

## ENZOOTIC BOVINE LEUKOSIS: PERFORMANCE OF AN INDIRECT ELISA APPLIED IN SEROLOGICAL DIAGNOSIS

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### ABSTRACT:

Bovine Leukosis Virus (EBLV) is a widely distributed pathogen agent in the bovine population of many countries, especially in dairy cattle. Once the bovine is infected, it remains as a virus carrier for life and such state is correlated with a specific antibody detectable level. In this study the evaluation of an indirect ELISA (Leucokit-La Plata) to detect antibodies against EBLV is presented. Comparing it with the Immunodiffusion as gold standard test, the sensitivity is 98.93%, the specificity 79.74%, the negative predictive value 99.56% and the positive predictive value 61.26%. The correspondence between both tests is 83.9% which is similar to the result mentioned by other authors (82.2%). The concordance was evaluated by calculating Kappa and Youden's J coefficients, obtaining values classified as good for both coefficients. Comparing Leucokit-La Plata and another commercial reference kit (Chekit Leucotest Bommeli AG, Bern Switzerland), the sensitivity (97.05%), specificity (94.11%), negative predictive value (92.30%) and positive predictive value (97.77%), were obtained. Applying Kappa and Jouden's Index (J) coefficients an almost perfect concordance was obtained between both kits. The Leucokit-La Plata is appropriate to apply to the commercialization of live bovines to export, bovine selection for hemo-vaccines and the implementation of control and eradication programmes.

**Key word:** bovine leukemia, ELISA evaluation.

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### INTRODUCTION

Bovine Leukosis Virus (BLV) is a pathogenic agent of significant importance in bovines from the American continent since it is widely distributed mainly in dairy cattle. This has also been pointed out in other continents and great efforts have been made in many countries for its control and eradication. These efforts are based on the application of tests such as immunodiffusion (ID), enzyme linked immunosorbent assay (ELISA), western blot (WB) and polymerase chain reaction (PCR) (2-10,13,15,20,24,25), following the separation or removal of the virus-carrier animals.

Serological test has been used for a number of years to identify infected cattle and traditionally the ID has been used. In more recent years, different ELISAs have been used in eradication programs and there are several commercial kits

available to detect antibodies against the principal viral proteins (gp51 and p24) (20). WB is an other serological test which is highly specific and suitable to be introduced as confirmatory test at late stages in the eradication programs (9).

PCR, a recent molecular biological tool, remains labour intensive and expensive to perform which limit the use to large commercial or research oriented diagnostic laboratories (20).

BLV infection is mainly transmitted horizontally, iatrogenic via, through exposure of the susceptible bovines to "B" lymphocytes virus carriers. Blood and milk, to a lesser extent, are the main sources of transmission and the vertical way can be responsible for approximately 5% of the positive calves born of carrier dams. Prevalence increases as from six months of age, with a greater incidence between 2 and 5 years, being greater in milk bovines than in beef bovines (4,13).

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Once infected, the bovine remains as a virus carrier for life and such state correlates with the specific antibody detectable level.

Serological tests applied in European countries to detect seropositive animals shall comply with the European Union Regulations (88/406/EEC) and the OIE Manual using the international reference serum E-4, which defines the inferior sensitivity limit for each case (7,11,19).

In the present study, the behavior of an indirect ELISA (Leucokit-La Plata) developed in the Virology Chair, Veterinary Sciences Faculty - La Plata National University (VSF – LPNU) is analyzed, comparing it with the ID test of recognised specificity and practicality, approved by the Office International des Epizootie (OIE) and still in use in several countries (5,12,24) as well as the commercial reference ELISA kit (Chekit Leucotest Bommeli AG, Bern Switzerland).

## MATERIALS AND METHODS

**Samples:** 1529 sera obtained from dairy farms from the provinces of Santa Fe and Buenos Aires, Argentina, were collected during the period 1999-2000. All of them were analyzed by the new commercial ELISA kit (Leucokit-La Plata) and ID (VSF - LPNU) tests.

Another 187 sera were analyzed using Leucokit-La Plata and the indirect reference ELISA (Chekit Leucotest Bommeli AG, Bern Switzerland).

**Immunodiffusion (ID):** all serum samples were analyzed using the commercial kit produced in the Chair of Virology (VSF - LPNU) (approved by SENASA, Service of Animal Health, File No 41, 285/87). The kit detects positive reference serum E-4 diluted 1/10 in negative serum following standard european directives (19).

**Indirect ELISA (Leucokit-La Plata):** the technique was carried out according to manufacturers instructions using a partially purified antigen obtained from a cell line (FLK) persistently infected with BLV (17), concentrated by centrifugation and precipitation. The crude antigen was available at a concentration between 33 and 60 mg/ml protein and stored in 0.1 ml aliquotes at -20°C until used. The protein constitution of the antigen was analyzed by Western Blot following the technique previously described by Gonzalez *et al.*, 1999 (9).

**Cut-off value (11):** mean of negative sera + 3 standard deviation (confidence interval: 99%) was established with 100 sera from three herds without disease data and seronegative by ID in three consecutive tests with a three month interval in each case. This cut-off value allowed us to define the reference control serum E-4 as positive, diluted 1/10 in negative serum. That value showed the best correspondence between our challenge test (Leucokit-La Plata) and the gold standard test (ID). According to the OIE (19) the analysis of the test results allows us the following considerations: the non infected individuals are classified as true negatives (TN) and as false positives (FP),

unless the test is perfectly specific (where only TN results are obtained). On the other hand, the infected individuals are classified as true positives (TP) and as false negatives (FN) unless the test is perfectly sensitive (where only TP results are obtained).

This information is passed onto a 2 x 2 contingency table, which constitutes the standard for presenting the results to calculate sensitivity, specificity, prevalence and predictive values (26).

**Statistical methodology:** the steps required to validate a diagnostic test according to what is specified in the OIE Manual (19,27) were followed.

The optical density average values for each serum were passed onto forms and processed using Epilinfo version 5.01 (Epilinfo version V.5.01, Centers for Disease Control Epidemiology Program Office, Atlanta Georgia, World Health Organization (CDC/WHO) and WinEpiscope version 2.0 Epidecon (Software WinEpiscope 2.0 Facultad de Veterinaria Zaragoza, Wageningen University, University of Edimburgh. CONS I+D P50/98).

## RESULTS

In Table 1, the columns refer to the “gold standard” ( ID test) and the rows refer to the challenge test ( Leucokit-La Plata, LK-LP). For the challenge test, the sensitivity was 98.93 %, the specificity was 79.74%, and the indexes of False negatives and False positives were 1.07% and 20.26%, respectively. These results are presented in Fig. 1.

The Apparent Prevalence was 39.50%, the Real Prevalence was 24.46%, the Negative predictive value was 99.56% and the Positive predictive value was 61.25%.

The correspondence between the two tests (Fig. 2) was 83.9%, which is similar to the result of 82.2% found by Fechner *et al.* (6).

**Table 1.** Results obtained in the analysis of 1529 sera samples for the detection of antibodies against Bovine Leukaemia Virus after application of *LEUCOKIT -La Plata* and Immunodiffusion.

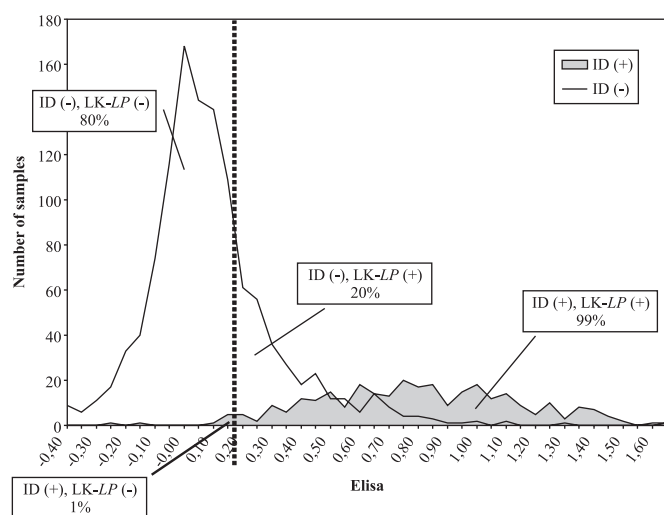
	ID(+)	ID(-)	Total
<b>LK-LP (+)</b>	370	234	604
	TP	FP	
<b>LK-LP (-)</b>	4	921	925
	FN	TN	
<b>Total</b>	374	1155	1529

LK-LP (+): LEUCOKIT -La Plata positive; LK-LP (-): LEUCOKIT -La Plata negative; ID (+): Immunodiffusion positive; ID (-): Immunodiffusion negative; ID nc: Immunodiffusion not conclusive; TP: true positive; FP: false positive; TN: true negative; FN: false negative.

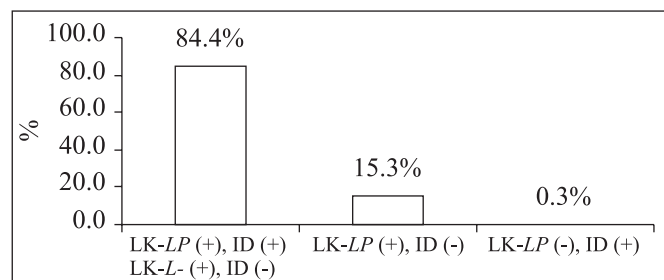
The Kappa coefficient (14,18) measures the agreement level when two tests classify the results in two or more excluding categories (infected, non infected). In this case the value was 0.65, considered a good concordance according to the following scale (16): <0 = no agreement; 0 - 0.20 = insignificant; 0.21 - 0.40 = medium; 0.41 - 0.60 = moderate; 0.61 - 0.80 = good; 0.81 - 1 = almost perfect.

The Youden's Index (J) (7) was 0.78. Values close to 0 denote discordance between the tests. Youden's J value 0.78 corresponds to a good concordance (1).

The results obtained for 187 analysed sera by Leucokit-La Plata and the reference kit (Chekit Leucotest - Bommeli) are



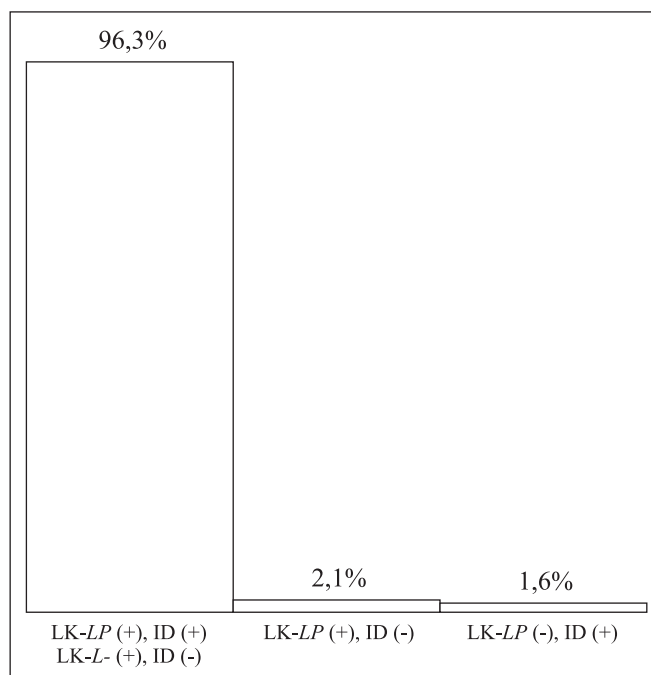
**Figure 1.** LEUCOKIT -La Plata and Imunodiffusion (FCV-UNLP). Results obtained after application of both tests on 1529 sera samples and their comparison with the cut-off value established for LEUCOKIT -La Plata; LK-LP (+): LEUCOKIT -La Plata positive. LK-LP (-): LEUCOKIT -La Plata negative; ID (+): Imunodiffusion positive; ID (-): Imunodiffusion negative.



**Figure 2.** Correspondence between LEUCOKIT -La Plata and Imunodiffusion applied on 1529 sera samples. LK -LP: LEUCOKIT -La Plata; ID: Imunodiffusion, (+): positive, (-): negative.

presented in Fig. 3 where a correspondence of 96.3% can be observed. This result is similar to that reported by other authors (97,7%) who compared different commercial ELISA kits (6).

In Table 2 the columns refer to the reference test (Chekit Leucotest Bommeli AG) and the rows to the challenge test



**Figure 3.** Correspondence between LEUCOKIT -La Plata and Chekit Leucotest Bommeli AG applied on 187 serological samples. LK -LP: LEUCOKIT -La Plata, CK: Chekit Leucotest Bommeli AG, (+): positive, (-): negative.

**Table 2.** Results obtained in the analysis of 187 sera samples for the detection of antibodies against Bovine Leukaemia Virus after application of LEUCOKIT -La Plata and Chekit Leucotest Bommeli AG

	CK (+)	CK (-)	Total
<b>LK-LP (+)</b>	132 TP	3 FP	135
<b>LK-LP (-)</b>	4 FN	48 TN	52
<b>Total</b>	136	51	187

LK-LP (+): LEUCOKIT -La Plata positive; LK-LP (-): LEUCOKIT -La Plata negative; CK (+): Chekit Leucotest Bommeli AG positive; CK (-): Chekit Leucotest Bommeli AG negative; TP: true positive; FP: false positive; TN: true negative; FN: false negative.

(Leucokit-La Plata). Applying a similar analysis to that showed before, the results obtained are: sensitivity 97.05%; specificity 94.11%; apparent prevalence 72.19%; real prevalence 72.72%; negative predictive value 92.30%; positive predictive value 97.77%; Kappa coefficient 0.90; Youden's Index (J) 0.91.

## DISCUSSION

The cut-off value of a diagnostic test is the scale point of measure from which the quantitative results are classified as positive or negative and it is the value from which the quantitative data are categorised. There are several methodologies to find that value depending of the type of values distribution (7,11,22). In our study we considered a normal distribution, according with the previous descriptive analysis of the original values.

The Leucokit-La Plata cut-off value has been confirmed using the reference serum E-4 diluted 1/10 in negative serum. It should be considered that any sera having a low tittle of antibodies can yield results close to the cut-off value, making it difficult to define them clearly as positive or negative. In these cases and as pointed out by Kozaczynska (15), sample analyses should be repeated, keeping the animals as no conclusive until the definite confirmation of the result is obtained.

In accordance with Reichel et al (20) the ID was considered as the "gold standard" test. It should be taken into account that the best "gold standard" test is a direct method or combination of methods that unequivocally identifies the virus carrier animal (23). In our case ID test has high specificity and unequivocally defines as positive the sample which is clearly reactive in the test.

From the results it can be seen that comparing Leucokit-La Plata and ID, high sensitivity (98.93%) and specificity (79.74%) with high predictive negative value (99.56%) were obtained. The Leucokit-La Plata antigen is constituted by two main immunogenic proteins of the virus (gp51 and p24) (10,11). The BLV infected bovines mainly yield antibodies against gp51. In early periods after infection and in animals with persistent lymphocytes a high tittle of antibodies against p24 (a core protein) is observed (15). For this reason, it is advantageous that both proteins constitute the antigen in Leucokit-La Plata. With reference to the application of Kappa coefficient or concordance proportion beyond chance, a value of 0.65 was obtained, considered as good or substantial according to Landis and Koch scale (16). The interpretation of the Kappa value in this case has restrictions since it was applied to two different serological tests (ID and indirect ELISA) and Kappa implicates that challenged tests are equivalent (28).

Applying Youden's Index (J) (or probability of correct classification independently of the prevalence) and with the same limitations previously mentioned (two different serological tests) a value of 0.78 was obtained and considered good according to the range established for this test (0: null and 1:

optimal). 83.9% of the sera were positive or negative in both tests. This correspondence is similar (82.2%) to the one found by Fechner *et al.* (6).

With respect to the comparison between Leucokit-La Plata and Chekit Leucotest Bommeli AG, Bern, Switzerland, (two similar diagnostic tests) a high concordance was obtained. The value of Kappa was 0.90, almost perfect according to Landis and Koch scale (16). Also in this case the Youden's Index (J) showed a high concordance (0.91). On the other hand, 96.3% of the sera were positive or negative in both tests.

Molecular diagnostic procedures (PCR) should not necessarily replace serological procedures, like ELISA, currently in use that had been proven to be cost-effective, rapid, sensitive and reliable and which are recognised as official test for commerce and trade purposes. The PCR is a technique especially appropriated for the diagnosis of virus infection where virus specific antibodies cannot be detected and the presence of viral genome is the only evidence of infection (21).

Finally we may conclude that Leucokit-La Plata has a high sensitivity (98.93%) and presents a high degree of concordance (Kappa 0.90) with the reference ELISA test (Chekit Leucotest Bommeli AG, Bern, Switzerland). The predictive Leucokit-La Plata values recommend its application in the commercialization of live cows (export) cow selection for hemo-vaccines and implementation of control and eradication programmes.

## RESUMO

### **Leucose bovina enzoótica: desempenho de um Elisa indireto empregado no diagnóstico sorológico**

O vírus da leucose bovina é um agente patogênico amplamente distribuído na população bovina de muitos países, principalmente naqueles com aptidão para leite. Uma vez infectado o bovino permanece como portador do vírus para toda a vida, e esse estado é correlacionado com o nível detectável de anticorpos específicos. Neste trabalho apresenta-se a avaliação de um ELISA indireto (Leucokit-La Plata) para a detecção de anticorpos contra VLB. Comparando com a prova de imunodifusão como teste de referencia a sensibilidade é de 98.93%; a especificidade de 79.74%; o valor preditivo negativo de 99.56% e o valor preditivo positivo 61.26%. A correspondência entre as duas provas é de 83.9%, semelhante aos resultados apresentados por outros autores (82.2%). A concordância entre as duas provas foi avaliada calculando-se os coeficientes Kappa e J de Youden e os valores obtidos foram classificados como bons. Fazendo-se uma comparação entre Leucokit-La Plata e outro kit comercial de referência (Chekit Leucotest Bommeli AG, Bern Switzerland), obteve-se uma sensibilidade de 97.05%, especificidade de 94.11%, um valor preditivo negativo de 92.30% e um valor preditivo positivo de 97.77%. Os coeficientes Kappa e index de Youden deram uma

concordância quase perfeita entre os dois testes. O equipamento Leucokit-La Plata é ideal para ser utilizado na comercialização de bovinos para exportação, para seleção de bovinos para hemovacinas e na implementação de medidas de controle e erradicação.

**Palavras-chave:** leucose bovina, ELISA avaliação.

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