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# Research Regarding the Diagnosis Certainty of Enzootic Bovine Leucosis

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**Abstract**: *Enzootic bovine leukosis (EBL) is a retroviral infection, common throughout the world, the evolution of which causes huge economic losses due to decreased milk production, weight loss and death of infected animals. Given the great economic importance of this disease, as well as the fact that the pathogenesis and dynamics of the immune response in EBL are not fully elucidated, the study aimed to investigate the presence of the bovine leukosis virus in animals that were declared healthy following clinical examination, by two classical serological methods, namely the enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID). It was also observed the correlation between the optical densities (OD) in the ELISA and the confirmation of the disease by gel immunodiffusion. For this, samples were taken from cattle, which were examined by ELISA (Ingezim BLV Compac 2.0 - Competition/Blocking Leukosis Kit), respectively by gel immunodiffusion (LEBCONTROL kit).*

## Introduction

The present study aimed to investigate the presence of enzootic bovine leukosis virus (EBLV) in blood samples collected from clinically healthy cattle using enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID). Furthermore, the study evaluated the proportion of ELISA-positive animals subsequently confirmed by AGID, as well as the correlation between optical density (OD) values obtained via ELISA and disease positivity determined through AGID in cattle exhibiting no clinical signs of infection.

## Materials and methods

Blood samples were collected from 175825 cattle from Alba County between 2021 and 2024 (Table 1). The 175825 cattle from Alba County came, mostly, from non-professional farms (households of the population), then from authorized person (AU) units and a significantly smaller number from farms within the county (Table 1).

**Table 1. Structure of cattle herds in Alba County according to the unit type**

| No. | Year | NPF* | AU units | Farms | Total bovines |
|---|---|---|---|---|---|
| 1 | 2021 | 18312 | 12984 | 9325 | **40621** |
| 2 | 2022 | 18143 | 13221 | 10082 | **41446** |
| 3 | 2023 | 22858 | 15590 | 8891 | **47339** |
| 4 | 2024 | 23327 | 14907 | 8185 | **46419** |
| TOTAL | | 82640 | 56702 | 36483 | **175825** |

Legend: *non-professional farms

For the laboratory analysis of the collected samples, a competitive or blocking ELISA was employed utilizing the INGEZIM BLV COMPAC 2.0 commercial diagnostic kit (Gold Standard Diagnostics Companies). This kit is characterized by a relative sensitivity and specificity of 100%. INGEZIM BLV COMPAC 2.0 is designed as a rapid, sensitive, and specific diagnostic tool for detecting antibodies against the enzootic bovine leukosis (EBL) virus in serum and plasma samples obtained from cattle. The resulting classification of sera is categorized as positive, equivocal, or negative.

For the confirmation of positive samples identified by the enzyme immunoassay test, the agar gel immunodiffusion (AGID) assay was employed. This method detects seroprecipitating antibodies present in infected animals. The principle of AGID involves the reciprocal diffusion of antigen and antibody within a gel matrix maintained at a pH of 7.2, initially devoid of reagents. As diffusion progresses, antigen-antibody complexes form precipitates at the zone of equivalence, manifested as distinct precipitation lines or arcs. The interpretation of the precipitation lines, characterized by a whitish appearance and intensity comparable to the positive control serum, determines the presence or absence of antibodies, classifying the sample as positive or negative. The LEBCONTROL kit (Romvac Company), which has a reported relative sensitivity and specificity of 100% along with established repeatability, was utilized to perform the AGID reaction. The kit includes a standardized antigen and reference sera for consistent analysis and reporting.

## Results and discussions

In 2021, 40621 samples were collected and tested for the detection of serum antibodies against enzootic bovine leukosis virus. In the first stage, following testing by the enzyme-linked immunosorbent assay (ELISA), 22 samples out of the 40621 samples came out positive. Subsequently, out of the 22 positive samples by ELISA and retested by agar gel immunodiffusion (AGID), 11 samples were confirmed (Table 2).

**Table 2. Results of testing samples taken in 2021, by ELISA and AGID**

| No. | Test results | | | No. | Test results | | |
|---|---|---|---|---|---|---|---|
| | ELISA | O.D. Unit. ELISA | AGID | | ELISA | O.D. Unit. ELISA | AGID |
| 1 | Positive | **0.295** | **Positive** | 12 | Positive | 1.053 | Negative |
| 2 | Positive | **0.068** | **Positive** | 13 | Positive | 0.805 | Negative |
| 3 | Positive | **0.097** | **Positive** | 14 | Positive | 0.742 | Negative |
| 4 | Positive | **0.102** | **Positive** | 15 | Positive | 0.733 | Negative |
| 5 | Positive | **0.091** | **Positive** | 16 | Positive | 0.737 | Negative |
| 6 | Positive | **0.100** | **Positive** | 17 | Positive | 0.841 | Negative |
| 7 | Positive | **0.094** | **Positive** | 18 | Positive | 0.979 | Negative |
| 8 | Positive | **0.104** | **Positive** | 19 | Positive | 0.973 | Negative |
| 9 | Positive | **0.070** | **Positive** | 20 | Positive | 1.045 | Negative |
| 10 | Positive | **0.114** | **Positive** | 21 | Positive | 0.931 | Negative |
| 11 | Positive | **0.101** | **Positive** | 22 | Positive | 1.053 | Negative |
| **X̄±SD** | | **0.11±0.06** | | | | **0.90±0.13** | |
| **CV(%)** | | **0.55** | | | | **0.15** | |

Legend: X̄ = arithmetic mean; SD = standard deviation; CV = coefficient of variability; OD = optical density

Compared to 2021, in 2022, although a larger number of samples were tested, collected from 41466 cattle, all 22 positive samples, resulting from the laboratory examination performed using the enzyme-linked immunosorbent assay, were negative upon reconfirmation by agar gel immunodiffusion (Table 3). We also mention, compared to the results recorded for the samples examined in 2021, that the average OD value from the 22 samples, positive by ELISA but negative by AGID, was 0.770, higher than the value recorded for the samples positive by AGID in 2021, respectively 0.110 OD units.

**Table 3. Results of testing samples taken in 2022, by ELISA and AGID**

| No. | Test results | | | No. | Test results | | |
|---|---|---|---|---|---|---|---|
| | ELISA | O.D. Unit. ELISA | AGID | | ELISA | O.D. Unit. ELISA | AGID |
| 1 | Positive | 0.711 | Negative | 12 | Positive | 1.003 | Negative |
| 2 | Positive | 0.600 | Negative | 13 | Positive | 0.805 | Negative |
| 3 | Positive | 0.891 | Negative | 14 | Positive | 0.742 | Negative |
| 4 | Positive | 0.893 | Negative | 15 | Positive | 0.733 | Negative |
| 5 | Positive | 0.926 | Negative | 16 | Positive | 0.737 | Negative |
| 6 | Positive | 0.622 | Negative | 17 | Positive | 0.978 | Negative |
| 7 | Positive | 0.708 | Negative | 18 | Positive | 0.484 | Negative |
| 8 | Positive | 0.490 | Negative | 19 | Positive | 0.706 | Negative |
| 9 | Positive | 0.972 | Negative | 20 | Positive | 0.814 | Negative |
| 10 | Positive | 0.687 | Negative | 21 | Positive | 0.931 | Negative |
| 11 | Positive | 0.424 | Negative | 22 | Positive | 1.053 | Negative |

**Table 4. Results of testing samples taken in 2023, by ELISA and AGID**

| No. | Test results | | | No. | Test results | | |
|---|---|---|---|---|---|---|---|
| | ELISA | O.D. Unit. ELISA | AGID | | ELISA | O.D. Unit. ELISA | AGID |
| 1 | Positive | **0.119** | **Positive** | 12 | Positive | 1.054 | Negative |
| 2 | Positive | **0.237** | **Positive** | 13 | Positive | 1.092 | Negative |
| 3 | Positive | **0.121** | **Positive** | 14 | Positive | 1.046 | Negative |
| 4 | Positive | **0.174** | **Positive** | 15 | Positive | 1.130 | Negative |
| 5 | Positive | **0.163** | **Positive** | 16 | Positive | 1.120 | Negative |
| 6 | Positive | 0.693 | Negative | 17 | Positive | 1.071 | Negative |
| 7 | Positive | 0.732 | Negative | 18 | Positive | **0.122** | **Positive** |
| 8 | Positive | 0.924 | Negative | 19 | Positive | **0.244** | **Positive** |
| 9 | Positive | 0.528 | Negative | 20 | Positive | 0.473 | Negative |
| 10 | Positive | 0.606 | Negative | 21 | Positive | 0.479 | Negative |
| 11 | Positive | 0.517 | Negative | 22 | Positive | 0.576 | Negative |

In 2023, out of the 22 positive samples, resulting from the ELISA examination of 47,339 samples, collected from cattle from most of the sanitary and veterinary districts in Alba County, seven samples were confirmed by agar gel immunodiffusion, all from females of different ages (Table 4). The values of OD units, recorded by ELISA, were significantly different between the positive samples, confirmed by AGID, and the negative ones. Analyzing statistically the results recorded in the two categories of animals, we find, in the positive animals (confirmed with EBL) an average OD of 0.17±0.05 and a coefficient of variability of 0.32%, and in the negative ones, an average OD of 0.80±0.26 and a much higher coefficient of variability, respectively 1.046%. We note that the values of the OD units, recorded in the samples tested in 2023, are quite close to those found in the samples analyzed in 2021, which allows us to conclude that based on these results, it is possible to preliminary classify the animals into negative and positive animals (Table 4).

**Table 5. Results of testing samples taken in 2024, by ELISA and AGID**

| No. | Test results | | | No. | Test results | | |
|---|---|---|---|---|---|---|---|
| | ELISA | O.D. Unit. ELISA | AGID | | ELISA | O.D. Unit. ELISA | AGID |
| 1 | Positive | **0.125** | **Positive** | 12 | Positive | 0.621 | Negative |
| 2 | Positive | **0.105** | **Positive** | 13 | Positive | 0.561 | Negative |
| 3 | Positive | 0.601 | Negative | 14 | Positive | 0.743 | Negative |
| 4 | Positive | 0.924 | Negative | 15 | Positive | 0.633 | Negative |
| 5 | Positive | 0.579 | Negative | 16 | Positive | 0.469 | Negative |
| 6 | Positive | 0.436 | Negative | 17 | Positive | **0.153** | **Positive** |
| 7 | Positive | 0.637 | Negative | 18 | Positive | **0.116** | **Positive** |
| 8 | Positive | 0.613 | Negative | 19 | Positive | **0.094** | **Positive** |
| 9 | Positive | 0.360 | Negative | 20 | Positive | **0.257** | **Positive** |
| 10 | Positive | 0.737 | Negative | 21 | Positive | **0.080** | **Positive** |
| 11 | Positive | 0.963 | Negative | 22 | Positive | 0.621 | Negative |

Analyzing the results recorded in the tests carried out during 2024, we note the existence of significant similarities with the values recorded in the determinations carried out in 2023, namely, out of the 22 positive samples by ELISA, seven cases of disease outbreaks were confirmed by immunodiffusion in agar gel (Table 5).

## Conclusions

The enzyme-linked immunosorbent assay (ELISA) is the main laboratory method used to detect contamination with the enzootic bovine leukosis virus in most countries where bovine leukosis occurs. Following the laboratory examinations performed by us and recorded as positive by ELISA, the confirmation or denial of the definitive diagnosis was made using the agar gel immunodiffusion reaction. In all determinations performed during the four years of study (2021-2024), the average OD unit values in ELISA-positive samples were significantly lower ($p<0.05$), compared to the average values from negative animals. Based on the results obtained, following the analysis of the 88 ELISA-positive samples, we can state that the OD unit values obtained in the enzyme-linked immunosorbent assay allow classification into positive or negative animals.