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Original article

Molecular detection of bovine leukemia virus in peripheral blood of Iranian cattle, camel and sheep

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Abstract

Bovine leukemia virus (BLV) is a *deltaretrovirus* which infects and induces proliferation of B-lymphocytes in the peripheral blood circulation and in lymphoid organs primarily of cattle, leading to leukemia/lymphoma. This study was carried out to investigate the presence of BLV in cattle, sheep and camels from the Chaharmahal va Bakhtiary and Isfahan provinces in Iran. A total of 874 blood samples collected from cattle, sheep and camels were used in this study to detect BLV using a nested-PCR. The results from this study indicated that 17.2% (n=874) of all blood samples collected were positive for BLV. The percentages of blood samples positive for BLV from cattle, sheep and camels were 22.1 (n=657), 5.3 (n=95) and 0 (n=122) respectively. The results from this study showed that BLV infected cattle and sheep. Camels seemed to be resistant to BLV infection. This study contributes to the nationwide effort to obtain baseline information on the prevalence of BLV, which will assist in planning the control strategy for the disease in Iran.

Key words: blood, BLV, camel, cattle, molecular detection, Nested-PCR, sheep

Introduction

Bovine leukemia (syn. Enzootic bovine leukosis), which was described for the first time in 1871, has attracted significant attention in several European countries due to the occurrence of high incidences suggestive of viral etiology (Fenner and White 2011). The etiological agent of bovine leukemia is the *Bovine leukemia virus* (BLV), a member of the Retroviridae family belonging to the genus *Deltaretrovirus* (Otto et al. 2007). The disease has been reported to occur

worldwide, and is a significant economic loss to the cattle industry (OIE 2012). Losses incurred in the dairy industry have been due to reduced milk production, increased replacement costs, reduced conception rates, and a greater susceptibility to other infectious diseases such as mastitis, diarrhea, and pneumonia (Ott et al. 2003).

The genome organization of BLV is similar to that of other retroviruses, where the structural proteins required for the production of infectious virions (from 5' to 3') includes the *gag* (encodes p24 and p15),

pro (encodes protease), *pol* (encodes polymerase) and *env* (encodes gp51 and gp30) gene proteins (Momtaz 2010, Moratorio et al. 2013). The BLV enveloped (*env*) gene coding for gp51 is highly conserved and both the gene and the antigen are present in the whole infected animal throughout the course of infection (OIE 2012). For this reason, this part of BLV is useful for molecular detection of the virus.

The serological tests used for diagnosis of bovine leukemia include the Agar Gel Immuno Diffusion (AGID) and the Enzyme-Linked Immuno Sorbent Assay (ELISA) (Momtaz et al. 2008, OIE 2012). Both tests are less sensitive when compared to polymerase chain reaction (PCR) described by several researchers (OIE 2012). The nested-PCR has shown to have greater sensitivity than the normal PCR (Gonzalez et al. 2000). Real-time PCR based on the conserved BLV *pol* gene proved to be highly specific and sensitive with a detection of up to 1 copy of an internal control plasmid (Heenemann et al. 2012, Rola-Łuszczak et al. 2013).

The main aim of this study was to detect the presence of bovine leukemia virus in the peripheral blood of cattle, camel and sheep by using nested-PCR.

Materials and Methods

Sampling and DNA Isolation

The present study was conducted in Isfahan and Chaharmahal va Bakhtiary provinces, Central and South-West Iran respectively. Samples were collected from randomly selected cattle, camels and sheep from slaughterhouses and farms. It was a cross-sectional study conducted from December 2012 and February 2014. A total number of 657, 95 and 122 blood samples were collected from cattle, sheep and camels respectively, using random sampling. Blood samples were analyzed at the Biotechnology Research Center of the Islamic Azad University, Shahrekord Branch after being transported in a cool box. At the center, samples were stored at -20°C for further use. Genomic DNA was then extracted from specimens using a DNA extraction kit (Cinnagen, Tehran, Iran) according to the manufacturer's protocol. The total amount of DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell (2001). DNA extracted from each sample was kept frozen at -20°C until used.

Gene Amplification

For the detection of BLV DNA, nested PCR was performed using the DNA extracted from the blood

samples. Two sets of oligonucleotide primer pairs were used for amplification of the DNA. The external primers specific to the BLV region were used as described by Wang et al. (2002). The sequence of primers for the first round was BLV-F1: 5-ATGGGAAATTCCCCCTCCTAT-3 and BLV-R1: 5-GTTTTTTTGATTTGAGGGTTGG-3 (Wang et al. 2002). The second amplification round was performed to amplify a 385 base pair (bp) fragment, using a pair of inner primers from the gag region (BLV-F2: 5-AACACTACGACTTGCAATCC-3 and BLV-R2: 5-GTTCCTTAGGACTCCGTCG-3) in the BLV genome (GenBank accession number K02120). The viral DNA from fetal lamb kidney cells infected with bovine leukemia virus (FLK-BLV) served as a positive control during amplification.

Two sets of nested-PCR program were 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 at 72°C and then by a final extension at 72°C for 5 min. Two µL from the first round amplicon was used as the template in a thermocycler (Eppendorf, Hamburg, Germany). The second round PCR was performed with inner oligonucleotide primers for 25 cycles with the same concentration of reagents and temperature conditions.

Analysis of PCR Products

The amplified PCR products were detected using agarose gel electrophoresis (1%). The electrode buffer used 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 thr components in sufficient H₂O and 10 µL Aliquots of PCR products were loaded to the gel. A constant voltage of 80 V for 30 min was used for product separation. After electrophoresis, images were obtained in UVIdoc gel documentation systems (UK).

Analysis

The prevalence analysis was computed in percentage and presented using simple frequency.

Results

Nested-PCR specimens producing a band of the expected size (385 bp) were considered positive (Fig. 1).

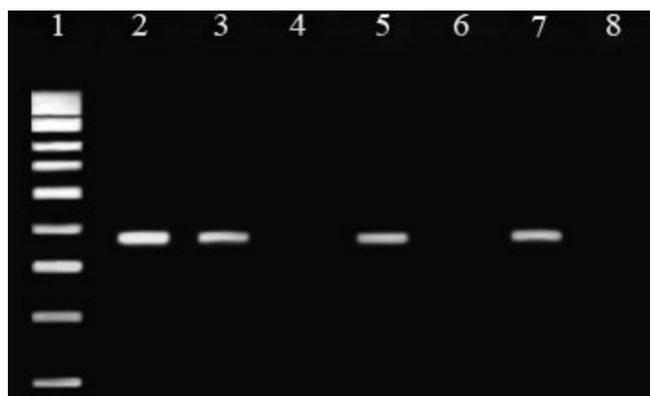


Fig. 1. Ethidium bromide-stained agarose gel electrophoresis of PCR products (385 bp) for detection of *BLV* DNA in blood samples from cattle, camel and sheep blood samples. Lane 1: 100 bp DNA ladder (Fermentas, Germany); Lane 2: positive control; lane 3, 5 and 7: positive samples (385 bp); lanes 4 and 6: negative samples; lane 8: negative control.

Table 1. Nested PCR positive blood samples for BLV in study sites.

Attribute		Nested PCR BLV positive samples (%)		
		Cattle	Camel	Sheep
Province	Chaharmahal va Bakhtiary	18.43 (40/217)	–	2.77 (1/36)
	Isfahan	23.86 (104/440)	0 (0/122)	6.77 (4/59)
Sex	Female	22.78 (134/588)	0 (0/58)	7.04 (5/71)
	Male	15.94 (11/69)	0 (0/64)	0 (0/24)

The positive control (FLK-*BLV* DNA) showed the expected amplification product specific for *BLV* (385 bp). The size of the bands matched that of the positive control. The proportions of positive individuals from each category and sex in the different provinces are shown in Table 1.

Discussion

Bovine leukemia virus which is mainly transmitted horizontally by direct exposure to biological fluids such as blood, milk, semen and saliva induces persistent lymphocytosis in cattle and sheep. The infection the animal with BLV leads to the development of immunity against envelope protein gp51, for which the antibodies can be detected serologically by immune diffusion tests and ELISA. The use of PCR in BLV diagnosis increases the detection of infected animals (Mohammadabadi et al. 2011).

Naturally, the disease occurs only in cattle but experimentally BLV can easily infect sheep which leads to the development of B-cell lymphosarcomas at higher frequencies and after a shorter latent period than cattle, and sheep are very susceptible to experimental inoculation and develop tumours more often and at a younger age than cattle (Mousavi et al. 2014). Sheep

can be infected by BLV only via iatrogenic accident. However, according to the Office International Des Epizooties (OIE), BLV is included in the list of reportable diseases of importance to international trade (OIE 2012, Giangaspero et al. 2013). Studies carried out in Brazil, Venezuela and Russia proved that sheep are naturally infected by BLV (Del Fava et al. 2010).

The herd prevalence of enzootic bovine leucosis (EBL) in Iran based on serological diagnosis in dairy cattle has been reported to be 41.3% (Mohammadi et al. 2011; Haghparast et al. 2012). The overall prevalence of BLV infection among animals in Iran has been documented as 29.9% and 32.8% by Mohammadi et al. (2011) and Kazemimanesh et al. (2012) respectively. There is high significant association between the seroprevalence of EBL and age, where animals with an age greater than 4 years have a higher chance of having BLV infection (Mohammadi et al. 2011, Morovati et al. 2012). The horizontal transmission of EBL in Iran has been reported to be exacerbated by the use of the same needle for herd vaccinations and treatment injections, and also the same gloves and sleeves for rectal palpations (Morovati et al. 2012). Other factors which have been reported to play a part in the transmission of the disease include the importation of unscreened heifers for diseases and frozen semen containing the BLV for artificial insemination.

nation (AI) (Morovati et al. 2012, Khamesipour et al. 2013).

In this study, a nested-PCR technique was employed to screen a total of 874 blood samples collected from cattle, sheep and camels from the Chaharmahal va Bakhtiary and Isfahan provinces in Iran. This study showed that 17.2% of all the blood samples tested were positive to BLV. If we take out the number of camels, of which all were negative, the percentage of animals affected rises to 20%. Serological studies worldwide have shown the prevalence to range from 0-100% (Otto et al. 2007, Kazemimanesh et al. 2012). Previous studies in Iran have shown that the prevalence of BLV infection in Iranian cattle based on ELISA continued to increase with time (Mohammedi et al. 2011). Previous studies also showed that several cities of Iran have been affected with BLV at different times (Haghparast et al. 2012, Kazemimanesh et al. 2012, Morovati et al. 2012). The prevalence of BLV infection reported in Iran based on serological techniques ranges from 0.5-88.8% from 1996 to (Kazemimanesh et al. 2012, Mousavi et al. 2014) with the average range being from 20-30%. This shows that efforts to intervene in the transmission of BLV and the importation of the virus through semen and the introduction of animals to Iran need to be put in place and work effectively.

Recent studies using molecular techniques have pinpointed imported semen for AI as one of the sources of BLV introduction in Iran (Khamesipour et al. 2013). This shows that screening of imported semen using nested-PCR will help in identifying and rejecting semen that contains the virus. Enzyme-Linked Immunosorbent Assay and nested-PCR, apart from being used for surveillance of BLV infection in Iran, can also be used for screening imported cattle and sheep in Iran. It is common practice in Iran to introduce sperm from foreign countries. The sperm imported from America, Canada and European countries with a higher amount of importation being from America, probably due to high milk production (Joezy-Shekalgorabi and Shadparvar 2009). Phylogenetic analyses of the BLV *gag* gene in Iranian isolates were significantly identical to those of European and American isolates (Momtaz 2010). This correlation of the molecular findings and the location from where semen is imported suggests that the BLV circulating in Iran came from those countries. This puts more emphasis on the screening of semen imported in Iran. Moreover, Holstein registered heifers were imported from Europe, the United States, and Canada during the 1970s and early 1980s as the precursor to the establishment of intensive dairy cow husbandry in Iran (Sadeghi-Sefidmazgi et al. 2012). This also could be another source of BLV introduction in Iran. There-

fore, any cattle or sheep introduced into the country needs to be screened for BLV to control virus introduction.

In this study we did not detect BLV in the 122 blood samples collected from camels despite the fact that camels are being grazed in the same environment as cattle and sheep. Camels can acquire the infection under natural conditions but this study showed that camels are resistant to BLV infection (they do not possess receptors specific for BLV). However, this study has indicated that BLV was circulating in the Chaharmahal va Bakhtiary and Isfahan provinces in Iranian cattle and sheep.

Conclusions

Bovine Leukemia virus infection in cattle and sheep in Iran remains a serious problem which requires great emphasis on control strategies such as screening of imported semen and heifers, and the use of sterile equipment such as needles, dehorning instruments and ear tags during routine farm management practices. Generally, the horizontal transmission of BLV which is the most common mode of transmission needs to be controlled to lower the rate of infection and finally eliminate the disease in Iran. Therefore, the information obtained in this study expands the understanding of the distribution of BLV in Iran and helps in planning the control strategies for EBL in this country.

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