COMPARATIVE ANALYSIS OF AN ELISA AND FLUORESCENT ANTIBODY TEST FOR THE DIAGNOSIS OF BOVINE LEUKAEMIA VIRUS INFECTION IN CATTLE

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Abstract

The study was performed on 60 Holstein-Friesian cows and 41 heifers. The results on the comparison of the efficacy of an ELISA and fluorescent antibody test (FAT) in the diagnosis of bovine leukemia, indicated that the FAT technique enables the identification of a greater number of bovine leukemia virus (BLV) carriers than the ELISA, thus suggesting that the FAT may be more useful than the ELISA for diagnosis of BLV infection.

Key words: cows, heifers, bovine leukosis, diagnosis, ELISA, immunofluorescence.

Bovine leukaemia virus (BLV; family Retroviridae, subfamily Orthoretrovirinae, genus Deltaretrovirus) is the aetiological agent of enzootic bovine leukosis (EBL), a chronic neoplastic disease. Viral genetic material integrates with the host cell DNA and remains in the host organism until its death. EBL is widespread, mainly in dairy cattle herds, and remains an important epizootic issue. The number of BLV infections in Poland, other European Union countries, and world regions (North America, Africa, Australia) remains high (14).

EBL is controlled by the elimination of BLV infection sources because there is no effective vaccine or treatment against this disease. Appropriate identification of BLV-infected animals is crucial. Such animals should be separated from the herd, as they can be the source of further infections. Although this disease has been controlled for more than 20 years with the application of many different diagnostic methods, the issue of EBL still requires further research. New infections in allegedly EBL-free herds are particularly troublesome. ELISAs are currently widely used in veterinary practice. They detect BLV antibodies in the serum and milk of infected animals. The ELISA is a highly sensitive and specific diagnostic test; however, the opinions of different authors are not unequivocal (4, 7, 11, 12). The aim of the present study was to compare ELISA with a fluorescent antibody test (FAT), which has not been used yet in EBL diagnostics.

Material and Methods

Material. This study involved 60 Holstein-Friesian cows and 41 heifers. Nineteen 3-month-old individuals were separated from the heifer group. The remaining twenty-two 6-month-old heifers constituted another group. The study animals originated from 3 large herds in the North-Eastern region of Poland. The herds were free from tuberculosis and brucellosis. Blood for analyses was sampled from the mammary or jugular vein. Heparin was used as an anticoagulant. The cows were examined in the first and third month after calving.

ELISA. The detection of BLV infection using ELISA was performed in a specialised diagnostic laboratory of the Veterinary Hygiene Department (HerdChec Bovine Leukaemia Virus Antibody Test Kit, IDEXX Laboratories, USA) while the FAT was performed on lymphocytes isolated as previously described (9). In this study, the Histopaque 1077 (Sigma Chemical Company) preparation was replaced with Gradisol L (Aqua-Medica, Poland).

Indirect fluorescent antibody staining. The FAT enables direct virus detection in infected lymphocytes. In the first stage of the reaction, cattle lymphocytes were incubated with a BLV3 monoclonal antibody (working dilution 0.3 µg/50 µl), specific for the viral major capsid protein p24 (VMDR Inc., Pullman). The binding of this primary antibody to viral antigen was detected using goat anti-mouse IgG (H+L) antibody conjugated with the fluorochrome phycoerythrin (PE) (orange signal; working dilution of 0.1 µg/50 µl) (Invitrogen, USA).

To perform the FAT, the isolated lymphocytes (1×10^6 cells in 1 ml) were suspended in a phosphate buffered saline, pH 7.4 (PBS), depleted of Ca^{2+} and Mg^{2+} ions (BioMed, Poland), containing 1% bovine serum albumin (BSA) and 0.02% NaN_3, and then incubated with the primary monoclonal antibody (30
min, 4°C) and washed 3 times (5 min, 200×g). The cell suspension was incubated with the secondary antibody (conjugated with PE; 30 min, 4°C) and washed 4 times (5 min, 200×g). The control was prepared with the same protocol but without the primary antibody. The smears were stored at 4°C and analysed at 400 or 1000× magnification with the use of a fluorescence microscope (Axiolab, Carl Zeiss, Germany) equipped with an appropriate filter. Six hundred cells were registered in each analysed smear.

**Statistical analysis.** The number and percentage of BLV-infected and -uninfected animals were determined and the significance of differences (P≤0.05, P≤0.01, and P≤0.001) between the two diagnostic methods was verified using the chi-square test. Calculations were made with STATISTICA 7.1 computer software (StatSoft, Poland).

**Results**

BLV–infected blood lymphocytes are presented in Figs 1A. and 1B. Infection was detected based on an orange signal emitted by PE, confirming the location of the viral major capsid protein p24. This protein was found in and on the lymphocytes. Moreover, using the FAT, in some cases the virus occurred only on the cell surface (Fig. 1C) leaving the cell interiors free from this pathogen. This seems to be an early BLV infection phase. Infection in this stage would not always result in viraemia and EBL development.

Based on the FAT results, almost all the studied cows (Fig. 2) and the majority of heifers (Fig. 3) were BLV-infected. In comparing the compatibility of the results obtained from the two methods, more negative results were obtained from the ELISA, both in the cows (44 results, 36.97%) and in the heifers (18 results, 43.9%). Consequently, the number of negative results was lower in the FAT (cows: 14 results, 11.76%; heifers: 9 results, 21.95%; Figs 2 and 3). In five 3-month-old heifers and in two cows, the results were reversed: positive in the ELISA and negative in the FAT. The results obtained with the methods in cows differed in the two analysed months and in heifers differed in only one of them. Based on the repeat examination of new portions of the same blood serum samples using the ELISA, the negative result obtained with the FAT was confirmed in both cows and in 3 out of 5 heifers.

The statistical analysis of the divergent results obtained with the ELISA and FAT methods showed the significance of the differences in the cows and older heifer groups. In cows, the largest differences between the number of negative results were found in the third lactation month (28.33% results; χ² = 14.60, P≤0.001), and slightly less divergent results were reported for the first month after calving (22.03% results; χ² = 7.11, P≤0.01; Table 1). Considerable differences were found in the 6-month-old heifers (50% results; χ² = 14.67, P≤0.001); however, in the 3-month-old heifers, the divergent results were sporadic and the differences were not statistically significant (10.53% results; χ² = 0.43, P=0.511).

![Fig. 1. Blood lymphocytes with the p24 protein present in the cells: A) 1 000x; B) 400x; C) p24 protein occupying the cell surface only, 1 000x; D) control, 1 000 x.](image-url)
Fig. 2. The number of EBL-positive and EBL-negative cows diagnosed in the first and third month of lactation with the use of the ELISA and FAT methods.

Fig. 3. The number of BLV-infected and BLV-uninfected heifers diagnosed in the third and sixth month of life with the use of both methods.

Table 1
The number of EBL-negative animals and differences between both methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Cows - month of lactation</th>
<th>Heifers - month of life</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>I (%)</td>
<td>III (%)</td>
</tr>
<tr>
<td>ELISA</td>
<td>23 (38.98)</td>
<td>21 (35.00)</td>
</tr>
<tr>
<td>FAT</td>
<td>10 (16.95)</td>
<td>4 (6.67)</td>
</tr>
<tr>
<td>Differences</td>
<td>13 (22.03)**</td>
<td>17 (28.33)**</td>
</tr>
</tbody>
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** P ≤ 0.01; *** P ≤ 0.001

Discussion

The percentage of BLV-infected animals detected with the employed FAT was greater than that obtained with the ELISA. Differences between the results obtained from the two studied methods were found in the 6-month-old heifers and the cows in the third month of lactation. All serologically positive results in cows and heifers, except two results in 3-month-old heifers, were confirmed using the FAT. The results of many authors indicate insufficient detection of BLV-infected animals with the use of the ELISA (3, 5, 6, 12). Consequently, unidentified virus carriers remain in the herd and are a source of further BLV infections.

This situation has an important effect on the eradicating of BLV infection and on the protection of leukosis-free herds against the introduction of BLV-infected animals. According to Kettmann et al. (10), false-negative results obtained in the ELISA can be obtained for animals in the early BLV-infection phase and when there is no detectable humoral response,
since the BLV antibody synthesis begins a few weeks after the infection. It can be suspected that such a situation occurred in the present study in the cows in the third month of lactation and in the 6-month-old heifers, in which low titres or even lack of antibodies could have caused the negative result in the serological test and a positive result in the FAT reaction.

According to some authors, the bovine leukaemia viral protein expression may have a temporary character and cause the periodical disappearance of antibody titres in blood serum to an undetectable level in a serological test (8). The lack of detectable specific antibodies in BLV-positive animals may also be caused by mutations within the env gene, which encodes antigen epitopes that determine the immunological properties of gp51 glycoprotein (2, 6, 13). These mutations may modify the antigenicity of the viral envelope protein and thus considerably reduce ELISA sensitivity, especially when the test detects only antibodies targeted against one epitope.

Moreover, ELISA results obtained in young calves may also indicate BLV infection of non-infected animals. False-positive results occur when non-infected calves are fed colostrum, which is the source of the gp51 antibodies (1, 5, 7). It seems that such a situation may have occurred in the present study in the case of two 3-month-old heifers, in which the positive results obtained in the ELISA were not confirmed in the FAT reaction.

The FAT indirect reaction applied in the present study allows for the detection of viral proteins directly in varied cells of an infected organism, regardless of the infection phase, concomitant diseases, animal age, pregnancy, and lactation month. Therefore, it is possible to detect BLV even in early infection phases when gp51 antibodies are not yet synthesised or when their level is low and below the detectability with the ELISA.

Results of this comparison indicated that the FAT technique enables the identification of a greater number of BLV carriers than the ELISA, thus suggesting that the FAT may be more useful than the ELISA for the diagnosis of BLV infection.

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**References**