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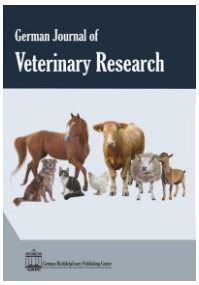
Short communication

A case study of enzootic bovine leukosis in an Albanian dairy herd based on serological and hematological test results

Majlind Sulçe[1], Anita Koni[2], Gerald Muça[1], P'ëllumb Zalla[1], Albana Munga[1], Valentin Shtjefni[2] and Xhelil Koleci[1]*

[1] Faculty of Veterinary Medicine, Agricultural University of Tirana, Tirana, Albania
[2] Food Safety and Veterinary Institute, Rruga Aleksand¨er Moisiu 82, Tirana, Albania



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***Corresponding author:**
Xhelil Koleci
E-mail:
xhelil.koleci@gmail.com

Abstract

Enzootic bovine leukosis (EBL) is a notifiable infectious disease with sporadic frequency in Albania. EBL is not a zoonotic disease, but it has an important impact on the cattle trade. Diagnosis of this disease can be performed by many diagnostic procedures, including agar gel immunodiffusion (AGID), enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) tests. In some cases where persistent lymphocytosis is present, cell count can turn useful. This study aimed to evaluate serological and hematological tests' suit-ability for the diagnosis of EBL and assess the manual and automatic hematological count technique diagnostic parameters compared to the serological test. Results suggest that the strategic use of hematological and AGID tests in parallel can increase the sensitivity of diagnosis and facilitate the detection of infected animals in different disease stages. Moreover, our results indicate that this approach is feasible in small-scale cattle herds size, which properly fits Albanian circumstances.

Keywords: Enzootic Bovine Leukosis, Cattle, Agar Gel Immunodiffusion, Hematology

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Introduction

Enzootic bovine leukosis (EBL) is an infectious disease of cattle caused by a virus of the "*Retroviridae*" family and can affect bovines at any stage of life (Ruiz et al., 2018). There is evidence that sheep, goats, and alpacas can be experimentally infected (Barez et al., 2015). The bovine leukosis virus primarily affects B cells, and in some cases, it induces persistent lymphocytosis and lymphosarcoma (Schwartz et al., 1994). Since the virus is present in the circulating peripheral blood, the transmissions can occur through the infected blood, and the iatrogenic route is important. Transmission of the virus can occur horizontally and vertically (Aida et al., 2013). The most common clinical signs include splenomegaly, lymph node enlargement, and gastrointestinal disturbances. Infected cows have a decreased yield of milk production compared with non-infected animals (Norby et al., 2016).

Mainly, clinical signs are due to B cell proliferation. Persistent lymphocytosis leads to immunological disorders that can make animals more susceptible to secondary and co-infection. Approximately 30-70% of infected animals do not show clinical signs and remain in a subclinical form throughout life. EBL is a notifiable disease and has a significant impact on the international cattle trade. Therefore, many countries have adopted different strategies to control and eradicate the disease, including different segregation measures to stamp out infected and in-contact animals. The availability and performance of diagnostic tests are important for diagnosing and controlling the EBL. There are different techniques in use for the diagnosis of EBL, involving hematology, agar gel immunodiffusion (AGID) (Simard et al., 2000b), enzyme-linked immunosorbent assay (ELISA) (Simard et al., 2000a; Walsh et al., 2013), and polymerase chain reaction (PCR) techniques (Klintevall et al., 1994; Nagy et al., 2003). Each of these techniques has its advantages and disadvantages. Hematology can be useful since several cases can develop persistent lymphocytosis, which can be observed when performing automatic and manual cell counts.

Since most EBL cases do not develop persistent lymphocytosis, only hematology can lead to a misdetection of a certain number of positive cases.

AGID is a technique used to detect the antibodies in bovine sera and is usually used for the routine operations of animal import and export (OIE, 2018). However, PCR and ELISA are better than AGID for their accuracy and sensitivity, respectively, in detecting bovine leukemia virus (Martin et al., 2001). On the other side, PCR or real-time quantitative PCR has the advantage of detecting the bovine leukemia virus several weeks before the appearance of antibodies in the blood. A combination of two or more tests will increase the chances of diagnosing the disease correctly. A decision on which technique should be used depends mainly on costs, availability, and time to generate results. In non-European Union countries, such as Albania, cattle herd circumstances do not always support the execution of relatively expensive tests such as ELISA and PCR; consequently, cheaper methods are more affordable.

Based on these considerations, the present study aimed to evaluate the suitability of serological and hematological tests for EBL diagnosis and assessment of the diagnostic parameters of the manual and automatic hematological count technique compared to the serological test.

Materials and methods

Sampling and sample processing

Blood samples were collected from coccygeal vena using a 9 ml plain vacutainer from a dairy farm situated in Durres district, Albania. From each blood sample, 2 ml whole blood was transferred immediately in a 2 ml K3 EDTA tube test for automatic and manual hematological testing. Collected blood samples were transported in a cold box to the infectious diseases laboratory at the Faculty of Veterinary Medicine, Agricultural University of Tirana. Blood samples collected in vacutainer without anticoagulants were preserved overnight at 4°C, and the blood sera were harvested by centrifuging at 3000 rpm for 5 minutes. Serological and manual tests took place in the laboratory of infectious diseases at the Faculty of Veterinary Medicine, Agricultural University of Tirana, while the automatic hematological analysis was conducted at a licensed private clinic in Tirana city. A month after blood sampling and testing, a positive cow was slaughtered, and the spleen and liver were available for macroscopic and microscopic examination.

Hematology

Hematology has been used as a means of detecting persistent lymphocytosis and as a tentative procedure for diagnosing EBL. The criteria of persistent lymphocytic- ptosis are an increased absolute lymphocyte count than the normal mean count plus 3 of the standard deviation (Radostits et al., 2007). Both automatic and manual tests were performed in parallel in all blood samples. An automatic blood analyzer (Pro Cyte DX Hematology Analyser) produced by IDEEX, USA, was used for Complete Blood Count (CBC) following the producer's manual. For the manual cytological evaluation, smears were prepared from the samples collected in K3 EDTA tubes, and May Grunwald Giemsa staining was performed in the laboratory. Briefly, after air drying, blood smears were fixated in absolute methanol for 20 minutes and stained with May Grunwald for 5 minutes, rinsed with phosphate buffer for 2 minutes, stained with Giemsa stain at a 1:20 ratio for 20 minutes, washed with distilled water. Cytological smears were examined under the microscope. At least ten microscopic fields with 400x magnification were evaluated by an expert M.S. cytologist, blinded to the other methods' results.

Serological evaluation

Serum samples preserved at -20°C were taken from the refrigerator and kept at room temperature for 2 hours before they were tested using the agar gel immunodiffusion assay (AGID) test. The test was conducted according to the OIE manual (OIE, 2018). A 1% agar solution in a buffer of 2 M Tris, pH 7, 2, with 8,5% NaCl was prepared. A 2.8 mm thick layer of 1% agarose in 0.85% NaCl with pH 7.2 was prepared for each Petri dish. Six circular wells 6.5 mm in diameter and 3 mm apart were cut out around a central well. Twenty- five microliters of viral antigen gp51In were placed in each plate's central well, and 50µl of one positive, one weak positive, and one negative sera control sample were placed in alternate wells. Simultaneously, 50 µl of tested sera samples were placed in the remaining wells. Plates were incubated at room temperature (20-25°C) in a humid chamber. The results were read every 24, 48, and 72 hours (OIE, 2018). Criteria used to evaluate positive and negative results were the formation of a well-defined line between antigen and sera samples. AGID was considered the gold standard test to evaluate hematological tests' diagnostic efficiency.

Statistical analysis

Concordance between manual and automatic methods was calculated using the Kendall W test. Contingency tables were used to observe hematological tests' analytical performance compared to AGID. The agreement between AGID and hematological analysis was assessed by employing the Chi-Square test. All statistical analysis was performed using a statistic online Statulator test (Dhand and Khatkar, 2014), and significance levels were set at $P<0.05$ for all tests.

Results

In total, 24 samples were collected and tested in parallel by using AGID and hematological tests. Based on the established cut-off for the lymphocytes, 12 samples were positive, and 12 were negative for the manual count, while for the automatic CBC, ten samples were positive and 14 negatives out of 24. Concordance between the manual and automatic methods was high (Kendall W test = 0.922, $p<0.05$), while concordance between all tests was not too high (Kendall W test = 0.572, $p>0.05$).

The mean number of lymphocytes for all samples was 66.9% (min 6 and max 99) for the manual count and 63.7% (min 35 and max 89) for the automatic count.

Table 1: Serological and hematological results based on agar gel immunodiffusion (AGID) test, manual Grunwald Giemsa staining, and IDEEX Pro Cyte DX automatic hematology analyzer.

		Manual count		Automatic count	
ID	**AGID Results**	Lymphocytes %	Result	Lymphocytes%	Result
1	Positive	99.0	Positive	88.9	Positive
2	Negative	87.0	Positive	77.8	Positive
3	Positive	92.0	Positive	79.9	Positive
4	Negative	29.0	Negative	53.5	Negative
5	Positive	29.0	Negative	46.5	Negative
6	Positive	6.0	Negative	35.0	Negative
7	Positive	99.0	Positive	53.7	Negative
8	Negative	64.0	Negative	47.8	Negative
9	Positive	71.0	Negative	71.8	Negative
10	Negative	73.0	Positive	81.3	Positive
11	Positive	65.0	Negative	63.6	Negative
12	Positive	24.0	Negative	35.7	Negative
13	Negative	34.0	Negative	42.3	Negative
14	Negative	89.0	Positive	80.9	Positive
15	Negative	85.0	Positive	72.4	Negative
16	Positive	90.0	Positive	77.2	Positive
17	Positive	96.0	Positive	89.3	Positive
18	Positive	96.0	Positive	82.7	Positive
19	Negative	28.0	Negative	49.9	Negative
20	Positive	91.0	Positive	86.6	Positive
21	Positive	96.0	Positive	73.1	Positive
22	Negative	50.0	Negative	45.3	Negative
23	Positive	62.0	Negative	53.5	Negative
24	Positive	51.0	Negative	41.1	Negative

Table 2: Contingency between serological results of agar gel immunodiffusion (AGID) test and hematological results of the automatic hematological test.

Automatic hematological method	**Results AGID**		**Total**	**confidence interval (95%)**	***P-value***
	Positive (%)	**Negative (%)**			
Positive	7 (70.00%)	3 (30.00%)	10	1.75 (0.31-9.75)	0.521
Negative	8 (57.14%)	6 (42.86%)	14		

Table 3: Contingency between serological results of agar gel immunodiffusion (AGID) test and hematological results of the manual hematological test.

Manual hematological method	**Results AGID**		**Total**	**confidence interval (95%)**	***P-value***
	Positive (%)	**Negative (%)**			
Positive	8 (66.67%)	4 (33.33%)	12	1.43 (0.27-7.52)	0.673
Negative	7 (58.33%)	5 (41.67%)	12		

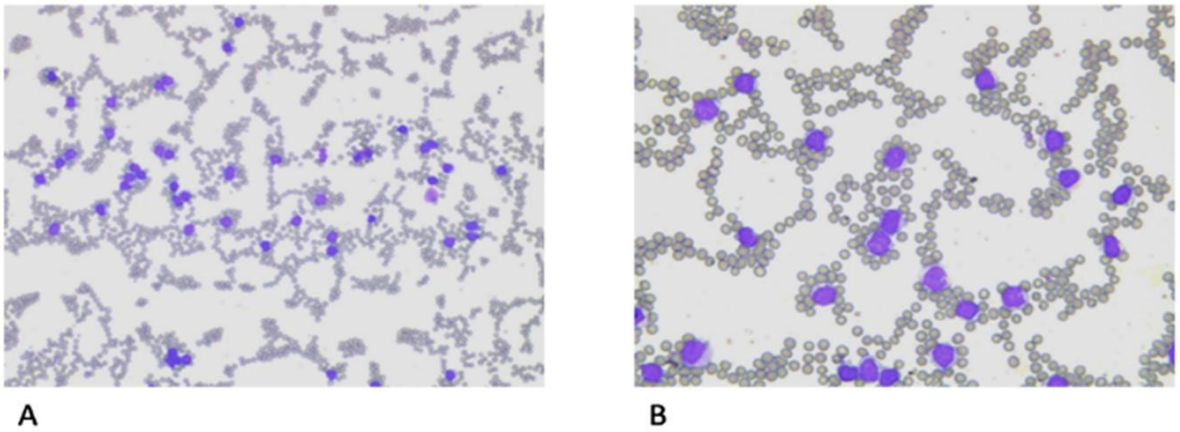

Figure 1: Cytological smears of a bovine leukemia virus (BLV) positive cow's blood samples. Note: high number of lymphocytes and the low number of red blood cells (A. 200× and B 400× magnification).

Results for all methods are presented in Table 1. The AGID test detected 15 (62.5%) positive and 9 (37.5%) negative out of 24 animals tested, revealing three and five positive animals more than manual and automatic test methods, respectively. The association between AGID and hematological methods (automatic and manual) was not significant ($p<0.05$).

Automatic CBC showed the following analytical values: sensitivity of 46.7%, specificity of 66.7%, positive predictive value of 70%, and negative predictive value of 42.8% (Table 2), compared to the manual count, which showed a specificity of 55.5%, sensitivity of 53.3%, diagnostic value of 54.2%, positive predictive value of 66.7%, and negative predictive value of 43.6% (Table 3). In Figure 1, the most representative case of persistent lymphocytes is presented.

Macroscopic examination of the liver and spleen indicates visible severe hepatomegaly and moderate splenomegaly. The liver structure also had a significant alteration with a visible change in color and consistency. Bile ducts were enlarged, while the spleen did not show such changes, except splenomegaly. Cytological evaluation of the liver showed typical lymphocytic infiltration, while few hepatocytes were seen, which explains the hardness of the liver tissues (Figure 2).

Discussion

In recent years, cases of EBL in Albania have rarely been reported. This study demonstrated that EBL is still circulating in dairy cattle and that different cost-affordable diagnostic tests can be performed with satisfactory results. In this study, the AGID test was chosen as the most sensitive and specific available test for bovine leukosis diagnosis, even though other serological tests, such as ELISA, have greater sensitivity (Malovrh et al., 2005). We compared the serological results with both manual and automatic hematological findings. The diagnostic tests used in this study measured different biomarkers, including antibodies and white cell linage (lymphocytes), which may explain at least in part the lack of full agreement between serological and hematological tests.

Nonetheless, the tests do not have a perfect agreement; it is useful to use them strategically in parallel as it will increase the sensitivity and facilitate the detection of infected animals in the different stages of infection. Despite the hematological test performed manually being time-consuming and possessing low sensitivity, it still could be used to diagnose EBL, especially when the molecular and serological tests are unavailable or unaffordable. Hence, the technique is more feasible, especially in Albanian circumstances, considering the very small size of cattle herds.

Referring to AGID as the gold standard test, the manual count (cytology) showed better sensitivity (53.3%) compared to the automatic CBC (46.7%). In comparison, specificity was higher for the automatic method (66.7%) than the manual one (55.6%). The manual count detected eight true positive cases compared to seven true positive cases detected by the automatic CBC. Despite these slight differences, both hematological tests showed a high agreement with each other. The automatic and manual methods de- detected five and three positive animals less than the AGID test, respectively. The established cut-off for persistent lymphocytosis was set as a mean value plus three standard deviations (Radostits et al., 2007).

The pathogenesis of EBL in affected animals may explain the visible disagreement between AGID and hematological methods. The animals usually start to become serologically positive after 3-8 weeks after infection, and limited numbers of the infected animals develop persistent lymphocytosis (Mammerickx et al., 1976). The negative hematological results of animals from positive herds must be interpreted carefully, and it is suggested to separate these animals from the affected herds.

Conclusions

To the best of our knowledge, this is the first study to use the automatic hematological method combined with the serological test to diagnose EBL in Albania. The results showed that tests are feasible and that performing AGID and hematological tests in parallel improves overall sensitivity and can detect more affected animals.

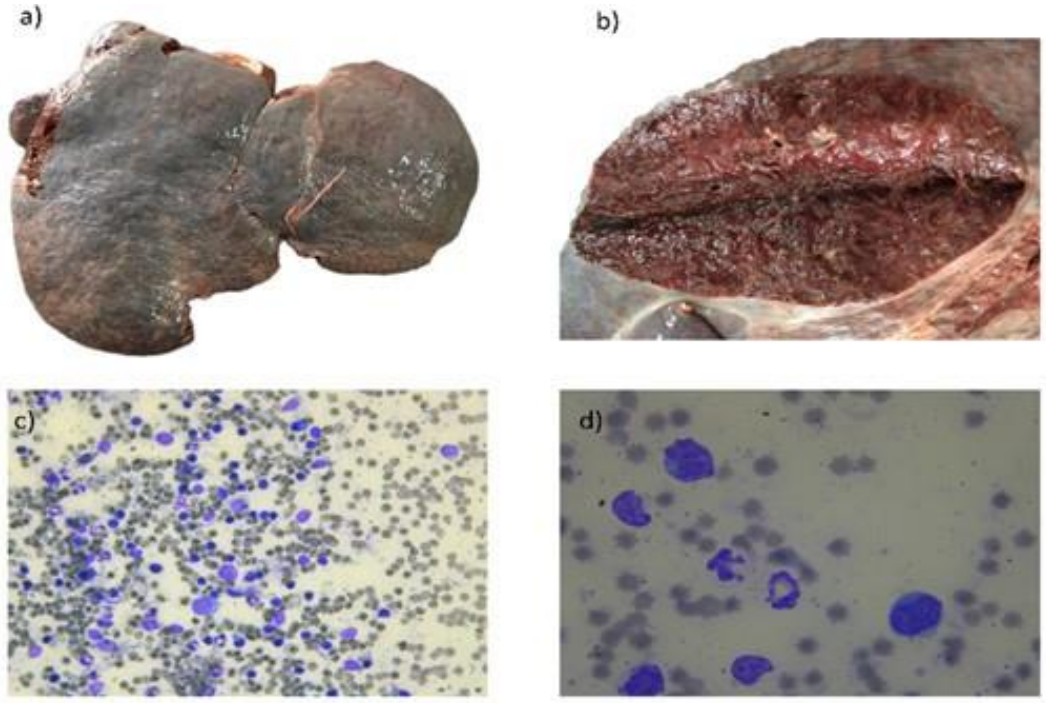

Figure 2: Macroscopic and microscopic liver findings of a cow who tested positive for EBL. Hepatomegaly (a) and alteration of hepatic tissue structure (b). Microscopic view: evidence of small and immature lymphocytic infiltration 400× (c) and 1000× (d) magnification.

This study has certain limitations, such as the modest number of individual animals and herds tested and the lack of comprehensive diagnostic methods, including molecular and ELISA tests. Those tests could confirm the presence of the EBL virus and improve diagnostic tests' overall performance. Further studies are needed to assess the EBL prevalence in the Albanian cattle population.

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