Laboratory Diagnosis of Enzootic Bovine Leukosis Using the ELISA Test

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Abstract: *Enzootic bovine leukosis (EBL) is a chronic viral disease of cattle caused by the bovine leukaemia virus (BLV), a member of the genus Deltaretrovirus within the family Retroviridae. The virus affects lymphocytes, leading to malignant transformations and the formation of tumours in hematopoietic organs such as the bone marrow, spleen, and lymph nodes. Infection can be transmitted horizontally through blood, body fluids, and insects, as well as vertically via colostrum, milk, or during intrauterine development. Once infected, animals remain lifelong carriers, which facilitates the spread of the virus within herds. Proviral BLV DNA has also been detected in milk and meat products, raising concerns about a potential risk to humans. Diagnostic methods for EBL include serological tests (ELISA, AGID) and PCR. In the present study, 43 blood samples were analyzed using ELISA to detect antibodies against BLV. Statistical analysis indicated an approximately normal distribution of the results and confirmed the stability of the laboratory process. Additionally, an Ishikawa cause-and-effect diagram was created to identify factors influencing the spread of the disease, and a flowchart was developed to illustrate the laboratory diagnostic protocol for BLV detection. Effective BLV control requires strict biosecurity measures, regular monitoring, and segregation of infected animals. This summary integrates current knowledge on the epidemiology, diagnosis, control, and prevention of EBL, providing practical guidance for veterinary professionals.*

Keywords*: BOVINE LEUKOSIS; BLV VIRUS; CATTLE; BLOOD SAMPLES; ELISA TEST; DIAGNOSIS; PREVENTION; CONTROL; BIOSECURITY*

1. Introduction

Enzootic bovine leukosis (EBL) is a chronic viral disease of cattle caused by the bovine leukemia virus (BLV), which belongs to the genus Deltaretrovirus within the family Retroviridae [10]. The virus affects lymphocytes, inducing malignant transformations that may lead to tumor formation and severe disorders of hematopoietic organs such as the bone marrow, spleen, and lymph nodes [1, 11]. The disease has a chronic course and low contagiousness and occurs most frequently in cattle populations.

BLV infection can be transmitted both horizontally - through contact with blood, body fluids, contaminated instruments, or blood-sucking insects - and vertically, via colostrum, milk, or intrauterine transmission [2, 5, 10]. Once infected, animals remain lifelong carriers, even in the absence of clinical signs, which facilitates the spread of the virus within herds [5]. BLV proviral DNA has also been detected in milk and meat products, raising concerns about potential risks to human health [3, 4].

In the experimental part of this study, 43 bovine blood samples were analyzed using the ELISA method to detect antibodies against BLV. In addition, an Ishikawa cause-and-effect diagram was constructed to systematize the main factors influencing the spread of leukosis, along with a flowchart illustrating the laboratory protocol for infection detection. These experimental approaches contribute to a better understanding of the disease and to the development of effective control and prevention strategies.

The control of BLV requires the implementation of strict biosecurity measures, regular monitoring, and the segregation of infected animals [8]. Current research efforts focus on the development of safe and effective vaccines aimed at limiting the global spread of the virus and supporting its eventual eradication [9].

The aim of the present review and laboratory protocol is to summarize current knowledge on the origin, epidemiology, diagnosis, control, and prevention of BLV, providing both practical and scientific guidance for veterinary professionals [10, 11].

2. Methodology

Sample Collection and Transport

- Blood samples are collected from animals under sterile conditions.
- The storage conditions are checked to ensure they are appropriate (temperature between 2 – 8 °C, no hemolysis).
- If the storage conditions are compromised, the sample is excluded from the analysis.
- Samples are transported to the laboratory in a cooled state.
- Upon receipt, the accompanying documentation is verified (animal identification, farm, collection date, veterinarian's signature).
- Samples with incomplete or invalid documentation are not accepted.
- Properly documented samples are registered in the laboratory logbook with a unique identification number.
- Serum is separated from the blood sample by centrifugation.
- The sterility of the serum is checked - only clean samples are accepted for testing.

Performing the ELISA Test

1. Load the samples into the microplate according to the manufacturer's instructions.
2. Incubate the plate for the time and temperature specified in the kit protocol.
3. Wash the plate four times with wash buffer to remove unbound substances.
4. Add the conjugate - an enzyme-linked antibody specific to the BLV antigen.
5. Incubate again under controlled conditions (temperature and time).
6. Wash the plate four more times to remove excess conjugate.
7. Ensure that the plate is completely dry before proceeding - this is crucial for accuracy.
8. Add TMB substrate (TMB-Complete) - the enzyme reaction will produce a blue color.
9. Incubate at room temperature for a specific period.
10. Stop the reaction by adding the stop solution.
11. Read the results using a photocolorimeter at a wavelength of 450 nm.

The optical density (OD) of each sample is compared to the control values:

- Negative control – indicates baseline reaction level.
- Positive control – indicates maximum expected signal.

Each sample is interpreted according to the cut-off values specified by the ELISA kit:

- OD < Cut-off → Negative result;
- OD = Cut-off ± 10% → Doubtful result (requires retesting);
- OD > Cut-off → Positive result (animal is infected with BLV).

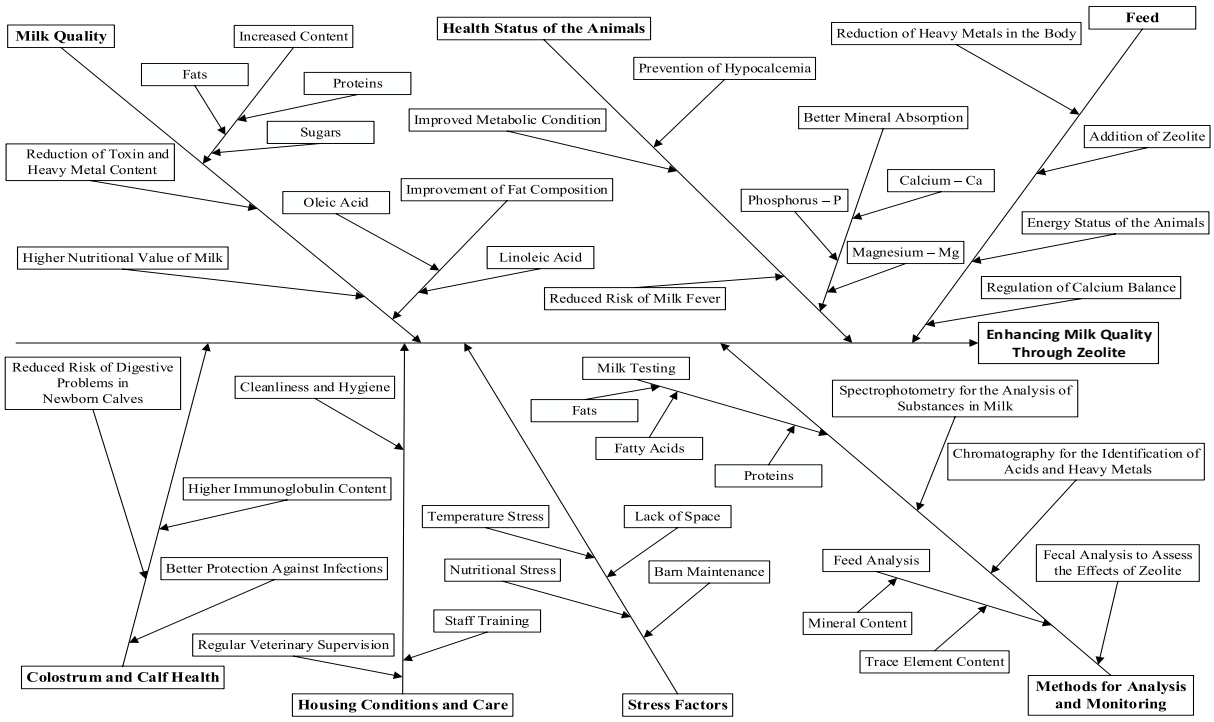

Fig. 1 *Ishikawa (Cause-and-Effect) Diagram.*

Quality Control

Each testing series must include positive and negative controls.

If the control results deviate from expected values, the test is considered invalid.

All data must be recorded in the laboratory logbook, including the date, operator's name, and sample number.

The ELISA method is a reliable, sensitive, and specific technique for detecting antibodies against the Bovine Leukemia Virus.

Strict adherence to temperature conditions, incubation times, and washing steps is essential, as deviations may lead to false-positive or false-negative results.

When performed correctly, ELISA enables timely identification of infected animals and implementation of control measures to manage and eradicate the disease in herds.

Optical Density (OD)

The decimal logarithm of the ratio between the intensity of incident light (I_0) and the intensity of light transmitted through the sample (I□) [12].

The test is valid if:

- OD P1 > 0.500 optical density (positive control);
- OD N1 < 1/3 × P1 optical density (negative control);
- OD C1 < 0.2 optical density (blank control).

3. Results and Discission

Enzootic bovine leukosis (EBL) is a viral disease caused by the bovine leukemia virus (BLV), which belongs to the genus Deltaretrovirus of the family Retroviridae [10]. The virus affects lymphocytes, inducing malignant transformations that can lead to tumor formation and severe disorders in the organism [1]. The disease is chronic and weakly contagious, occurring most frequently in cattle. The classical clinical manifestations include enlargement or formation of tumors in hematopoietic organs such as the bone marrow, spleen, and lymph nodes [11].

BLV infection is transmitted through infected blood lymphocytes to healthy animals. The transmission can be horizontal - via blood-sucking insects, contact with blood and body fluids - or vertical - through intrauterine infection, colostrum, or milk [2, 5, 10]. Proviral BLV DNA has been detected in milk and meat products, raising concerns about a potential risk to humans, as some studies associate the infection with an increased risk of cancer and other malignancies [3, 4].

After infection, cattle remain lifelong carriers and continuously produce antibodies against the virus, although most animals do not show obvious clinical signs [5]. The effect of BLV on milk production and animal longevity is controversial - some studies have found no significant impact, while others report reduced milk yield and fertility [5, 10].

Two main types of tests are used to detect BLV infection: serological tests (for antibodies) - AGID and ELISA - and PCR tests for viral DNA [6, 7]. Serological methods determine whether the animal has responded to the virus by producing antibodies, while PCR detects the viral genome and allows for early diagnosis. Colostrum containing antibodies against BLV may protect calves from infection [6].

The control of BLV requires the implementation of biosecurity measures and good herd management practices, which are labor-intensive, require strict compliance, and are influenced by environmental factors [8]. Safety remains a major concern, as vaccination may increase the risk of infection or restore viral pathogenicity. Epigenetic mechanisms and environmental conditions also influence the course of infection, making it difficult to use a single gene as a reliable marker for selecting resistant breeds [8]. Currently, the development of a safe and effective vaccine is considered the most appropriate approach to globally limit the spread of BLV [8, 9].

Infected animals are slaughtered separately in specialized abattoirs. If all lymph nodes are affected, the meat is used for technical purposes; if only certain lymph nodes are involved, the meat may be consumed after boiling. Meat from animals that test positive but show no lesions in the lymph nodes or internal organs is considered safe for consumption. When tests for Salmonella are

negative, disinfection is performed by boiling, and the intestines and internal organs are destroyed [11].

The purpose of this review is to summarize current data on the origin, genetic diversity, epidemiology, risk factors, and strategies for prevention and control of BLV, providing both practical and scientific guidance for veterinary professionals [10].

Fig. 1 presents an Ishikawa cause-and-effect diagram illustrating the main factors associated with the spread and prevention of bovine leukosis within a farm. The diagram is divided into seven main categories:

- Human factors;
- Animal factors;
- Biological factors;
- Transmission;
- Environment;
- Control through monitoring, testing, and isolation;
- Prevention.

Human factors – This category includes risks related to improper storage of test kits, insufficient staff training, poor hygiene during sample collection, and the need for training in good veterinary practices. Errors in these areas can lead to misdiagnosis and disease spread.

Animal factors – This includes the immune status of the animal, age (younger or older animals), and their role as virus carriers, which facilitates virus transmission.

Biological factors – These involve genetic predisposition to the disease and the potential for virus transmission between animals.

Transmission – This describes the different routes of viral spread: perinatal (from mother to calf), via blood, body fluids, milk, urine, and saliva.

Environment – Factors include stressful rearing conditions, improper sample handling, the presence of viral outbreaks in the region, and the need for regular testing of all animals. The importance of isolating newly introduced or infected animals is emphasized.

Control through monitoring, testing, and isolation – Measures include testing prior to vaccination or introducing new animals, separating BLV-positive and negative animals, using disposable needles and instruments, and ensuring proper sterilization.

Prevention – This encompasses strategies to minimize animal-to-animal contact, isolate infected animals, and implement other preventive measures that reduce the risk of leukosis transmission.

In Fig. 2 is shown the flowchart of Bovine Leukosis Laboratory Procedure, which begins with the collection of a sample for leukosis testing. Once the sample arrives at the laboratory, it is first checked to ensure that it has been stored correctly. At the same time, the accompanying documentation is reviewed. If the documents are in order, the sample is registered in the laboratory system. After registration, the serum is separated and prepared for use in determining whether the sample contains indicators of leukosis.

Before the analysis begins, the sterility of the plate used for the test is verified. Once sterility is confirmed, the serum is dispensed into the appropriate wells. A first incubation is carried out, after which it is checked whether the plate has remained under the required conditions for the proper amount of time. Upon completion of the incubation, the plate is washed four times to remove unbound components, and conjugate is then added. A second incubation follows, after which the plate undergoes another series of four washes.

After washing, the plate is examined to ensure that it is properly dried. Once ready, the TMB-Complete reagent is added, and the plate is left to incubate at room temperature. After this incubation step, the substrate necessary for visualizing the reaction is added. In the final step, the results are read and the process is completed.

The objective of this laboratory procedure is to determine the presence of antibodies against the Bovine Leukemia Virus (BLV) using the enzyme-linked immunosorbent assay (ELISA) method.

This method allows for the identification of seropositive animals and assessment of the epizootiological situation in livestock farms.

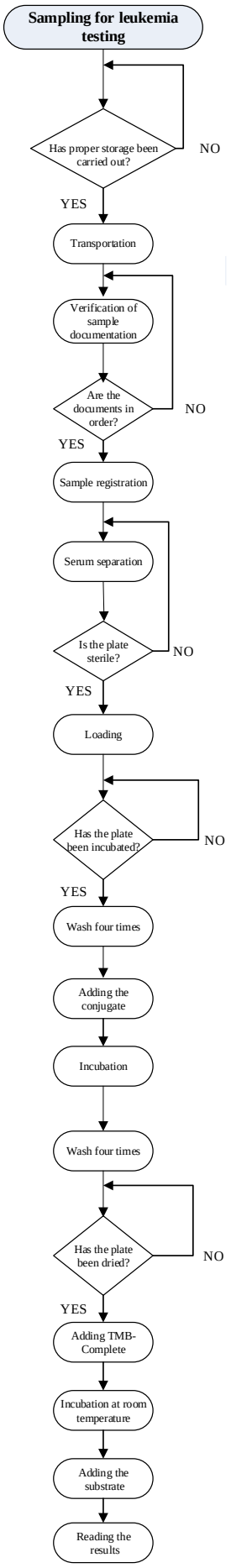

Fig. 2 Flowchart of Bovine Leukosis Laboratory Procedure.

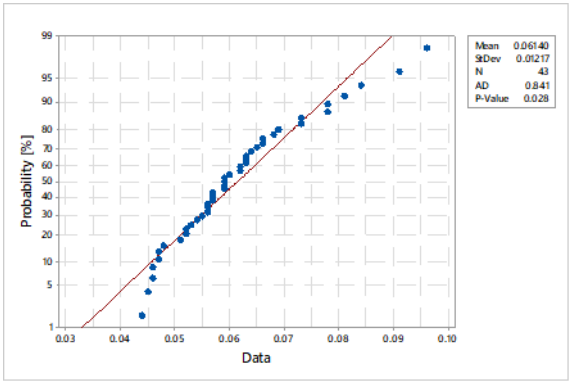

Fig. 3 *Verification of the normal distribution of blood sample data for Leukosis from the year 2025.*

Fig. 3 shown the normal probability of the blood sample data from 2025. It can be seen that the distribution of blood sample growth can be regarded as a random variable following a normal distribution. From the figure, it can be seen that the values are AD = 0.841, the Anderson–Darling coefficient is p = 0.028, and the number of observations is N = 43. The mean value of all data is = 0.06140, and the standard deviation is S = 0.01217. Based on the Anderson–Darling test, since p = 0.028 > 0.01, it can be concluded that the distribution is normal.

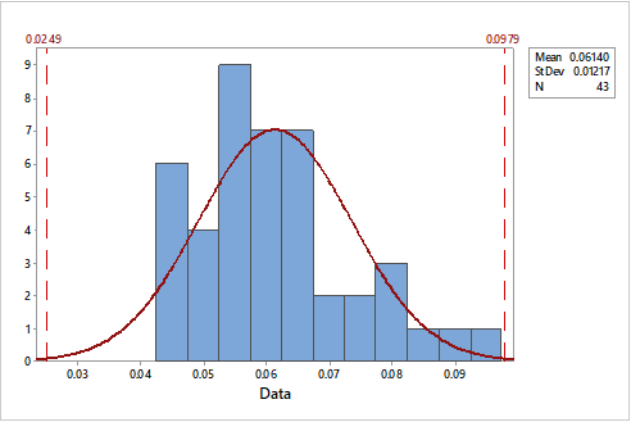

Fig. 4 *Histogram of the blood sample data from 2025 analyzed for Leukosis.*

In Fig. 4 the histogram of blood sample data collected in 2025 is presented. The normal probability density curve is also plotted on the histogram, illustrating the shape of the normal distribution corresponding to the sample values. The natural tolerance limits LNTL and UNTL were calculated according to the three-sigma rule.

These limits define the range within which approximately 99.7% of the values are expected to fall, assuming a normal distribution of the data.

From the Fig. 4, it can be seen that all of the samples fall within the interval between LNTL and UNTL, indicating that the data have a normal distribution.

No observations fall outside these natural tolerance limits, confirming that there are no extreme deviations among the samples.

The control chart in Fig. 5 shows the individual values of blood samples from the 2025 dataset. From the control chart can be seen that there is a point on the UCL and one that cross it and show that the process is not under statistical control. In same time there is not blood samples that are above the specification limit (0.632), and that shows that all the probes are negative for bovine leukemia virus and all tested livestock are healthy.

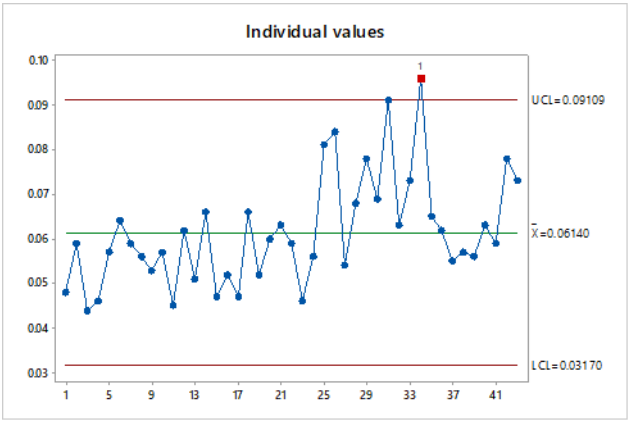

Fig. 5 *Individual values chart for the analysis of blood samples from 2025.*

4. Conclusion

Enzootic bovine leukosis (EBL) is a disease in cattle caused by the bovine leukemia virus (BLV). It is a chronic condition that spreads slowly among animals. The virus affects white blood cells and can cause serious changes in the hematopoietic organs. Once infected, cattle remain carriers for life. Because of this, eradicating the disease is difficult, and early detection and preventive measures are of great importance.

In the experimental part of the study, 43 blood samples were tested using the ELISA method. The results showed the presence of antibodies against BLV, indicating that the animals had been exposed to the virus. The test proved to be reliable and accurate for identifying infected animals. Data analysis demonstrated stability, with only one sample standing out and requiring further verification. The control chart confirmed that the laboratory process was properly conducted and that the results can be used confidently for preventive purposes.

The diagrams and charts used in the study help to understand how the disease spreads and how safety, control, and isolation measures for infected animals can be effectively applied.

In summary, combining knowledge about the virus with laboratory testing enables continuous monitoring of the infection and the development of effective strategies for prevention and control - protecting both livestock and humans.

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