, South Korea), DNA was extracted based on the kit protocol with brief modifications. Regarding that BLV virus belongs to retrovirus family, and also presenting the reverse transcriptase enzyme, there was no need to make cDNA. Genomic DNA was extracted and stored at - 20.00 °C.²⁴

Positive control. A blood sample of infected cow with clinical symptoms was used as a positive control. Also, the infection was confirmed using ELISA test kit (IDEXX Laboratories, Westbrook, USA). DNA of the sample was included in the molecular analysis as a positive control.

Nested-PCR amplification of partial gag gene. The method was applied in the present study due to the low number of the virus in milk samples as well as high sensitivity and specificity of nested-PCR method. Positive and negative controls, containing all PCR materials other than template DNA, were included in each round of the test. The primary PCR was performed to amplify a fragment of gag gene using outer primers, described previously,² including Forward: 5'-ATGGGAAATTCCTCTAT-3' and Reverse: 5'-GTTTTTGTATTTGAGGGTTGG-3'. Each PCR reaction was done in a final volume of 20.00 µL containing 10.00 µL of master mix (2x), 0.40 mM of each of outer specific primers, and 3.00 µL of template DNA. The cyclical conditions were as follow: Initial denaturation at 94.00 °C for 3 min, 30 cycles of 94.00 °C for 60 sec, 55.00 °C for 60 sec, and 72.00 °C for 120 sec, and a final extension at 72.00 °C cycle for 10 min. The product size of the primary PCR was 1175 bp. The products of the primary PCR, several unknown samples and positive and negative control samples, were visualized at 1.50% agarose gel. The secondary PCR was performed using inner primers, described previously,² including Forward: 5'-AACACTACG ACTTGCAATCC-3' and Reverse: 5'-GTTCCTTAGGACTCCGT CG-3'. The secondary PCR was also done in a final volume of 20.00 µL containing 10.00 µL of master mix (2x), 0.40 mM of each of inner specific primers, 3.00 µL of the template from the primary PCR product. The secondary step conditions were as follow: Initial denaturation at 94.00 °C for 3 min, 30 cycles of 94.00 °C for 45 sec, 59.00 °C for 45 sec, and 72.00 °C for 40 sec, followed by a cycle at 72.00 °C for 5 min. The product size of the secondary PCR was 384 bp and visualized at 1.50% agarose gel.

Sequencing and phylogenetic analysis. Two PCR products, a positive sample and positive control, were sequenced after the clean-up at both strands using an automatic sequencer (ABI 3500; Pishgam Biotech Co.,

Tehran, Iran). Pair-wise alignment of both DNA strands, chromatograms evaluation and sequences editing was performed by Sequencher software (version 5.4.6; Gene Codes Corp., Ann Arbor, USA). Then, the edited sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) program of the US National Center for Biotechnology Information (NCBI). Phylogenetic analysis was performed using maximum likelihood, maximum parsimony and minimum evolution methods of Molecular Evolutionary Genetics Analysis Software (MEGA 7.0; Biodesign Institute, Tempe, USA) based on gag gene.

Statistical analysis. The data were analyzed using SPSS software (version 16.0; IBM Corp., Armonk, USA). Chi-square test was used to determine the relationship between nominal qualitative variables.

Results

In the present study, among 419 raw milk samples, 403 (96.20%) samples were included in the study. Sixteen samples for a variety of reasons such as low sample volume, contamination with animal feces during sampling, and mixing with other samples were excluded from the experiment process. Out of 403 raw milk samples, 40 (9.93%) samples showed expected PCR product (Fig. 1). Based on dairy type, most positive cases were observed in semi-industrial dairy farms (23.49%) and the least prevalence was detected in the traditional farms (0.65%). There was a significant relationship between the type of husbandry and prevalence of BLV infection ($p < 0.001$), (Table 1). On the other hand, the highest rate of infection was diagnosed in hybrid Sarabi cows (21.43%) followed by hybrid Holstein cows (13.09%). The infection was only found in one native cow (0.88%). The findings revealed that breeds had a significant effect on the infection rate

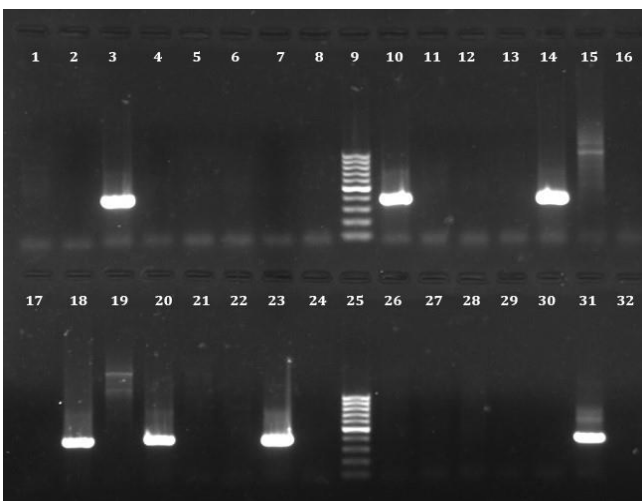


Fig. 1. Gel electrophoresis of the secondary PCR of some samples. Lanes 3, 10, 14, 18, 20 and 23: positive for BLV, lanes 9 and 25: DNA ladder, lane 31: positive control, lane 32: negative control. Other lanes are negative samples.

($p < 0.001$), (Table 1). Also, most positive cases were detected from West of Zanjan province (47.62%). Geographical distribution of BLV infection was statistically significant ($p < 0.001$), (Table 1).

Table 1. Prevalence of BLV infection based on husbandry type, breed and its geographical distribution in Zanjan province.

Parameters	Sample size	BLV + (%)	BLV - (%)
Husbandry type			
Traditional	153	1 (0.65)	152 (99.35)
Industrial	101	4 (3.96)	97 (96.04)
Semi-industrial	149	35 (23.49)	114 (76.51)
Breed			
Native	113	1 (0.88)	112 (99.12)
Hybrid Holstein	275	36 (13.09)	239 (86.91)
Hybrid Sarabi	14	3 (21.43)	11 (78.57)
Simmental	1	-	1 (100)
Sampling region			
North	132	1 (0.76)	131 (99.24)
South	58	7 (12.07)	51 (87.93)
East	67	1 (1.49)	66 (98.51)
West	21	10 (47.62)	11 (52.38)
North east	64	10 (15.63)	54 (84.37)
North west	61	11 (18.03)	50 (81.97)
Total	403	40 (9.93)	363 (90.07)

Phylogenetic analysis was conducted using maximum likelihood, maximum parsimony, and minimum evolution methods, topology of the phylogenetic tree of which was similar (Fig. 2). The sequencing of the two samples showed that these sequences corresponded to 99.00% homology with the sequences recorded in the GenBank® belonging to the gag gene of BLV. The sequences of the products, a positive sample and the positive control, are shown in Figure 3. The two sequences were deposited in the GenBank® under the accession numbers MN410793-MN410794.

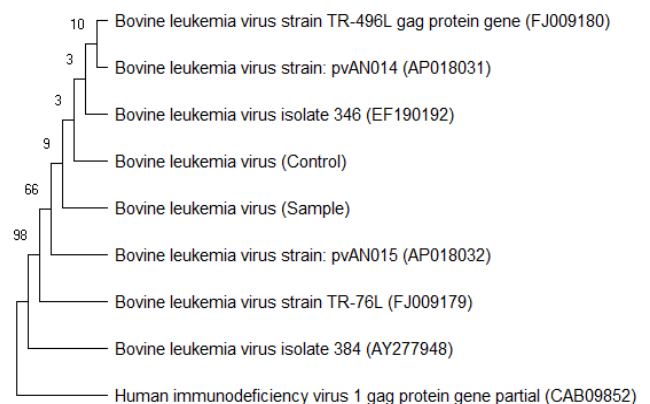


Fig. 2. Molecular phylogenetic analysis by maximum likelihood method using sequence of gag protein gene. Numbers at the nodes represent bootstrap values based on 1000 replicates. BLV (Control): Sequence of gag protein gene of control isolate confirmed by ELISA test. BLV (Sample): Sequence of gag protein gene of a positive raw milk isolate. Human immunodeficiency virus 1 gag protein includes as outgroup.

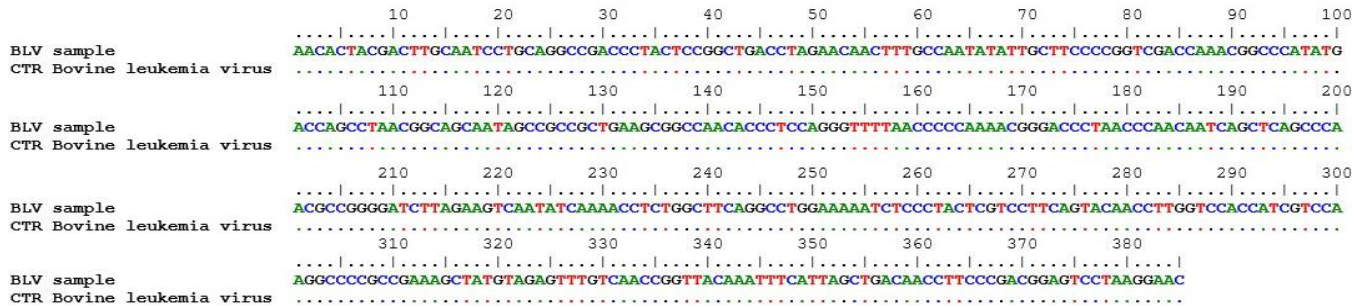


Fig. 3. The sequences of gag gene of the isolated BLV virus (BLV sample; sequence of the milk isolate, CTR Bovine leukemia virus; sequence of the positive control).

Discussion

In this study a relatively high rate of BLV infection was found in dairy cows in Zanjan province, northwestern of Iran. Moreover, nested-PCR method was successfully applied for the detection of the virus in raw milk samples.

Bovine leukemia virus is one of the most important carcinogenic viruses that infects B lymphocytes,¹ and commonly causes lymph glands tumors in cattle. Also, carcinogenic viruses contribute to the development of several types of cancer in human beings including liver cancer, cervical cancer, viral lymphatic leukemia, Burkitt lymphoma, and Kaposi's sarcoma.^{10,15,21} The only virus known to cause breast cancer in animals is mouse mammary tumor virus (MMTV) that causes breast cancer in mice. It is known that this type of virus is transmitted by the mother to the infant.^{15,21}

According to previous studies, the infection of the BLV is seen in most countries of the world. Due to the latent nature of the disease in most cases, absence of significant clinical symptoms in initial clinical cases, and the complexity of laboratory diagnosis, the disease is relatively widespread in the cow's breeding industrial systems in most countries of the world. In general, there are primary reports in each country based on serology tests. According to the surveys in Iran, the disease is present in most parts of the country.^{22,23}

In a previous study, a relationship was found between breast cancer and BLV infection. In a study by Buehring *et al.*, it was found that one of the risk factors for breast cancer in women is the accumulation of BLV transmitted by livestock through the consumption of contaminated food, in the breast tissue of women.²¹ In the study, the prevalence of the virus in breast tissue cells of women with breast cancer was 59.00% and the prevalence in women without the disease was 29.00%. In a case-control study conducted by Giovanna *et al.* in Columbia, 53 tissue samples of breast cancer patients aged 18-80 years old and 53 healthy subjects, as the control group, were studied for the presence of BLV.²⁴ In the study, 43 samples (40.50%) of the total of 106 samples were positive for the presence of BLV gene, 19 cases in the patient group and 24

cases in the control group. It seems that the reason for the presence of the virus in the body of the control group, women without disease, is the virus ability to stay in the latent phase in the host's body.²⁴ The results of the studies determine the importance of considering the virus in terms of public health. The study conducted by Lee *et al.* in Korea on the relationship between the presence of the virus in the people involved in the production of meat and meat products and the incidence of lung cancer showed no relationship between them.²⁵

Some studies conducted in Iran have reported the prevalence of BLV in herds in some of the geographical areas of the country.^{22,23} Nekoei *et al.* reported that BLV was detected in 22.10% of blood samples of cattle from Isfahan and Chaharmahal and Bakhtiari provinces using nested-PCR method.²² In another study, BLV infection was detected in 25.40% of cattle from northeast of Iran by ELISA method.²³ Here, using nested-PCR method as the most sensitive method, 9.93% of cows were infected with BLV in Zanjan province. However, only raw milk samples were studied here. It seems that further studies on other dairy and meat products or food products made from these materials may be useful in estimating the actual risk level of these products. On the other hand, it is recommended to study more about the role of BLV in breast cancer.

In this study, a relatively high rate of milk samples from Iranian dairy cows was contaminated with BLV. Considering the ability of the virus to be transmitted from the infected to healthy animal as well as to humans through the consumption of infected and non-pasteurized milk, dairy and meat, preventive measures should be proposed to prevent the transmission of infection to humans.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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