Performance assessment of three tests applied in enzootic bovine leukosis diagnosis

Received for publication, May 20, 2014
Accepted, September 21, 2014

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Abstract

The aim of the present paper was the comparative assessment of two ELISA tests on serum and milk samples and agar gel immunodiffusion test (AGID) regarding ability to detect enzootic bovine leukosis (EBL) in Timis County, according to farm type, age, breed and stage of lactation, and how these parameters affect the humoral immune response of positive cattle. Serum samples were analyzed using blocking ELISA and AGID, and the milk samples by indirect ELISA, calculating quality parameters of applied tests. The obtained results showed that AGID detected 43 positive cattle and the two ELISAs 51 positive animals. Performances of AGID were influenced by farm type, age and serum antibody level. It was established that cattle aged 3-6 years were most prone to develop EBL. The level of serum antibodies has been influenced by farm type, age and lactation phase, and milk antibodies level strictly by the phase of lactation; the lowest values of milk antibodies were registered between 2nd and 8th month of lactation. Consumption of insufficiently heat-treated milk from BLV positive cattle in 2-8 months of lactation is associated with increased risk of infection in humans.

Keywords: enzootic bovine leukosis, serum and milk ELISA, AGID, level of antibodies.

1. Introduction

Enzootic bovine leukosis (EBL) is a malignant neoplastic disease of the reticuloendothelial system, specific for adult cattle, expressed by complex clinical signs induced by various locations of neoplastic B cells aggregations (M. Spînu [1]). It is caused by bovine leukemia virus (BLV), a retrovirus that belongs to the Deltavirus genus of the family Retroviridae, related to human T-lymphotropic virus (HTLV) and simian T-lymphotropic virus (F.A. Murphy et al. [2]). The genomes of BLV and HTLV I and II show 58, respectively 59% similarity regarding their nucleotide sequences (S. Dube et al. [3]).

Domestic cattle are natural hosts for BLV, and water buffaloes are considered wild reservoir of the virus (N. Gillet et al. [4], Office International des Epizooties [5]). Natural infection is usually asymptomatic (without clinical or hematological changes), approximately one third of infected animals develop persistent lymphocytosis, and only 0.1-10% tumors (Office International des Epizooties [5]). Even if it is a chronic disease associated with a relatively low mortality, EBL causes significant economic losses due to morbidity and increased susceptibility to other infections, decreased milk and meat production, costs of prevention and control measures, including compensations, and restrictions on the sale and export of dairy and meat products etc. (M. Spînu [1], L. Nuotio et al. [6], Z. Trainin & J. Brenner [7]).
BLV is horizontally transmitted, usually through the transfer of infected cells from blood and milk (direct contact, iatrogenic transmission, transfer via hematophagous insects, and milk consumption) (S.G. Hopkins & R.F. Di Giacomo [8]). Transmission via colostrum is considered negligible due to the protective role of maternal antibodies (S.M. Rodriguez et al. [9]). In utero transmission was demonstrated in cattle with persistent lymphocytosis (S.G. Hopkins & R.F. Di Giacomo [8], M.L. Lassauzet et al. [10]).

Although experimental transmission has been demonstrated in a number of species, including primates (M.J. Burridge [11]), only sheep, goats, rabbits and rats develop the disease (leukemia) (N. Gillet et al. [4], A. Florins et al. [12], M. Mammerickx et al. [13], P. Dimitrov et al. [14]).

Regarding risks to human health, a long time it has been considered that BLV is not pathogenic to humans (Office International des Epizooties [5], T. Burmeister et al. [15], R. Kettmann et al. [16]), but some new evidence contradicts this statement. Thus, anti-BLV antibodies were detected in 12.5 to 74% healthy people evaluated (Gh. Nikbakht et al. [17], G.C. Buehring et al. [18]), and 12.3% of tissue samples collected from seropositive people contained BLV proviral DNA (Gh. Nikbakht et al. [17]). Also, proviral DNA was detected in 35.8% of breast tissue samples collected from women with breast cancer (M. Giovanna et al. [19]). It was previously demonstrated that BLV can infect human myeloma cells (K. Slavikova et al. [20]) and human cells of neural origin (C. Altaner et al. [21]), causing also cytopathic effect in four cell lines of human origin (C.A. Diglio & J.F. Ferrer [22]). All this suggests a possible implication of this virus in the development and evolution of various types of cancer and neurological diseases in humans.

Due to the great economic and health importance, EBL is the subject of intensive eradication programs, implemented nationally and worldwide, based on early detection of positive cattle. Our country implements the measures specified in the Program of supervision, prevention, control and eradication of animal diseases, of those transmissible from animals to humans, animal welfare and environmental protection, identification and registration of cattle, pigs, sheep and goats of National Sanitary Veterinary and Food Safety Authority (NSVFSA [23]).

Early diagnosis is essential for EBL control, in this purpose being used enzyme linked immunosorbent assays (ELISA on serum samples and milk) and agar gel immunodiffusion test (AGID). These diagnosis methods led to the eradication of EBL in many European countries, such as Spain (1994), Cyprus (1995), Ireland (1997), Luxembourg (1999), Austria (2001), Switzerland (2005), United Kingdom (2006), Slovenia (2006), The Netherlands (2007), Sweden (2007) and Slovakia (2008) (Office International des Epizooties [5]).

This study aims to evaluate two ELISAs, on serum and milk samples, and AGID in terms of antibodies anti-BLV detection in cattle in Timis County, depending on farm type, age, breed and lactation phase. Furthermore, the influence of the four mentioned parameters on level of anti-BLV antibodies in serum and milk was assessed.

2. Material and methods

Cattle and biological specimens. We assessed 368 female clinically healthy cattle from 11 localities of the Timis County where, between 2008 and 2009 outbreaks of EBL were declared (the epidemiological data were obtained through the courtesy of the Timis Sanitary Veterinary and Food Safety Directorate). Cattle, selected based on a random cluster sampling program, were:
- from households (n = 283) and private farms (n = 85);
- aged between two and ten years, with the following categories: 2 years (n=51), 3 years (n=67), 4 years (n=81), 5 years (n=67), 6 years (n=53) 7 years (n=31), 8 years (n=12) and 9 years old (n=6);
- Romanian Spotted Cattle - RSC (n=122), Romanian Black Spotted Cattle - RBSC (n=96) and Holstein-RBSC crossbred (n=150);
- in different lactation phases: first month (n =54), 2-3 months (n=73), 4-6 months (n=55), 7-8 months (n=65), dry period (n=53) and 1-2 weeks before parturition (n=68).

Jugular venous blood samples were taken using sterile tubes without anticoagulant and serum separator. After clotting at 4°C for 5 hours, sera were obtained using standard procedure (C. Jury et al. [24]): the tubes were centrifuged for 10 min. at 1200rpm, the supernatant serum was transferred into another tube and centrifuged again, in the same condition, and the obtained sera were transferred into labeled cryotubes. Cryotubes were kept at -80°C until analysis.

Milk samples were collected using 10 ml sterile tubes, labelled in correspondence with blood samples, and were defatted according to instructions manual provided by the manufacturer of the milk ELISA kit: tubes were centrifuged for 15 min. at 2500g and skim milk below the fat layer was transferred in labelled cryotubes. Cryotubes were kept at -80°C until analysis.

**AGID** was conducted using the *Kit for serological diagnosis of EBL* (SN "Pasteur Institute" SA, Bucharest, Romania). The kit contains the following reagents: lyophilized ELB antigen, lyophilized ELB control (positive) serum and diluent. Agar gel and plate preparation, distribution of reagents and sera, reading and interpretation of the results were performed according to the standard procedure (Office International des Epizooties [5], J.M. Miller et al. [25]). Briefly, gel diffusion plates (0.8% noble agar and 8.5% NaCl) with wells filled with reagents and test sera were kept at room temperature in humid chamber for 72 h before the final reading. Test sera were considered positive when the precipitation line with the antigen was clear and equidistant from the wells and formed a line of identity with the control serum.

**ELISA.** Blocking ELISA for the detection of antibodies against BLV in cattle sera was performed using **ELISA Enzootic Bovine Leukosis Virus (BLV) Antibody Test** (IDEXX Laboratories, Inc., Netherlands). Instructions manual of the kit complies with the standard procedure of Office International des Epizooties [5]). Briefly, serum samples were thaw in water bath at 37°C; buffer solution 2 was added in microtiter plate precoated with BLV gp51, and after that test sera and positive and negative control were transferred; the plates were incubated 60 min. at 37°C, washed, and conjugate diluted 1:100 in buffer solution 1 was added in wells; before substrate addition, the plates were incubated 30 min. at 37°C and washed; the reaction was stopped by adding stop solution; microtiter plates were read at 450 nm. For each sample, antibodies level (%E/P) was calculated: %E/P = (OD sample / OD of positive control) × 100. Any sample whose % E/P was less than or equal to 100% was scored as BLV negative.

Indirect ELISA for the detection of antibodies against BLV in cattle milk was performed according to standard method of Office International des Epizooties [5], with some modifications specified in the instructions manual provided by the manufacturer of **DRG® Bovine Leukemia Virus p24-gp51 Ab serum, milk** kit (DRG International Inc., USA) used for assessment. Briefly, the milk samples were thaw in water bath at 37°C and diluted 1:2 in deionized water; diluted milk samples and negative and positive controls were added in washed microtiter plates precoated with p24 and gp51 viral antigens and monoclonal antibodies anti-BLV-p24 and anti-BLV-gp51; the plates were incubated for 60 min. at 37°C; after washing, HRPO conjugate, diluted 1:100 in HRP conjugate buffer, was added; after another incubation, in the same condition, and washing, substrate (equal parts of buffer A and
buffer B) was added, and plates were incubated for 15 min. at room temperature; thereafter, stop solution was added, and microtiter plates were read at 450 nm. A sample was scored BLV negative if the OD value was below or equal to the average OD value of the negative control plus 0.150 OD units (OD samples \( \leq \) mean OD negative control + 0.150 OD units).

**Comparison between serum ELISA, milk ELISA and AGID.** For each test, the proportion of positive and negative results, related to the farm type, age, breed and lactation phase of animals and relative to total samples assessed, were determined. The results of the three tests, in terms of positive and negative animal number, were passed onto a 2 x 2 contingency table, which is used to calculate sensitivity, specificity, prevalence and predictive values. Quality parameters were calculated using standard formulas, AGID being considered reference test (K.Y. Choi et al. [26], E.T. González et al. [27]). Also, apparent prevalence (% of positive samples only by serum and milk ELISA) and true prevalence (% of positive samples by ELISA and AGID) and Youden's index (shows correlation between sensitivity and specificity), and the correspondence between tests were determined (González et al. [27]).

**Statistical analysis** was performed using the Statistical Package for Social Sciences (SPSS, version 21, Chicago, IL, USA). In order to establish the influence of farm type, age, breed and lactation phase of assessed animals on tests performance and level of anti-BLV antibodies in serum and milk, data were classified as nominal and quantitative. Chi-square test was used to compare nominal variables. For quantitative data, mean, standard deviation, minimum and maximum values were determined. Differences of the mean of various categories were analyzed using one-way ANOVA test. Differences were considered significant when \( P \) values were less than 0.05.

3. Results

The two ELISA tests on serum and milk samples were 100% concordant, detecting an equal number of positive animals (n = 51) in total of 368 examined, with 8 more than the AGID. The comparative results of the three tests, according to farm type, breed and age of cattle are listed in Tables 1-3.

**Table 1.** Comparative results of AGID and serum and milk ELISAs according to farm type

<table>
<thead>
<tr>
<th>No.</th>
<th>Farm type</th>
<th>No. of assessed cattle</th>
<th>ELISA+ AGID+</th>
<th>ELISA+ AGID-</th>
<th>ELISA- AGID+</th>
<th>ELISA- AGID-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>1.</td>
<td>Households</td>
<td>283</td>
<td>34</td>
<td>12.01</td>
<td>7</td>
<td>2.47</td>
</tr>
<tr>
<td>2.</td>
<td>Private farms</td>
<td>85</td>
<td>9</td>
<td>10.58</td>
<td>1</td>
<td>1.17</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>368</td>
<td>43</td>
<td>11.68</td>
<td>8</td>
<td>2.17</td>
</tr>
</tbody>
</table>

**Table 2.** Comparative results of AGID and serum and milk ELISAs according to cattle age

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>No. of assessed cattle</th>
<th>ELISA+ AGID+</th>
<th>ELISA+ AGID-</th>
<th>ELISA- AGID+</th>
<th>ELISA- AGID-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>1.</td>
<td>2 years</td>
<td>51</td>
<td>3</td>
<td>5.88</td>
<td>1</td>
<td>1.96</td>
</tr>
<tr>
<td>2.</td>
<td>3 years</td>
<td>67</td>
<td>12</td>
<td>17.91</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.</td>
<td>4 years</td>
<td>81</td>
<td>21</td>
<td>25.92</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.</td>
<td>5 years</td>
<td>67</td>
<td>5</td>
<td>7.46</td>
<td>2</td>
<td>2.98</td>
</tr>
<tr>
<td>5.</td>
<td>6 years</td>
<td>53</td>
<td>2</td>
<td>3.77</td>
<td>1</td>
<td>1.88</td>
</tr>
<tr>
<td>6.</td>
<td>7 years</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3.22</td>
</tr>
</tbody>
</table>
Table 3. Comparative results of AGID and serum and milk ELISAs according to cattle breed

<table>
<thead>
<tr>
<th>No.</th>
<th>Breed</th>
<th>No. of assessed cattle</th>
<th>ELISA+ AGID+</th>
<th>ELISA+ AGID-</th>
<th>ELISA- AGID+</th>
<th>ELISA- AGID-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RSC</td>
<td>122</td>
<td>15</td>
<td>12.29</td>
<td>5</td>
<td>4.09</td>
</tr>
<tr>
<td>2</td>
<td>RBSC</td>
<td>96</td>
<td>14</td>
<td>14.58</td>
<td>1</td>
<td>1.11</td>
</tr>
<tr>
<td>3</td>
<td>H-RBSC</td>
<td>150</td>
<td>14</td>
<td>9.33</td>
<td>2</td>
<td>1.33</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>368</td>
<td>43</td>
<td>11.68</td>
<td>8</td>
<td>2.17</td>
</tr>
</tbody>
</table>

The eight cattle scored ELISA+ AGID- had the following characteristics regarding:
- farm type: seven were from households and one from private farms;
- age: one was 2 years old and the remaining seven were aged 5 years and above;
- breed: five were RSC, one RBSC and two H-RBSC;
- lactation phase: five were in dry period and three in 7-8 months of lactation.

The majority of the positive detected cattle by both serum and milk ELISA were from households (n = 41) RSC (n = 20) and aged between 3 and 5 years (n = 33).

Table 4 summarizes data used for the calculation of serum and milk ELISA quality parameters. The columns refer to standard test (AGID) and the rows refer to the challenge tests (serum and milk ELISA), that had the same results.

Table 4. 2 x 2 contingency table for quality parameters of serum and milk ELISAs calculation

<table>
<thead>
<tr>
<th>ELISA</th>
<th>AGID</th>
<th>AGID</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AGID+</td>
<td>AGID-</td>
<td></td>
</tr>
<tr>
<td>ELISA+</td>
<td>a = 43</td>
<td>b = 8</td>
<td>a+b = 51</td>
</tr>
<tr>
<td>ELISA-</td>
<td>c = 0</td>
<td>d = 317</td>
<td>c+d = 317</td>
</tr>
<tr>
<td>Total</td>
<td>a+c = 43</td>
<td>b+d = 325</td>
<td>N=a+b+c+d = 368</td>
</tr>
</tbody>
</table>

After applying the standard formulas, the following values of the two ELISAs quality parameters were obtained: sensibility = 100%, specificity = 97.53%, positive predictive value = 84.31% and negative predictive value = 100%. The apparent prevalence was 13.85% and true prevalence 11.68%. Youden’s index was 0.97 and correspondence between tests 84.31% (Figure 1).
According to the farm type, there were significant higher OD and %E/P values (P = 0.003) of anti-BLV serum antibodies in cattle from private farms compared to positive cattle from households (Table 5), but the level of milk antibodies was not significant affected by the farm type (P=0.504).

As it can be seen from Table 6, age groups analyzed were characterized by varying levels of serum anti-BLV antibodies, but the significant differences (P = 0.005) were recorded only between 3 years old cattle on the one hand, and 4 years and 5 years old cattle, on the other hand. The differences between these groups regarding milk antibodies were not significant (P = 0.49 and 0.71). The other age groups were not statistically analyzed due to the low number of positive cattle.

The differences in milk and serum antibodies level for the three local breeds were not significant (P = 0.57) (Table 7). After adding a second discrimination element, respectively farm type, we obtained significant differences (P = 0.001) between H-RBSC from households and farms in the serum antibody level (OD = 2.23±0.49 and %E/P = 125.24±28.16, respectively OD = 3.10±0.22 and %E/P = 174.14±12.75). Significant differences (P = 0.001 and P = 0.002) were also obtained between serum antibodies level of H-RBSC from farms on the one hand, and RBSC (OD = 2.60±0.53 and %E/P = 145.15 ± 33.29) and RSC (OD = 2.60±0.53 and %E/P = 145.66±30.60) from households, on the other hand. Differences between breeds considering milk antibodies level were insignificant, whether related or not to the farm type.
Table 7. OD values and level of anti-BLV antibodies in serum and milk according to breed of positive cattle

<table>
<thead>
<tr>
<th>Breed</th>
<th>No. of positive cattle</th>
<th>Serum antibodies</th>
<th>Milk antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OD values</td>
<td>Antibodies level (%E/P)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mean±SD</td>
<td>Limits</td>
</tr>
<tr>
<td>RSC</td>
<td>20</td>
<td>2.59±0.59</td>
<td>1.79-3.42</td>
</tr>
<tr>
<td>RBSC</td>
<td>15</td>
<td>2.60±0.53</td>
<td>1.79-3.44</td>
</tr>
<tr>
<td>H-RBSC</td>
<td>16</td>
<td>2.77±0.54</td>
<td>1.79-3.45</td>
</tr>
</tbody>
</table>

Anti-BLV serum antibodies reaches maximum values before parturition and during the first month postpartum, are maintained at an average level between 2nd and 6th month of lactation, and then declines in late lactation and during the dry period (Table 8, Figure 2). This trend is not influenced by the farm type, age or breed, differences between groups being insignificant. The difference between the mean values of serum antibodies in various phases of lactation is significant (P < 0.05), except for late lactation and dry period, when similar values were obtained.

Table 8. OD values and level of anti-BLV antibodies in serum and milk according to lactation phase of positive cattle

<table>
<thead>
<tr>
<th>Lactation phase</th>
<th>No. of positive cattle</th>
<th>Serum antibodies</th>
<th>Milk antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OD values</td>
<td>Antibodies level (%E/P)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mean±SD</td>
<td>Limits</td>
</tr>
<tr>
<td>1th month</td>
<td>6</td>
<td>2.98±0.33</td>
<td>2.61-3.44</td>
</tr>
<tr>
<td>2nd-3rd month</td>
<td>13</td>
<td>2.80±0.46</td>
<td>1.79-3.45</td>
</tr>
<tr>
<td>4th-6th month</td>
<td>10</td>
<td>2.58±0.35</td>
<td>2.20-3.147</td>
</tr>
<tr>
<td>7th-8th month</td>
<td>5</td>
<td>2.24±0.58</td>
<td>1.79-3.07</td>
</tr>
<tr>
<td>Dry period</td>
<td>12</td>
<td>2.29±0.67</td>
<td>1.79-3.07</td>
</tr>
<tr>
<td>1-2 weeks before parturition</td>
<td>5</td>
<td>3.25±0.12</td>
<td>3.12-3.45</td>
</tr>
</tbody>
</table>
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Dynamics of anti-BLV milk antibodies is slightly different from that of serum antibodies: high values are recorded before parturition and during the first month of lactation, the average level in dry period and the minimum values during between 2nd and 8th month of lactation (Table 8, Figure 2). These differences are statistically significant ($P < 0.0001$).

4. Discussion

We consider AGID as reference test because, according to the Program of supervision, prevention, control and eradication of animal diseases of National Sanitary Veterinary and Food Safety Authority (NSVFSA [23]), ELISA positive cattle are subject to confirmation by the AGID test. If animals are ELISA positive and AGID negative, reconfirmation is done by AGID, 30 days after the first blood sampling.

Comparative assessment of AGID and both serum and milk ELISAs, in terms of their value in EBL diagnosis, revealed superiority of two ELISAs in relation to all monitored parameters (farm type, age, breed and lactation phase). These findings are supported also by numerous studies (E.T. González et al. [27], G. Florent et al. [28], G. Gutierrez et al. [29], P. Have & R. Hoff-Jorgensen [30], K. Knapen et al. [31], M. Mammerickx et al. [32], K. Murakami et al. [33], V.K. Nguyen & RF. Maes [34], C. Platzer et al. [35]).

As mentioned above, AGID detected eight less positive animals than ELISAs, seven from households and one from private farms. This difference can only be explained by the low level of serum anti-BLV antibodies in those samples (OD = 1.79±0.004 and %E/P = 100.59±0.25). These lower values also corresponded to the two lactation phases – 7-8 months and dry period – in which AGID did not allow the detection of positive animals.

ELISAs superiority is reflected also in relation to age categories of tested cattle. Most AGID false negative cattle belong to age groups over five years old. This confirms previous findings, that the AGID detects a smaller number of BLV positive animals in the upper age categories compared to ELISA (S.R. Pop [36]) and the maximum effectiveness of AGID occurs in age ranges between 4 and 7 years (M. Mammerickx et al. [32], C. Platzer et al. [35], S.R. Pop [36]).

The fact that most BLV positive cattle were aged between 3 and 6 years (40 out of 51) suggests an age predisposition for the disease. Our findings are within the limits specified in the literature, some authors considering that cattle aged 3-8 years are most prone to develop EBL (M. Spînu [1], F.A. Murphy et al. [2]).
The absence of significant differences between the breeds regarding the number of positive animals, did not allow us to establish, at least for cattle population studied, a breed predisposition to EBL.

Sensitivity, specificity, positive predictive value and negative predictive value of the two ELISA kits used are similar to those reported by Pop [36] and Sălceanu [37], for commercial blocking ELISA kits compared to AGID in Bihor and Iași Counties.

The four quality parameters are prone to suffer variations depending on the type of ELISA technique (indirect or blocking) used in the EBL diagnosis, as well as the target antibodies. Diagnostic kits for simultaneous detection of two types of antibodies (anti-gp51 and p24) have a low sensitivity and specificity; it also provides a large number of false positive and false negative results which, in turn, affects positive and negative predictive values. This phenomenon occurs because the tests allow, at least theoretically, an early diagnosis of the disease, when the humoral immune response is dominated by anti-p24 antibodies. For example, Leucokit kit-La Plata, LK-LP and Chekit Leucotest Bommeli AG have sensitivity of about 97%, specificity of 76%, positive predictive value of 61% and negative predictive value of 98% (E.T. González et al. [27]).

However, applying indirect ELISA on milk samples (using DRG® Bovine Leukemia Virus p24-gp51 Ab serum, milk, DRG International Inc., USA), we obtained similar results to those of blocking ELISA on serum (ELISA Enzootic Bovine Leukosis Virus (BLV) Antibody Test (IDEXX Laboratories, Inc., Netherlands). The results accuracy can be explained by the fact that the screening kit used allows the analysis of pools up to 15 milk samples compared to other milk ELISA kits, which are able to detect anti-BLV antibodies in pools of 50-100 samples. Also, in our study, the milk samples were tested individually and diluted 1:2. The majority of the indirect ELISA kits for detection of anti-p24 and gp51 antibodies contains polyclonal antibodies, as opposed to DRG® Bovine Leukemia Virus p24-gp51 Ab serum, milk used, in which microtiter plates are coated with monoclonal antibodies.

Correspondence between AGID and ELISAs was 84.31%, comparable to values reported by other authors (E.T. González et al. [27], V.K. Nguyen & RF. Maes [34], H.M. Naif et al. [38]).

Youden’s index (probability of correct classification independent of prevalence) was 0.97, close to the optimal upper limit (Y = 1), which demonstrates a high diagnosis value of ELISA Enzootic Bovine Leukosis Virus (BLV) Antibody Test (IDEXX Laboratories, Inc., Netherlands) and DRG® Bovine Leukemia Virus p24-gp51 Ab serum, milk (DRG International Inc., USA).

The more intense humoral immune response in BLV positive cattle from private farms can be attributed to fodder supplements (minerals, vitamins, probiotics, prebiotics, etc.), whose general stimulating effects of the immune system are widely recognized (C.M. Cope et al. [39], J.W. Spears [40]). This intense immune response should be regarded with some caution, because a high level of anti-BLV antibodies not necessarily means an optimal humoral immune response to the virus (Z. Trainin & J. Brenner [41], H. Ungar-Waron et al. [42]). The phenomenon is explained by some "defects" in the structure of immunoglobulins M and G synthesized in BLV presence, which prevents them from reacting effectively with viral structures (Z. Trainin et al. [43]).

The age influence on serum antibodies level should be also regarded with caution, because the majority of the positive cattle from private farms belong to age categories with elevated levels of anti-BLV antibodies. This phenomenon was also found in case of local breeds, the differences between their antibodies level being statistically significant only if farm type is associated in the analysis.

One aspect that deserves a special attention is the dynamics of anti-BLV antibodies presence in milk. Their level drops significantly from the second month of lactation and
reached minimum values in the peak of lactation. As mentioned above, milk from BLV infected cattle, particularly of those with persistent lymphocytosis, contains viable virus (C.J. Kuckleburg et al. [44], G. Gutierrez et al. [45]), so the transmission via milk consumption is possible (S.G. Hopkins & R.F. Digiacomo [8]). It was also demonstrated that BLV present in food products of bovine origin can not be completely inactivated by pasteurization or cooking (G.C. Buehring et al. [18]). Thus, low levels of neutralizing antibodies in milk and, possibly, structural defects of immunoglobulins (Z. Trainin et al. [43]), promotes human infection, phenomenon with potential risks for developing cancer and neurological disorders (M. Giovanna et al. [19], C. Altaner et al. [21]).

5. Conclusions
AGID does not allow detection of BLV seropositivity in cattle with low titers of anti-gp51 antibodies regardless of age, breed or lactation phase. ELISAS allowed detection of a higher number of animals with EBL compared to AGID test, taken as reference. Cattle aged 3 to 6 years old are more prone to develop EBL. The humoral immune response in BLV positive cattle from private farms is more intense, at least quantitatively, than in positive cattle from households. The level of milk anti-BLV antibodies is conditioned by the lactation phase. Consumption of insufficiently heat-treated milk from BLV positive cattle in 2-8 months of lactation is associated with increased risk of infection in humans.

Abbreviations
BLV – bovine leukemia virus
EBL – enzootic bovine leukosis
ELISA – enzyme linked immunosorbent assay
AGID – agar gel immunodiffusion test
HTLV – human T-lymphotropic virus
H-RBSC – Holstein-Romanian Black Spotted Cattle crossbred
OD – optical density (absorbance)
RBSC – Romanian Black Spotted Cattle
RSC – Romanian Spotted Cattle
%E/P – level of anti-BLV serum antibodies

References


Performance assessment of three tests applied in enzootic bovine leukemia diagnosis


