EVALUATION OF SERUM-ELISA & NESTED PCR   FOR BOVINE LEUKEMIA VIRUS (BLV) DIAGNOSIS IN DAIRY HERDS AROUND BABOL CITY (NORTH OF IRAN) AS A CASPIAN CLIMATE

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ABSTRACT

Bovine leukemia virus(BLV),the causative agent of enzootic bovine leukosis,is an exogenous, B-Lymphotropic retrovirus belonging to *the Orthoretrovirinae* subfamily and *the retroviridae* family that induces persistent lymphocytosis (PL) in cattle. This survey investigated the presence of anti-BLV antibodies and BLV provirusin Babolsmall province(North of Iran)cattle blood samples. In this survey, sampled from 6 herds of Babolsmall province industrial dairy cattle emphasis randomly sampling. A total of 234 whole blood samples from cattle were studied. An enzyme linked immunosorbent assay (ELISA) was used to detect anti-BLV antibodies and nested PCR was employed to increase of care in allcattle blood samples specially the samples that shown doubtful serological results obtained by ELISA. Then epidemiological aspects of BLVare reviewed with emphasis on diagnostic tests(ELISA and nestedPCR). Overall prevalence of anti-BLV antibodies in dairy cattle blood samples were 0%and15.38%,respectively.When using ELISA as a gold standard,sensitivity and specificity for nestedPCR were 100% and 97%,respectively.The predictivevalue of a positive test was 70% and the predictive value of a negative test

was100%. Interpretation of kappa scores for two methods accounted substantial 0.85. The percentage of accuracy between two tests accounted 97%. We find a direct relationship between aging and percent of infection (p<0.05) and also between number of parturition and percent of Infection (p<0.05).

Keywords: Bovine Leukemiavirus, Epidemiologic, ELISA, Nested PCR, Dairy Herds, Babolsmallprovince

INTRODUCTION

BLV, the causative agent of enzootic bovine leukosis,is an exogenous, B-Lymphotropic retrovirus belonging to *the Orthoretrovirinae* subfamily and *the retroviridae* family that induces persistent lymphocytosis (PL)incattle**(Radostits*et al.*, 2007)**. This survey investigated the presence of anti-BLV antibodies and BLV provirus in Babol small province cattle blood samples. An enzyme linked immunosorbent assay (ELISA) was used to detect anti-BLV antibodies and nested PCR was employed to increase of care in all cattle blood samples specially samples that shown doubtful serological results obtaine by ELISA. Then epidemiological aspects of Bovine Leukemia Virus (BLV)are reviewed with emphasis on diagnostic tests(ELISA and nestedPCR).

MATERIALS AND METHODS

In this survey, sampled from 6 herds of Babolsmall province industrial dairy cattle emphasis randomly sampling. A total of 234 whole blood samples from cattle were studied. The ELISA and nested PCR tests were performed on all samples and were used according to manufactures instruction. ELISA testkit was from INDEXX Laboratories. Bovine genomic DNA was extracted and was directly subjected to a nestedPCR that amplifies the *env*gene between nucleotides 5029 and 5376. The sequence used for designing the primers is available from GenBank, accession No.K02120**(Ballagi-Pordany, Belak, 1996;Belak, Ballagi-Pordany, 1993)**.

Blocking enzyme-linked immunosorbent assay - Serum ELISA

Test procedure

i) Coating the plate

All wells are coated with BLV antibody, prediluted in coating buffer (100 μl/well), the plate is sealed and incubated for 18 hours at 4°C. A wash cycle (standard wash) is performed, which is three washes fillingwells to the top, with a 3-minute soak in between each wash, and then the plate is blotted. BLVantigen is added, prediluted in wash buffer (100 μl/well), the plate is sealed and incubated for 2 hours at 37°C.

A standard wash cycle is performed.

ii) Preparation and addition of samples and controls

The positive and negative control sera are prediluted (1/2) in wash buffer and the solution is added to four wells per control (100 µl/well). For testing pooled samples, 80 sera may be bulked then diluted (1/2) using wash buffer and the solution is added to two wells (100 µl/well) per sample. Single samples should be diluted 1/100 using wash buffer and the solution added to two wells (100 µl/well) per sample.

After plating out the samples, the plate is sealed and incubated for 18 hours at 4°C. A brief wash is performed by filling the wells and immediately emptying them.

iii) Preparation and addition of conjugates and substrate

Predilutedbiotinylated antibody is added (100 µl/well) to all wells – predilute using wash buffer+ 10%fetal calf serum – the plate is sealed and incubated on a rocking table for 1 hour at 37°C. A standardwash is performed as described earlier. The peroxidase-conjugated avidinis prediluted in wash buffer and the solution is added to all wells (100 µl/well). The plate is sealed and incubated on a rocking table for 30 minutes at37°C. A standard wash is performed. 100µlorthophenylaminediamine substrate is added to all wells, the plate is covered and left in the dark for 9 minutes. The reaction is stopped with   100µl   of 0.5M sulphuric acid per well

(**Beier*et al*., 2004; OIE terrestrial manual 2012; Rola & Kuzmak 2002; Teifke & Vahlenkamp, 2008; Vandermaaten & Miller, 1976; Venables & Martin, 1997**).

Reading and interpretation of results

The plate reader is blanked on air and the absorbance is read at 490 nm. For dual wave-length readers a reference filter between 620 nm and 650 nm is used. Results are read within 60 minutes after the addition of stop solution (**Beyer *et al*., 2002; Miller, Vandermaaten, Schemerr, 1985**).

The absorbance of the negative control should be about 1.1 ± 0.4; if the absorbance is below0.7, the colour development time in step iii above (preparation and addition of conjugates and substrate) should be increased. Conversely, the time should be shortened if the absorbance is above 1.5. The absorbance of the positive control should be less than the absorbance of the negative control $\times 0.25$.

A sample is positive when the absorbance of each of the two test wells is identical with or less than the mean absorbance of the four negative wells $\times 0.5$.

A sample is negative when the absorbance of each of the two test wells is identical with or higher than the mean absorbance of the four negative control wells $\times 0.65$.

For samples giving values between the absorbance of the negative control × 0.5 and × 0.65 it is recommended to retest the animal, using a sample taken 1 month later (**Beier *et al*., 2004; Venables & Martin, 1997**).

DNA extraction

I. Purification of total DNA is a prerequisite for achieving optimal sensitivity. Special precautions should be taken during all steps to minimize the risk of contamination (**Ballagi-Pordany, Belak, 1996; Belak, Ballagi-Pordany, 1993;Beyer *et al*., 2002;Dimmok, Rodwell, Chung, 1987**).

   i) Approximately 100µl chelating resin (Sigma C-7901 or Chelexfrom Bio-Rad) is added for each sampleina 1.5 ml eppendorf tube.

   ii) 100 µl of the samples and 10µl of the mimic are added to the tubes with chelating resin. The samples are vortexed.

   iii) The eppendorf tubes are closed and incubated at 56–60°C for 20 minutes.

   iv) The tubes are vortexed for 10 seconds.

   v) The tubes are incubated at 98°C for 8–10 minutes.

   vi) The tubes are vortexed for 10 seconds and immediately put on ice.

   vii) Optional:all samples are equilibrated to a standard amount of DNA (500 ng/reaction) applying, for example, the Beta Globin-method.

   viii) The tubes are centrifuged at 15,000 *g*for 2 minutes.

   ix) 5 µl isused in the PCR assay (**Beier*et al*., 2004; Venables & Martin, 1997**).

Nested PCR procedure

i) Primer designand sequences

The BLV region used as target is the gp51 (*env*) gene. The sequence used for designing the primers is available from GenBank, accession No.K02120 (**Ballagi-Pordany, Belak, 1996; Belak, Ballagi-Pordany, 1993**). The sequences of the primers are:

Oligo	Sequence	Position in K02120
OBLV1A	**(5'-CTT-TGT-GTG-CCA-AGT-CTC-CCA-GAT-ACA-3')**	**5029**

OBLV6A	(5'-CCA-ACA-TAT-AGC-ACA-GTC-TGG-GAA-GGC-3')	**5442**
OBLV3	(5'-CTG-TAA-ATG-GCT-ATC-CTA-AGA-TCT-ACT-GGC-3')	**5065**
OBLV5	(5'-GAC-AGA-GGG-AAC-CCA-GTC-ACT-GTT-CAA-CTG-3')	**5376**

PCRI-product size: 440bp; PCRII-product size: 341bp; Mimic-product size: 761 bp.

ii) Reaction mixtures

Reaction mixtures are blended (except sample and mimic) before adding to the separate reaction tubes. One negative control (double distilled H2O) per five samples, and one positive control should beadded. Total volumes of mixtures are calculated by multiplying the indicated volumes by the total number of samples, including controls, plus one. Taq polymerase is used in a premade 1/10 dilution (**Beyer *et al*., 2004; Theilen *et al*., 1982**).

DNA samples and mimic1 (2) should be added in separate rooms in the laboratory: laboratory room 1for DNA preparations and mimics, and laboratory room 2 for PCRII-products, to minimize contamination.

a) **Reagents added in clean laboratory room**

This mixture may be prepared in advance and stored at 4°C for up to 1 month.

Reagents per reaction(conc.)	PCRI reaction	PCRII reaction
Double-distilled H2O (standardised)	**21 μl**	**21 μl**
10 × PCR buffer (Perkin Elmer)	**5 μl**	**5 μl**
dNTP(10 mM)	**4 × 1 μl**	**4 × 1 μl**
Bovine serum albumin (1 mg/ml)	**5 μl**	**5 μl**
***Primers(10 pmol/μl)*:**		
OBLV1A	**1.5 μl**	**–**
OBLV6A	**1.5 μl**	**–**
OBLV3	**–**	**1.5 μl**
OBLV5	**–**	**1.5 μl**
In total:	**38 μl**	**38 μl**
The following shouldbe added just before starting the PCR		
Reagents per reaction (conc.)	**PCRI reaction**	**PCRII reaction**
MgCl2 (25 mM)	**5 μl**	**5 μl**
Taq polymerase (1 unit/reaction)	**2 μl**	**2 μl**
Mineral oil	**2 drops**	**2 drops**
In total:	**45 μl**	**45 μl**
b) Reagents added in laboratory room 1(DNA)or 2 (PCRII)		
Reagents per reaction (conc.)	**PCRI reaction**	**PCRII reaction**
DNA sample* (or water*)	**5 μl**	**–**
PCRI product	**–**	**5 μl**
In total:	**50 μl**	**50 μl**

iii) **PCR thermoprofiles**

PCRI-thermoprofile

5 × 94°C/45 seconds, 60°C/60 seconds, 72°C/90 seconds

30 × 94°C/45 seconds, 55°C/60 seconds, 72°C/90 seconds

1 × 72°C/420 seconds ≥20°C

PCRII-thermoprofile

5 × 94°C/45 seconds, 58°C/60 seconds, 72°C/90 seconds

30 × 94°C/45 seconds, 53°C/60 seconds, 72°C/90 seconds

1 × 72°C/420 seconds ≥20°C

iv) **Laboratory procedure**

Mix PCRI-reagents as described in step ii. Use separate gloves or tube openers for each individual tube when adding the DNA samples. Put the samples on ice. Heat the thermoblock to 80°C. Put samples in the thermoblock and start the PCRI-programme (step iii).

Mix PCRII –reagents as described in step ii. Use separate gloves or tube openers for each individualtube when adding the PCRI - product. Put the samples on ice. Heat the thermoblock to 80°C. Put samples in the thermoblock and start the PCRII- programme (step iii)**(Beier***et al***., 2004; Venables & Martin, 1997)**.

Agarose gel electrophoresis

Take the PCRII-products to the electrophoresis laboratory. Load approximately 10–15 μl of thesamplesand 23 μl loading buffer on a 2%agarose gel containing ethidiumbromide at 0.01%. Using 0.5 ×Tris/borate/EDTA (TBE) buffer, electrophoresis is performed with 90 mA for 2 hours. To control the size of the amplification products, a 100 bp ladder is recommended. Analysis of PCR products is done byUV illumination**(Beier***et al***., 2004; Venables& Martin, 1997)**.

Interpretation of the results

i) Positive samples

Positive samples should have PCR products of the expected size (341 bp), similar to the positivecontrol.

ii) **Negative samples**

Negative samples should have no PCR products of the expected size (341 bp), but mimic product (144bp) should be present.

iii) Unclear results

The assay must be repeated if the positive controls (mimic or external positive control) are negative, orif the negative water controls are positive**(Beier***et al***., 2004; OIE terrestrial manual 2012; Vandermaaten& Miller, 1976; Venables& Martin, 1997;**Monti et al., **2005;Theilen***et al***., 1982)**.

STATISTICAL ANALYSIS

The validity of the nested PCR technique for the detection of BLV in cattle was evaluated using ELISAas gold standard.

ELISA and nested PCRresultsfor234 cattle samples were constructed in a **2-by-2 Table** in which the final nested PCR result was cross tabulated with the ELISA results, thus defining true positive, false-positive, false-negative, and true negative values. They were used to calculate the standard diagnostic accuracy indices of sensitivity, specificity, negative predictive values, and positive predictive values. To determine the level of inter-rateragreement between ELISA and nested PCR in cattle samples, kappa values were calculated, and the strength of agreement was interpreted using a criteriain which a value of 0 to 0.20 is slight,0.21 to 0.40 is fair, 0.41 to 0.60 is moderate,0.61 to 0.80 is substantial, and 0.81 to 1 is almost perfect, with a significant difference between observers at a P value of<0.05.

RESULTS

Overall prevalence of anti-BLV antibodies in dairy cattle blood samples were 0% and15.38%, respectively. When using ELISA as a gold standard, sensitivity and specificity for nested PCR were 100% and 97%, respectively. The predictive value of a positive test was 70% and the predictive value of a negative test was 100%.Interpretationofkappa scores for two methods accounted substantial 0.85. The percentage of accuracy between two tests accounted 97%.We find a direct relationship

between aging and percent of infection(p<0.05) and also between number of parturition and percent of Infection (p<0.05).

DISCUSSION

The causative agent is BLV, an exogenous C-type oncovirusin the Retroviridae family. It isone of the important cattle viral diseases that lead to permanent infection (PI) and also permanent lymphocytosis and lymphosarcoma (**Radostits *et al.*, 2007**).

AGID test is a good screening test to determine the presence of infectionin an individual animal or herd(**Johnson &Kaneene, 1992**). The estimated specificity of 99.8% and the sensitivity of 98.5% indicate that the test is are liable and accurate method to detect BLV infection (**Radostits *et al.*, 2007**).

In more recent years, ELISA-based testing has replaced the AGID in eradication programs in several countries. The superior sensitivity of the ELISA for pooled serum samples allows detection of antibodies in herds with aprevalence of less than 1%, whereas the AGID test detected only 50% of the herds detected by the ELISA (**Johnson & Kaneene, 1992**). Two commercially available ELISAs and the polymerase chain (PCR) were evaluated and compared with the AGID to detect antibodies to BLV or its nucleic acid (**Reichel*et al.*, 1998**). The ELISA tests

detected about 10% more reactors than the AGID and the electrophoretic immunoblotting results (**Radostits *et al.*, 2007; Michaelzadeh & Badiei, 2005**).

Fechner*et al.,* compare the PCR and AGID methods. They demonstrated that this methodsensitivity is 10% more than ELISA and 17.7% more than AGID (**Fechner *et al.*, 1997; Fechner *et al.*, 1996**).

Martin *et al.,* also make a comparison between PCR as a direct method and ELISA and AGID as indirect methods. In that survey a specific primer is used for *env pol* and*tax*.PCR done on DNA extracted from peripheral blood monocytes or milk leukocytes. The high positive reaction is obtained by PCR and *env* or*pol* primers, then*tax*primer and finally by ELISA and AGID(**Martin*et al.*, 2001**).

Nagy *et al*., (2003)evaluated the PCR usage in recognizing of bovineleucosis in adult dairy cows. They demonstrated sensitivity of this test 67% and 1%, respectively. The predictive value of positive test was 1% and its negative predictive value was 42%. The cattle percentage which is correctly recognized by this method is 73.5%. However, the sensitivity and negative predictive value of this test is low, totally. Consequently, it is not suggested to employ PCR for recognizing of BLV in herds with high prevalence alone (**Nagy *et al.*, 2003**).

Serological surveys in cattle in the United States indicate prevalence rates within herds ranging from 0-100%. The disease does not spread rapidly and the number of herds containing positive reactors to the AGID test is usually small (**Radostits *et al.*, 2007**). However, in infected herds the number of sero positive animals may be as high as 80%. Infection with the virus is estimated to be at least20% in the adult dairy cow population of the United States, 6-11 % in Canada, 27%in France, 37% in Venezuela; in the United Kingdom the prevalence of infection is low. In New Zealand, it is estimated that about6.5% of the dairy herds have infected cattle, with an estimated within herd prevalence of 3.7% (**Reichel *et al.*, 1998**). The prevalence of infection in beefcattle in Australia is 0.22% (**Vanleeuwen *et al.*, 2001**).

In a nationalsurvey in Canada, 40% ofthe herdscontained BLV-infected cows. In PrinceEdwardIsland in Canada, 49.2% of theherds tested had at least one positivereactor, and 5.5% of all the cows testedwere positive. In maritime Canadian dairycattle, the individual cow prevalence was21% and the herd prevalence 70% (**Vanleeuwen *et al.*, 2001**).The seroprevalence of BLV infection in breedingbeed bulls under 2 years of ageoffered for salein Kansas was 8.5% (**Gnad *et al.*, 2004**). This indicates that

young bulls purchased for entry into recipient herds could be infected with the virus (**Radostits** *et al.***, 2007**).

In Argentina the individuals ero prevalence is 33%, while the percentage of infected herds with one or more infected animals is 84% (**Trono** *et al.***, 2001**).

An outbreak of enzootic bovin eleucosis in Egypt was associated with the importation of Holstein Friesian heifers and bulls from Minnesota in 1989 to form a closed dairy herd in Upper Egypt (**Zaghawa** *et al.***, 2002**).

In Iran, Haddadzadeh studied 4797cattle blood samples in dairy herds near Tehran that 471 of them (9.81%) recognized positive by the AGID test and the total 33 herds were tested,27 of them (81.88%) were infected (**Haddadzadeh & Bazargani, 1984**).

On the other study in Iran, Mashhadi studied 5 dairy herds around Shahriyar small provincethat the rate of contamination was 38.6%. The highest percentage was for the ages 6 through 11years old by 83% contamination and the lowest one was for the ages below 2 years old by 6% contamination (**Mashhadi & Badiei, 2001**).

On another survey which was done by Michaelzadeh in dairy herds near Tehran of Iran, 100% of the 6 studied herds were infected with BLV that from 4164 studied animals, 2006 of them (48.4%) were infected with BLV. The lowest infection was for the ages 6through 12 months (24.71%) and the highest infection was for the ages 61 through 72 months (77%) (**Michaelzadeh & Badiei, 2005**).

CONCLUSION

On the basis of our obtained results from the 6 herds with more than 50 dairy cattle around Babolsmall province(north of Iran), only one herd of daniyal company was not infected with BLV and from 234 studied cattle blood samples, 20 cases of them (8.5%) were infected with BLV. The lowest infection was for the ages 28 through 36 months with 0% contamination and the highest infection was for the ages 55 through 63 months with 14.3% contamination. The epidemiological aspects of BLV are reviewed with emphasis on diagnostic tests(ELISA and nested PCR).The definite and meaningful difference was also observed among the various studied ages by the Chi square test (p<0.05). We find a direct relationship between aging and percent of infection(p<0.05) and also between number of parturition and percent of Infection(p<0.05).

Our obtained results have conformity and agreement to the result of another papers and references.

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