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Design and implementation of a TaqMan® real-time PCR method for detection and quantification of bovine leukemia virus

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Abstract

The bovine leukemia virus (BLV) is an important infectious agent transmitted from cattle to humans. It is considered one of the oncogenic viruses in breast cancer, so an accurate detection of this virus is important. The study aimed to design a specific and sensitive method based on TaqMan® real-time polymerase chain reaction (RT-PCR) for BLV detection. Probes and primers were designed using bioinformatics software for a 108 pairs region of the BLV *tax* gene. Criteria employed for determining analytical sensitivity were prepared using *in-vitro* RNA transcriptions. The National Center for Biotechnology Information (NCBI), basic local alignment search tool (BLAST) databases various viral panels and genomic samples from healthy individuals (Qom Province, Iran in 2023) were used to verify analytical specificity and clinical specificity, respectively. This method can measure a minimum of 10 copies of DNA and RNA mL^{-1}. Moreover, the assay is linear in the range of 10^0 - 10^9 copies mL^{-1}. By testing negative specimens, the method specificity was 100%. The reproducibility results of the reaction were examined at the intra- and inter-assay comparison. In fact, 10 technical replicates of each concentration of the control sample were analyzed in each working reaction. Due to the locally made kit, exact sensitivity and specificity, rapid analysis, and relatively low cost, as compared to commercial kits of other countries, the method introduced in the present study could be suitable for accurate detection of the BLV. Also, the TaqMan® real-time PCR method could be detected in cattle and human and before malignant changes of breast cancer which could reduce infection and breast cancer.

Introduction

Bovine leukemia (B-cell leukemia/lymphoma), first reported in Lithuania in 1871, is an infectious disease that can spread through cattle herds. The cause of bovine leukosis was a virus isolated from cattle in an affected herd of cultured lymphocytes in 1969. Bovine leukemia virus (BLV) is an RNA virus belonging to the subfamily orthoretrovirinae, family retroviridae and order ortervirales. There are several studies on BLV as a genus delta retrovirus associated with human T-cell leukemia virus type 1, 2 (HTLV-1, 2) and with typical retroviral genomic areas: Group-specific antigen, capsid region (*gag*), long terminal repeat, promoter region (*LTR*), envelope (*env*) and polymerase (*pol*). Nonetheless, in contrast to other oncogenic retroviruses, deltaretroviruses possess an additional area, *tax* (trans-activating region of the *X* gene) that is oncogenic to host cells with regulatory functions.[1,2]

The cattle are the main host of BLV, however, BLV infects other animals such as sheep, water buffalo, alpacas, rabbits, mice, goats, and pigs.[3] The BLV infection prevalence is high in dairy herds (39.00 to 100%) in milk as well as in beef herds. The BLV is easily transmitted through contaminated blood and milk, but it can cause the disease in < 5.00% of infected cattles. It is linked to chronic lymphocytic leukemia and mammary cell infection can lead to mammary tumors in the host.[4]

The mechanism by which BLV is transmitted to humans is unknown, however, BLV is transmittable to humans by consumption of unpasteurized raw milk, milk-products and meat from infected cattle. The *tax* protein of the BLV virus has many regulatory functions (transcriptional activation) and may be linked to transformation by disrupting tumor suppressor genes and inhibiting the DNA repair system.[2,5] Clinical leukemia occurs in < 5.00% of affected cattle. Nonetheless, lymphocytes with BLV are

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observed in the milk and blood of sub clinically infected cows. Cattle herds with BLV are found worldwide. In Iran, a large proportion of herds are infected with BLV. The herd prevalence of enzootic bovine leucosis based on serological diagnosis in dairy cattle has been reported to be 41.30% and its prevalence is reported 32.80% among animals and 80.00% among different provinces based on molecular detection.[6,7] The BLV genome and antibodies to the capsid protein (P24) are detected in blood specimens from females. It may take several decades for the oncogenic effect of BLV to translate into breast cancer, therefore, a virus detection kit with high sensitivity and specificity is essential.[2] Oncogenic viruses such as hepatitis C and B viruses, human leukemia virus types 1 and 2, Epstein-Barr virus, human herpesvirus 8 and papillomavirus are involved in all types of cancers.[8] The BLV causes breast cancer in humans.[9]

For many years, serologic methods have been used primarily in the diagnosis of viral infections, and although direct and rapid culture and search methods have been emphasized in such infections, serologic methods can still aid in the diagnosis of infections. Serological BLV diagnostic techniques such as antibody-oriented tests, target antibodies that recognize the extracellular *gp51* protein encoded by *env-gp51* and the *p24* capsid protein encoded by the *gag* gene.[10] Limitations of serologic testing include antigenic changes, viral infections with different viral serotypes, immunologically silent carriers or hidden carriers,or the absence of antibodies in the early stages of the disease. According to the new guidelines, determining the number of RNA copies of this virus is of great importance as an indicator for diagnosing acute infection, predicting the likelihood of transmission of the virus, predicting the disease progression rate in chronically infected cases and evaluating the effect of treatment in persons treated with antiretroviral drugs.[11] One of the newest quantitative measurement methods currently receiving attention is Real-time detection systems or Real-time polymerase chain reaction (PCR) method. This research was conducted to develop the TaqMan® real-time PCR method with specific primers and probes for a region of the BLV *tax* gene which is one of the conserved regions of the viral genome.[12]

Antibodies to these proteins are formed soon following BLV infection, so developing a method based on molecular detection such as real-time PCR can be very efficient. Thus, cattle that have high proviral load (PVL) are the major source of risk for the virus spread and the development of enzootic bovine leucosis. However, cattle that have low PVL are not highly prone to transmit BLV to others. Reportedly, BLV provirus is found in the nasal mucosa, milk and saliva of dairy cattle with PVLs > 10,000, 14,000, and 18,000 copies *per* 10^5 cells in blood specimens, suggesting a PVL of approximately 10,000 copies *per* 10^5 cells. It is considered an index of the efficient spread of BLV in the body confirmed by quantitative polymerase chain reaction (qPCR) of BLV-tax.[13-15]

As the genome sequence of microorganisms increasingly changes, pathogens like viruses can gradually escape available detection methods targeting their genes.[16] Therefore, improving detection techniques for mutants is different from time to time. Yang *et al*., reported many novel mutations in the probe and primer areas of Real-time PCR assays applied for human influenza A viruses and subsequently developed a real-time PCR method that uses degenerate nucleotide bases in these areas and increases vulnerability to the point that new types of human influenza A viruses can be detected.[17]

The study aimed to design a specific and sensitive method based on TaqMan® real-time PCR for BLV detection. The time as well as geographical district of the study was Qom Province, Iran in 2023.

Materials and Methods

Designated primers and probes. To design the primers and probes, the sequence of the conserved *tax* gene in different sequences was obtained from a reliable database such as National Center for Biotechnology Information (NCBI) nucleotide and a database for the *tax* gene (108 bp) by accession number LC728442.1. Then, alignment analysis using bioinformatics software such as MEGA Software (version 10.0; Biodesign Institute, Tempe, USA)[18], and ClaustalW multiple sequence alignment program (UCD, Dublin, Ireland) was performed and the best region of *tax* gene was selected. Primer 3 and Beacon Designer™ software were used to design the primers and probes. Specificities and characteristics of each primer were evaluated using NCBI and Gene Runner™ software (version 6.0; Frank Buquicchio and Michael Spruyt Institute, Florida, USA), and OLIGO Primer Analysis Software (version 7.0; Molecular Biology Insights Inc., Cascade, USA), respectively. Also, the sequence of human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control. Sequences for primers were as follows: (a) *tax* F (5′-TTGTTCCCTCGACTG CATG-3′); (b) *tax* R (3′-ATTGGCATTGGTAGGGCTG-5′) and (c) probe (FAM-GATCAACTGCCCCCTTCCG-BHQ1). The endogenous control was tested by amplifying a 120 bp sequence of the *GAPDH* gene using primers GAP F (5′-CGAGATCCCTCCAAAATCAA-3′; GAP R (3′-TTCACACCCAT GACGAACAT-5′), and GAP probe (HEX-TGGAGAAGGC TGGGGCTCAT-TAMRA). Cycling conditions were 95.00 ˚C for 1 min, 58.00 ˚C for 30 sec, and 72.00 ˚C for 30 sec for 40 cycles. The location of the internal BLV TaqMan® probe, regions of the BLV genome, is 7,511 - 7,529 in the near 5′ sequences of forward primer.

Viral RNA extraction. Viral RNA was extracted from the positive sample using FavorPrep™ kit (Ping Tung Biotechnology Park, Taipei, Taiwan) and then reverse

transcription reaction was immediately performed using cDNA synthesis kit (Yekta Tajhiz Azma, Tehran, Iran) according to the manufacturer's protocol. Subsequently, a quantitative and qualitative analysis of the extracted genome was performed. The produced cDNA was applied as a template for the next step.[19]

Cloning the desired fragment in the plasmid. For quantitative measurement of the viral genome, cDNA molecule with certain values should be used as a standard. This standard was prepared by cloning the PCR product into a cloning vector. Thus, the desired sequence obtained from the application of the primers was cloned into the standard pTZ57 vector using cutting enzymes. To propagate the recombinant vector containing the desired gene, the reaction product was transferred into TOP10 *Escherichia coli* strain predisposed by $CaCl_2$ and then cultured in an Luria Broth (LB) agar medium (HiMedia, Maharashtra, India) with ampicillin (100 µg mL^{-1}). The recombinant clones confirmed plasmid containing the target gene (*tax* gene) by colony PCR technique with designed primers.[19] This was both as a positive control of kit compared to the genome extracted from samples of BLV virus-suspected and also to determine (Validation Primers) primers in the kit optimization stages.

cDNA synthesis. The cDNA synthesis was done in a volume of 20.00 mL with 1.25 mM random hexadeoxy-ribonucleotide, 5.00 mM $MgCl_2$, 10.00 mM Tris + HCl (pH = 8.30), 50.00 U MuLV reverse transcriptase, 50.00 mM KCl, 0.50 U mL^{-1} RNase inhibitor, and 1.00 mM dNTPs (Amersham Pharmacia Biotech, Amersham, UK). The mixture was annealed at 42.00 ˚C for 60 min and quenched at 95.00 ˚C for 5 min. The ultimate volume was adjusted to 100 mL using RNase-free water.[20] Since the DNA used as a template contained a promoter section, we would get a false positive response if the obtained RNA products were directly entered into the Real-time reaction.

Determination of the sensitivity and specificity of primers. In order to determine the analytical sensitivity of the reaction, serial dilutions (log dilutions) method of DNA by a factor of 10^0 - 10^9 copies *per* mL was prepared, and then the Real-time reaction was repeated three times for each dilution to determine the lowest dilution (Technical reproducibility method).[21] To determine the diagnostic method specificity, besides verifying the correctness of the specific binding of the primers to the desired pattern in the NCBI nucleotide basic local alignment search tool (BLAST) database, several genomic samples of viruses such as human T-cell lymphotropic virus type 1 (HTLV-1), Hepatitis B virus (HBV), Epstein-Barr virus (EBV) and mouse mammary tumor virus (MMTV) were used. Fifty negative animal and 50 human samples were used in which the absence of BLV virus was confirmed by nested-PCR method to determine the clinical features.

Real-time PCR reaction to detect BLV-tax. RealQ Plus 2.00 X Master Mix for Probe (Ampliqon, Odense, Denmark) was used to generate a standard curve with 10 different concentrations of cDNA obtained from RNA prepared by *in vitro* transcription method. The 10^0 - 10^9 copies *per* mL were selected. The amplification reaction was performed in a volume of 25.00 µL containing 12.00 µL of Master Mix, 5.00 µL of cDNA from serial dilutions prepared in a ratio of 10.00 to 1.00, 0.40 µL of each primer and 0.20 µL of the probe prepared using the template from.[10] The immunohistochemical pathology and examination of PBMC cells clearly showed that the number of BLV proviral copies led to an increase in the severity of the disease.

Statistical analysis. The determination of the concentration of similar replicates in a cycle of real-time PCR is displayed as standard deviation (SD) for different cycling thresholds (Ct). For this purpose, 3 replicates of each concentration of the sample were analyzed in each working reaction and the values of the coefficient of variation (CV) for Ct values, the R2 coefficient and the slope of the line for the standard samples were calculated. Data are reported as mean + SD using SPSS Software (version 16.0; SPSS Inc., Chicago, USA), and one-way analysis of variance compared to the results. A p-value < 0.05 was considered significant.

Results

Quantitative and qualitative analysis of extracted RNAs. The absence of protein and phenol contamination of the extracted RNAs were confirmed by NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The 260/280 absorbance ratio for all samples was between 1.80 and 2.00 indicating the absence of protein contamination, and the 260/230 absorbance ratio for all samples was in the range of 2.00 indicating minimal phenol contamination. The quality of the samples was examined using 1.00% agarose gel electrophoresis.

Cloning of BLV *tax* gene sequence in PTZ57 vector. The PCR product containing the adenine end was linked to pTZ57RT vector to its transfer into Top10 *E. coli* strain. Cloning was confirmed by PCR on colonies grown on an antibiotic-containing medium using *tax*-specific primers (Fig. 1A).

Result of PCR determination of specificity. The genomes of HTLV-1, HBV, EBV and MMTV viruses and the genomic sample of BLV as a positive control were subjected to PCR analysis after cDNA extraction. The results showed that no cross-reaction was occurred for any of the viruses (Fig. 1B).

Sensitivity assessment. The results showed that none of the serum samples from healthy individuals had a false-positive reaction and the specificity of this method for BLV-tax detection was calculated to be 100%. The reproducibility tests were performed with the prepared serial dilutions (10^0 - 10^9 copies *per* mL).

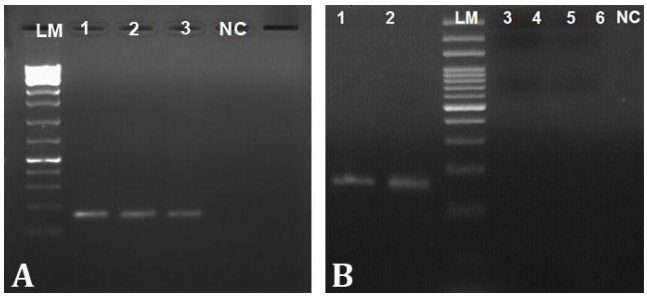

Fig. 1. A) Polymerase chain reaction (PCR) result for the extracted plasmid containing the *tax* gene sequence. LM: 100 bP Ladder Marker, Lanes 1 - 3: Plasmids extracted from recombinant bacteria containing gene bovine leukemia virus -*tax* and NC: Negative control. **B)** The result of PCR electrophoresis for the genomes of HTLV-1, HBV, EBV, mouse mammary tumor virus (MMTV), and bovine leukemia virus (BLV). Lanes 1 and 2: *BLV-tax* gene, LM: 100 bP Ladder Marker, Lane 3: HTLV-1, Lane 4: HBV, Lane 5: EBV, Lane 6: MMTV, and NC: Negative control.

The variation coefficient of Ct determined in the reproducibility intra- and inter- assays for all serial dilutions was lower in each case and it ranged from 3.00 to 5.00% that this method has high reproducibility (Table 1).

In this area, all criteria of R2, intercept and slope were included in the confirmed range of Real-time PCR. The y-intercept parameter was equal to 35 out of 54, which showed the sensitivity and accuracy of the developed method. The slope was 3.28 and the R2 coefficient was equal to 1 indicating the high efficiency of the reaction (Fig. 2). Infection with BLV was characterized by three progressive stages of disease: Asymptomatic stage, persistent lymphocytosis and lymphoma. The *tax* protein of BLV immortalized a part of BLV-infected cells and induced polyclonal proliferation of the cells. However, the *tax* protein did not have the ability to transform the cells. For lymphoma to develop, a malignant transformation was needed to occur with the help of host factors such as p53 mutation, TNF-α activities or bovine leukocyte antigen class II phosphorylation. The provirus load was increased with disease progression. Most studies of BLV-induced leukemogenesis have focused on the *tax* protein because it is believed to be a potent transcriptional activator of viral gene expression. It is considerable that increasing the proviral load of BLV virus is important for the ability of *tax* protein to induce host cell oncoproteins.

Table 1. The results of the internal (first row data) and inter-rater (second row data) measurement repeatability tests.

BLV-tax (copy number per mL)	Coefficient of variation (%)	Average cycling threshold values	Standard deviation
10^9	2.30	5.78	0.80
	2.70	5.92	0.18
10^8	1.00	9.39	0.10
	1.20	8.25	0.22
10^7	1.20	12.47	0.14
	1.60	12.00	0.14
10^6	1.00	15.63	0.13
	1.30	15.32	0.13
10^5	2.20	19.11	0.80
	2.50	19.01	0.78
10^4	1.10	22.56	0.18
	1.70	22.44	0.85
10^3	1.00	5.72	0.19
	0.9	5.30	0.24
10^2	2.10	29.19	0.80
	2.30	28.10	0.82
10^1	2.40	32.47	0.60
	2.60	33.00	0.35
10^0	2.30	19.01	0.55
	2.70	18.98	0.28

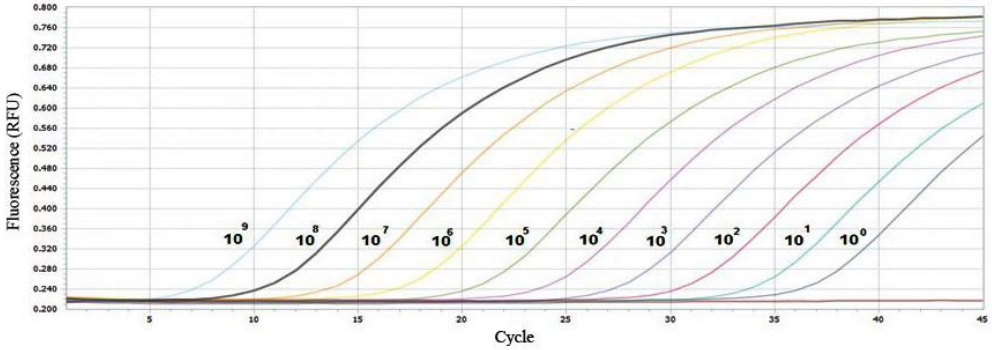

Fig. 2. The *tax* gene amplification curve with successive dilutions of 10^0 - 10^9 copies *per* mL.

Discussion

The leading cause of breast cancer is not known and many factors cause its progression and development. Infectious agents, particularly viruses, may be associated with the development of breast cancer**.** Some meta-analysis and cross-sectional studies on the relationship between HPV, EBV, BLV and MMTV and breast cancer showed the prevalence of such viral infections in breast cancer patients compared to the control group.[2, 22-24]

The use of an accurate and specific diagnostic method for BLV can play an important role in the diagnosis and treatment of breast cancer caused by this virus. Compared to serological methods, the real-time PCR method was not only very sensitive and specific but also simple and time-saving to use.

To monitor and find the treatment status of patients, the method used for quantitative measurement must provide accurate and repeatable results. In this study, reproducibility was investigated at the intra- and inter-assay levels. Internal measurement reproducibility examined both user and non-calibration errors of the instruments used. This was the case when inter-assay reproducibility, laboratory errors including equipment used, enzymes or master mixes and non-calibration of standards could check the stability and durability of the reaction. According to the criteria and guidelines of the University of American Pathology, the coefficient of variation for intra-assay reproducibility should not exceed 5.00%, and for inter-assay reproducibility should be less than 10.00% indicating the adequate reproducibility of an assay which our method was less than 2 and 6, respectively.[25]

Methods based on nucleic acid (nucleic acid testing) detection have been developed to identify the nucleic acid of viruses. The TaqMan® PCR assay, developed by Holland *et al*., utilizes the 5′-nuclease activity of Taq DNA polymerase. A fluorogenic probe that binds specially between the two PCR primers experiences degradation during every PCR cycle which releases the fluorophore leading to cancelation of the quenching of the two fluorescent markers, and the target gene amplification is monitored as an increasing fluorescent signal.[26,27]

The introduction of this method, which is also inexpensive, can help individuals with breast cancer caused by contamination with the BLV virus to complete their treatment process simultaneously with chemo-therapy and antiretroviral drugs. Lew *et al*, have developed a TaqMan® real-time PCR method for detecting BLV proviruses based on the conserved region of the *gag* gene. Results showed that this method was 100-fold more sensitive than conventional PCR assays and its lowest diagnostic rate was about 45 copies *per* mL of the viral genome.[28] Jimba *et al*. were able to identify a wide range of BLV mutant viruses using our newly developed BLV CoCoMo-qPCR method.[13] They also used degenerate primers based on the long terminal repeat (*LTR*) gene sequence of BLV virus and TaqMan® probes to measure viral load. The diagnostic range was a virus with less than 155 copies *per* mL of virus in positive samples from infected cows with different strains.[13,29] According to Dao *et al.* three out of 33 specimens in northern Vietnam for the BLV *pol* gene by real-time PCR were negative using the TaKaRa CycleavePCR™ system.[30]

Tang *et al*. developed a quantitative method to identify the *pol* gene of the Human immunodeficiency virus type 1 (HIV-1) virus based on the probe. This method had high sensitivity with a linear range between 1.00×10^7 - 4.00×10^7 copies *per* mL.[31] In another study, Kamangu *et al*. applied a method to find the *LTR* gene with a minimum detection limit of 10^5 copies *per* mL.[32] The results of their study compared to methods developed for the detection of HIV-1 virus showed that the *INT* gene was used for the first time as a target for the detection of this virus.[31-33] Acharya *et al*. have developed a proprietary real-time PCR method to identify subtype C of HIV based on the conserved regions of the *gag* gene. The linearity of this method was in the range of 50 copies of the viral genome to 10^7 copies of the virus *per* mL of plasma sample, and the lowest detection range was about 50 copies *per* mL of the viral genome.[33] The linear range and minimum detection limit of this developed method were also comparable to other methods presented.

In the USA, 83.90% of dairy cattle and 39.00% of beef cattle were infected with BLV and in Iran, 22.10 - 34.70% of dairy cattle were reported BLV-positive.[2] Buehring *et al*. reported that the BLV antibodies and genome for the capsid protein (P24) were detected in blood specimens from women.[3] Also, they detected BLV DNA in the breast tissue of 80.00% of females with breast cancer compared to 41.00% in the negative control group.[34] Khalilian *et al*. showed an association between breast cancer and BLV in Iran that the frequency of BLV DNA based on the *tax* gene in women with breast cancer was 30.00%.[2]

In addition to high sensitivity and specificity, this diagnostic method is easy to use and can determine the level of BLV RNA in a shorter time, and it can be easily used in diagnostic centers. The standards that are available in the form of commercial panels are very expensive, and in terms of their availability, there are many limitations that plague researchers. The standards produced in the present research were much cheaper in the laboratory, and since the present method had a wide dynamic range, it could be useful in diagnosing acute cases of disease and tracking treatment outcomes in patients undergoing treatment who have different viral loads. Because the Real-time PCR method based on the TaqMan® probe did not require post-amplification steps, it not only reduced costs but also minimized the transmission of technical contaminants that led to false positive results.

This test used the special *tax* gene which was conserved among the different genotypes of BLV viruses and was not the case in other methods. Therefore, the sensitivity and specificity of the method were greatly increased and improved compared to the existing methods. It also bears other advantages such as increasing the speed by reducing the replication time and eliminating the detection step after replication.[35]

Therefore, according to the results of the present study, it seems that the above method could be used in diagnostic centers as an auxiliary method in the treatment and diagnosis of breast cancer and also in the diagnosis of BLV infection in cattle.

Infection with BLV causes a severe economic burden in the cattle industry. The infection spreads insidiously because there is no successful treatment or vaccination. It is hoped that BLV infection in farms can be eradicated or prevented as soon as possible. Also, previous studies showed an association between BLV and breast cancer. Our current qPCR system was regarded as a highly accurate, specific and sensitive tool to detect the internal control gene (*Human GAPDH*) and all BLV strains in a tube separator medium.

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Conflict of interest

The authors declare no conflict of interest.

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