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Adaptation of an indirect enzyme-linked immunosorbent assay by purified gp51SU for detection of antibodies to bovine leukemia virus

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Summary

The objective of this study was to compare an indirect ELISA, based on a purified 60 kDa envelope glycoprotein (gp51SU), with a Pourquire indirect ELISA for the detection of antibodies to the bovine leukemia virus. For conducting this research, 340 serum samples were collected from two different breeds of cows (Sarabi and Holestin) in different herds. Commercial ELISA revealed positive results in 17 (7%) Holstein cows. An appropriate ELISA cut-off was determined by receiver operating curve (ROC) analysis in comparison with commercial indirect ELISA. Results showed a relative sensitivity and specificity of 97% and 92%, respectively, for a cut-off value of 0.34 in the domestic ELISA. In conclusion, the results of the present study showed that domestic developed kit can be used for diagnosis of bovine leukemia virus with appropriate sensitivity and specificity. In addition, a comparison of the results from a native breed, Sarabi, with Holstein showed that there was no significant ($P>0.05$) difference in the frequency of infection with BLV between the two breeds.

Key words: Bovine leukemia virus (BLV), gp51SU, ELISA, Relative specifity, Relative sensitivity

Introduction

Bovine leukemia virus (BLV), the etiological agent of enzootic bovine leucosis (EBL) in adult cattle, is an oncogenic B-lympho-tropic retrovirus. The disease is divided into three stages: serologically positive, but negative for lymphosytosis (SP); serologically positive with persistent lymphocytosis (PL) and leukemia (Kettmann *et al*., 1994; Domenech *et al*., 2000; Van den Heuvel *et al*., 2003). There is no detectable viremia in infected cows but instead there is a strong and persistent response to BLV structural proteins, especially the envelope surface glycoprotein 51 (gp51SU) and core protein 24 (p24) (Kittelberger *et al*., 1996). In experimentally infected animals anti-gp51SU antibodies seem to appear earlier and at consistently higher titres than the anti-p24 antibodies (Simard *et al*., 2000; Trono *et al*., 2001).

The importance of BLV is based on the economic losses primarily due to condemnation of cattle with leukosis, reduction of milk production, and loss of trade opportunities with countries restricting the importation of BLV-infected cattle (Martin *et al*., 2001). In this regard, developing a reliable test for the early detection of infection is a requisite, especially for testing the large number of animals and lowering the cost of the assay.

The methods used most widely for serological diagnosis of BLV infections are agar gel immunodiffusion (AGID) test (Simard *et al*., 2000) and several variations of the enzyme-linked immunosorbent assay (ELISA) (Klintevall *et al*., 1991; Martin *et al*., 2001). The AGID antigens usually

contain both p24 and gp51SU, but this test detects primarily antibodies to gp51SU (Kettmann *et al*., 1994). Most of the commercially available ELISA kits are designed to detect antibodies to gp51SU.

ELISA technique has numerous advantages over the AGID. Besides giving an objective interpretation of the test result, the turn around time is shorter (a few hours compared to 48-72 h for the AGID test), the reagents are ready to use (no need to pour agar onto plates), and the test is easy and automated, which reduces the technicians hands-on time. Also, software is available to analyze and store data. Furthermore, in countries where the prevalence of BLV is low, the ELISA test was found to be sensitive enough to be performed on pooled serum samples, an approach that considerably reduces the work and cost of testing (Simard *et al*., 2000).

There are considerable reports regarding the technical modification and practical uses of ELISA test (Van den Heuvel *et al*., 2003; De Giuseppe *et al*., 2004; Monti *et al*., 2005; Juliarena *et al*., 2007). However, some unsatisfactory results have restricted its routine usage in clinical laboratories. One of them demonstrated false negative readings for some herds, even with a new generation kit (Naif *et al*., 1992).

The objective of this study was to develop an indirect ELISA kit; using a purified 60 kDa envelope glycoprotein (gp51SU); and compare the sensitivity, specificity and agreement of domestic indirect-ELISA kit to a well known commercial ELISA kit (from Pourquire Co., France) which is routinely used for diagnosis of BLV infection.

Materials and Methods

Sera

Positive serum: E05 reference serum was used as positive control serum.

Negative sera: 45 sera, confirmed negative by commercial ELISA kit.

Sample sera: 340 serum samples obtained from a native (Sarabi) herd and from eleven Holstein herds, in east Azarbaijan province, Iran.

Antigen preparation

Whole virus particles were harvested from FLK-BLV tissue culture medium through several steps of density gradient centrifugation. Purified virions were lysed in Tris-HCl buffer, 0.01 M, pH = 7.2 containing 1% (w/v) Triton X-100 and 10^{-4} M PMSF., gp51SU was purified by chromatography on a diethylaminoethyl (DEAE) anion-exchange column. Briefly, 400 µl of 7.5 µg/ml whole viral proteins were loaded on top of a hydrated DEAE-cellulose column (pH = 7.2) and incubated at 4°C for 2 h. Then, the column was washed by Tris-HCl buffer and gp51 was eluted by 3×10^{-3} M NaCl. The purity of gp51SU was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and immunostaining. The concentration of purified gp51SU was measured by Bradford method as described before (Devare and Stephenson, 1977).

ELISA

Each well of 96-well ELISA plates (CovaLink, Nunc, Denmark) was coated, in duplicate with 2 µg, 1 µg, 500 ng, 250 ng, 0 ng of antigen in 100 µl of coating buffer (carbonate-bicarbonate buffer 0.05 M, pH = 9.6) and incubated at 4°C overnight. The plates were then washed twice with washing buffer (phosphate buffer saline (PBS) pH = 7.2 containing 0.05% Tween 20). Excess binding sites were occupied by adding 300 µl of 3% bovine serum albumin (Merck-Germany) to each well and incubating for 60 min at room temperature. Sample sera were added to each well in 100 µl volumes and in two fold dilutions ranging from 1:10 to 1:320 and incubated at 37°C for 2 h. After washing three times, 100 µl of 1:4500 dilution of horseradish peroxidase (HRP) conjugated rabbit anti bovine IgG (Koma biotech, South Korea) was added and incubated for 2 h at 37°C. The plates were then washed four times with washing buffer prior to adding 100 µl of tetramethyl benzidine (Sigma, USA). After 5 to 15 min, the reaction was stopped by adding 100 µl of 2 N H_2SO_4 to each well. The absorbance at 450 nm for each sample was measured by ELISA reader (Dana 3200, Iran).

The average of two negative control serum readings was subtracted by the average of two positive control and/or serum samples and it was considered the result for each sample.

Commercial ELISA (Pourquier, France) was used according to the manufacturer's instructions.

Statistical analysis

The results of different diagnostics methods were submitted to statistical analysis using the SPSS and NCSS software for windows to determine the relative sensitivity and specificity of the test. Pourquire ELISA was used as the reference test to calculate the relative sensitivity and relative specificity of domestic indirect-ELISA. All ELISA values were analyzed using a receiver operating characteristic test (ROC, SPSS 16 software, SPSS Inc).

The relative sensitivity and specificity were calculated according to the following equations:

% relative sensitivity = (No. positive by both methods) / (No. positive by both methods + No. positive by reference test and negative by the method being compared with the reference test)*100

% relative specificity = (No. negative by both methods) / (No. negative by both methods + No. negative by the reference test and positive by the method being compared with the standard)*100.

In order to evaluate the agreement between the two diagnostic methods, Kappa measurement was employed. The kappa statistic varies between 0 (chance agreement) and 1 (perfect agreement), where

$0.00<\kappa<0.20$ as "slight,"

$0.21<\kappa<0.40$ as "fair,"

$0.41<\kappa<0.60$ as "moderate,"

$0.61<\kappa<0.80$ as "substantial,"

$0.81<\kappa<1.00$ as "almost perfect" agreement.

Levels of anti-BLV antibodies in Sarabi and Holstein breeds were compared by Student's t-test. The difference was considered statistically significant when p-value was less than 0.05.

Results

Affinity purification of gp51SU resulted in an antigen that was almost free from other viral proteins (Fig. 1).

To detect the optimum antigen concentration for coating, the same dilution of positive control serum was applied to the ELISA wells coated with different concentrations of antigen. The concentration of antigen in the well with the highest absorbance value was considered as the optimum amount of antigen for coating. According to this experiment, a concentration of 1 µg/ml of antigen was selected for development of domestic ELISA.

An ELISA OD of 0.34 was selected as the most appropriate cut-off in which diagnostic sensitivity and specificity were 97 and 92%, respectively. Using this ELISA cut-off, 28 (8.2%) of the 340 tested sera were positive (Table 1).

Table 1: A comparison of the detection of antibody against bovine leukemia virus (BLV) in 232 sera from Holstein breed and 108 sera from Sarabi breed by a commercial indirect-enzyme linked immunosorbent assay and a domestic indirect-enzyme linked immuno-sorbent assay

	Domestic indirect-ELISA		Commercial indirect-ELISA	
	+	-	+	-
Holstein	20 (9%)	212 (91%)	17 (7%)	215 (93%)
Sarabi	8 (7%)	100 (93%)	8 (7%)	100 (93%)

For the estimation of a negative cut-off for developing the test, 45 negative serum samples, which were confirmed by commercial ELISA, were applied in developing ELISA and their absorbance values were recorded. The frequency of observed absorbance values for negative serum samples is shown in Fig. 2.

On the assumption that data from the reference test and domestic ELISA are distributed normally (as shown in Fig. 2 for negative controls) the area under the plot of ROC was 0.980. The principle of ROC analysis is to generate a plot in which a grid of possible cut-off values is produced and for each cut-off value, the resulting sensitivity and specificity were calculated (Fig. 3).

The kappa value between domestic indirect-ELISA and commercial indirect-ELISA was 0.881.

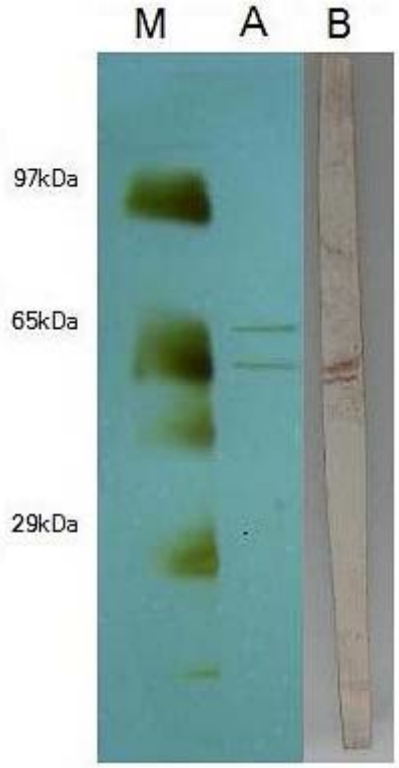

Fig. 1: Lane A: Purified gp51SU on SDS-PAGE after silver staining, Lane B: gp51SU detection in western blotting, and Lane M: Protein size marker

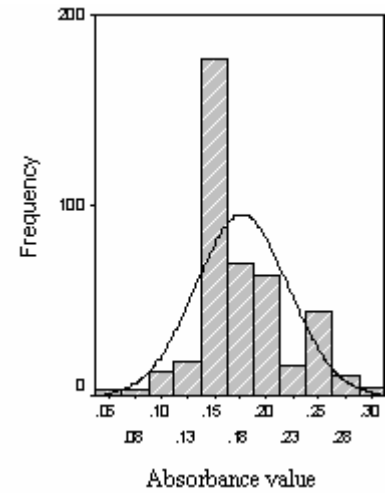

Fig. 2: Frequency of absorbance values of negative sera when 1 µg of coated antigen in each well

Discussion

Antibodies against gp5l are formed at a higher titer than are antibodies to the internal protein, p24, and serologic diagnosis of BLV infected animals has been based mainly on detecting antibodies to gp5l. As it

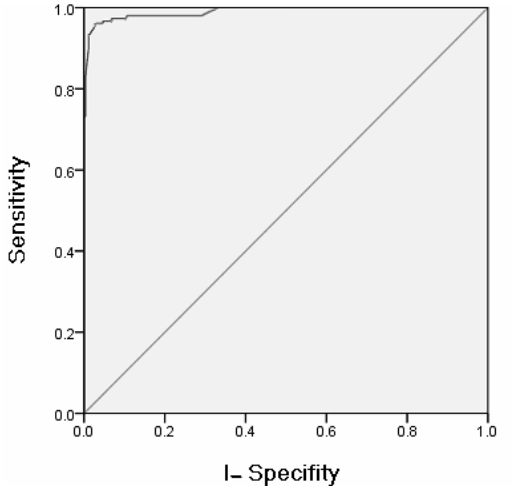

Fig. 3: ROC plot for BLV-gp51SU ELISA when Pourquire ELISA assumed as reference test. Area under plot = 0.980, Standard error = 0.016, Confidence interval = 0.95

was demonstrated before, anion exchange chromatography is a reliable method for purification of gp51SU, but it requires harvesting the virus particles from the cell culture (Devare and Stephenson, 1977). Although using pure antigens and specific antibodies considerably improve the sensitivity of ELISA, it also significantly increases the cost of the test (Van den Heuvel *et al*., 2003; De Giuseppe *et al*., 2004; Juliarena *et al*., 2007). For any given serological test, sensitivity and specificity are determined by the cut-off value (Swets, 1988). To consider a cut-off value, we assumed the highest left point on ROC plot as an appropriate cut-off point with respect to the relative sensitivity and specificity. Since making a cut-off point is a subjective issue, the selection of other cut-off points and comparison regarding relative sensitivities and specificities are also possible, specially for saving valuable animals in the case of high frequency diseases (Ridge and Vizard, 1993). The area under the ROC plot (0.980) shows the high accuracy of the domestic indirect-ELISA in distinguishing between positive and negative sera (Swets, 1988).

The sensitivity and specificity of domestics ELISA that is developed in the present study is comparable to the commercial ELISA kit and ELISA kits developed in other studies (Klintevall *et al*., 1991; Gonzalez *et al*., 1999; Monti *et al*., 2005). Furthermore, due to its high

sensitivity, it might, preferentially, be used in herds with a low frequency of the disease. Moreover, results of the kappa value show that the domestic ELISA may perform almost as well as the commercial ELISA, so it might be considered as a diagnostic test for BLV.

In summary, the results of the present study showed that domestic developed ELISA can be used for the diagnosis of BLV infection with appropriate sensitivity and specificity.

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