Evaluation of diagnostic tests to bovine leukemia virus

Avaliação de testes de diagnóstico para o vírus da leucemia bovina

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Summary: Bovine leukemia virus (BLV) is the causative agent of enzootic bovine leukosis (EBL). After infection with BLV there is no detectable viremia but there is a strong and persistent humoral immune response to structural proteins, essentially the gp51 envelope glycoprotein and the major core protein p24. Polymerase chain reaction (PCR) with primers used to amplify part of env gene, agar gel immunodiffusion (AGID) which employs a crude antigen preparation derived from concentrated cell culture fluid, two commercial immuno enzymatic assays (ELISAs) and one ELISA with recombinant gp51 antigen were used to detect proviral DNA and BLV antibodies in DNA and serum from bovines of Goiás (GO), Mato Grosso do Sul (MS), Minas Gerais (MG) and Paraná (PR) States of Brazil. The evaluated tests presented good results. The ELISA tests were more sensitive than AGID, and highly specific. PCR sensitivity should be improved, nevertheless it is a good complementary tool for serologic diagnosis.

Resumo: O Vírus da Leucemia Bovina (BLV) é o agente etiológico da Leucose Enzoótica Bovina (LEB). Após a infecção não se observa viremia, mas desenvolve-se uma resposta imune humoral forte e duradoura às proteínas estruturais, principalmente a glicoproteína do envelope gp51, do envelope, e à proteína do cerne p24. A reação em cadeia pela polimerase (PCR), com iniciadores direcionados à amplificação de fragmentos do gene env do BLV, a imunodifusão em gel de agar (IDGA), que emprega antígeno bruto derivado de sobrenadante de cultivo celular concentrado, dois ensaios imunoenzimáticos (ELISAs) comerciais e um ELISA que emprega a proteína gp51 recombinante foram utilizados para detectar, respectivamente, o DNA proviral e anticorpos para o BLV em amostras de DNA e soro de bovinos dos estados de Goiás, Mato Grosso do Sul, Minas Gerais e Paraná. Os testes de ELISA foram mais sensíveis que a IDGA e altamente específicos. A PCR revelou-se útil como ferramenta de diagnóstico complementar aos testes sorológicos.

Introduction

Bovine leukemia virus (BLV), the responsible for a disease known as Enzootic Bovine Leukosis (EBL), is a bovine Deltaretrovirus with worldwide distribution. BLV infections were associated with economic losses for milk producers (Motton and Buehring, 2003; Ott et al., 2003).

During the interaction BLV-host, the extern viral glycoprotein gp51 and the major structural protein p24 are the main target to the immune response, and thereafter, good reagents for diagnostic purposes (Van der Maaten and Miller, 1990; Merza et al., 1991). Antibodies to gp51, however, were detected with higher titles than antibodies to p24 protein (González et al., 2001).

Diagnostic tests have important applications in Veterinary Medicine, including research, epidemiological surveillance, certification of areas free of diseases and prevalence estimates studies. EBL diagnostic is made by serologic methods based on the detection of antibodies to gp51 and to p24. Agar gel immunodiffusion (AGID) was considered for many years the test of election due to its high specificity and acceptable sensitivity. In the last few years, however, ELISA tests were developed for use in serum and milk samples (Reichel et al., 1998). ELISA tests are based in the use of gp51 protein partially purified and of monoclonal antibodies to epitopes of this protein (Portetelle et al., 1989). The fetal lamb kidney cell line (FLK) infected by BLV is used for the production of gp51. Unfortunately, this procedure is expensive and, besides that, the FLK cells are susceptible to infections by Bovine Virus Diarrheaa Virus (BVDV) (Bolin et al., 1994; Beier et al., 2004).

The production of recombinant antigen to BLV diagnostic has been attempted in heterologous system like Escherichia coli (Choi et al., 2002), Saccharomyces cerevisiae (Legrain et al., 1989), the Vaccinia Virus (Portetelle et al., 1991) and, recently, baculovirus (De Giuseppe et al., 2004).
AGID is not able to detect infected animals from pooled serum samples and it is not practical to be used to test a large number of samples. Some commercially available ELISA are useful in these cases, but non specific reactions to BVDV virus are frequent (De Giuseppe et al., 2004). The test specificity depends on antigen quality. Impurities are responsible for high background values and for non specific reactions. Usually, high purification of antigens increases productions costs (González et al., 1999a). The polymerase chain reaction (PCR) is a useful and sensitive tool for fast and early detection of BLV proviral DNA in blood, organs and tumoral samples (González et al., 1999b).

The goal of this work was to compare methods (AGID, ELISA and PCR) used in the diagnosis of BLV in field sera samples from Brazil.

Blood samples were taken from bovines with more than 7 months of age, originated from four Brazilian states: Goiás, Minas Gerais, Mato Grosso do Sul and Paraná, giving a total number of 288 serum and 114 DNA samples. The samples came from naturally infected herds. The venopunction was done in jugular vein, using vaccutainer tubes with and without EDTA. After clot formation, the tubes without EDTA were centrifuged at 1,000 xg for 10 minutes and the sera separated and stored at -20 ºC until use. The DNA extraction was done from the blood collected in tubes with EDTA, using GFX Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech, USA).

Antibody detection was done by AGID (Miller and Van der Maaten, 1977), using gp51 protein produced in FLK cells as antigen, following protocol of Laboratorio Veterinario Miguel C. Rubino MGAP (DILAVE, Uruguay), by rgp51 ELISA, whose antigen is the gp51 protein produced by baculovirus (De Giuseppe et al., 2004) and by commercially available ELISA kits Bovine Leukemia Virus Antibody Test Kit Indirect ELISA (VMRD, EUA) e HerdChek® Bovine Leukemia Virus Antibody Test Kits (IDEXX, EUA), which detect antibodies to the gp51 protein, according to producers instructions.

The PCR was done as described by Camargos et al. (2003), using primers of BLV env gene to amplify fragments with 521 pairs of bases (bp): BLV1 5’-GGGCCATGTCACATGATGTT-3’ (5128-5149) e BLV2 5’-CCGGTGGCCCTTGGAAACAGCTTGAAAC-3’ (5627-5649). The numbers correspond to those of BLV sequences published by Sagata et al. (1985).

Due to lack of a gold standard the following criteria was used to classify samples for serologic tests comparison (Table 1): Negative samples, samples with negative results in at least three tests; Positive samples, samples with positive results in at least three tests; Indeterminated samples, samples which showed positive in two tests and negative in other two tests (De Giuseppe et al., 2004).

The relative sensitivity, relative specificity, Youden’s index and confidence interval of the tests were calculated considering the interpretation criteria and the software Win Episcope 2.0. The analytical sensitivity and specificity was done with OIE reference sera NR33 and E4 diluted 1/10, kindly donated by, respectively by Dr. Mark Horigan (VLA Weybridge) and Dr. Rikke Hoff-Jørgensen (Danish Veterinary Laboratory), respectively. NR33 serum does not contain antibodies to BVDV, Herpes Virus Bovine 1 (HVB1) and BLV. E4 serum does not contain antibodies to BVDV, HVB1, Parainfluenza 3 e Respiratory Sincicial Virus.

In consequence of the criteria used, 173 of 288 available samples were considered negative, and 106 were considered positive by the serological tests. Nine samples which were considered inconclusive were excluded from the analysis.

The commercially available ELISA tests, the AGID and the rgp51 ELISA had analytical sensitivity and specificity in accordance with OIE requirements (2004).

The number of samples correctly identified as positives and negatives are presented in Table 2, which also shows the number of samples with false diagnosis (Seven false negatives in AGID, and three false positives in VMRD and IDEXX elisas) in accordance with the criteria described in the Table 1. Two hundred and seventy-nine samples were tested. AGID did not detect 7 seropositive bovines, and VMRD and Idexx ELISA tests had 3 false positives each. These results are similar to the observed by Simard (2000).

### Table 1 - Classification criteria of samples for diagnostic tests comparison

<table>
<thead>
<tr>
<th>Sample classification</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Test 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Positive</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Negative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Negative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Indeterminated</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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### Table 2 - Evaluation of serologic tests in Brazilian samples

<table>
<thead>
<tr>
<th>TESTS</th>
<th>ELISA</th>
<th>VMRD</th>
<th>IDEXX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results</td>
<td>rgp51</td>
<td>AGID</td>
<td>VMRD</td>
</tr>
<tr>
<td>Negatives</td>
<td>173</td>
<td>173</td>
<td>170</td>
</tr>
<tr>
<td>Positives</td>
<td>106</td>
<td>99</td>
<td>106</td>
</tr>
<tr>
<td>Incorrect</td>
<td>0</td>
<td>7</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 3 shows the relative sensitivity, relative specificity, Youden’s and confidence interval (95 %) values for each serological test. The rgp51 ELISA had 100 % sensitivity and specificity. AGID had the lowest sensitivity value. The ELISA tests presented similar sensitivity, specificity and execution time. As observed by De Giuseppe et al. (2004), rgp51 ELISA
presented the highest specificity which is probably due to difficulties in purification of antigen originated from FLK cells and the occurrence of cross reactions with non specific antigens, and/or by the use of FLK cells infected with BVDV (Bolin et al., 1994; Beier et al., 2004). From the practical point of view, IDEXX ELISA has one disadvantage, which is the need to retest the positive samples in one confirmation plate. The high prevalence of BLV in dairy herds in Brazil (Camargos et al., 2002), may render it not viable in the current format.

Table 3 - Relative sensitivity, relative specificity, Youden's and confidence interval of serologic tests for BLV

<table>
<thead>
<tr>
<th>Tests</th>
<th>rgp51</th>
<th>VMRD</th>
<th>IDEXX</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGID</td>
<td>93,4</td>
<td>98,3</td>
<td>98</td>
</tr>
<tr>
<td>ELISA</td>
<td>(88,7-98,1)</td>
<td>(96,3-100)</td>
<td>(96-100)</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>100,0</td>
<td>100,0</td>
<td>100,0</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>100,0</td>
<td>100,0</td>
<td>93</td>
</tr>
<tr>
<td>Youden's index (%)</td>
<td>0,93</td>
<td>0,98</td>
<td>0,98</td>
</tr>
<tr>
<td>Confidence interval (95 %)</td>
<td>(0,89-0,98)</td>
<td>(0,96-1)</td>
<td>(0,96-1)</td>
</tr>
</tbody>
</table>

Although AGID has the lowest sensitivity and the FLK cells used for AGID antigen production had been infected by BVDV (data not shown), this test continues to be the most practical and inexpensive, when compared with commercially available ELISA kits. For the realization of epidemiological studies and control programs in Brazil, where a large number of samples is used, it is necessary to develop ELISA tests with the capacity to detect antibodies in serum and milk, preferentially using recombinant antigens, or those originated from cell lines free of BVDV, since BVDV is capable of inducing false positive reactions (Bolin et al., 1994; Beier et al., 2004). The rgp51 antigen produced in baculovirus proved to be a good alternative to antigen produced in FLK cells.

Table 4 shows the results of serological and PCR tests applied on 114 samples and following criteria of Table 1. The criteria used to classify samples for comparison of serologic tests was not applied for serologic and PCR tests comparison.

Table 4 - Evaluation of serologic and PCR tests

<table>
<thead>
<tr>
<th>Tests</th>
<th>rgp51</th>
<th>VMRD</th>
<th>IDEXX</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>ELISA</td>
<td>ELISA</td>
<td>PCR</td>
</tr>
<tr>
<td>Negatives</td>
<td>51</td>
<td>55</td>
<td>47</td>
</tr>
<tr>
<td>Positives</td>
<td>63</td>
<td>59</td>
<td>67</td>
</tr>
</tbody>
</table>

When serologic tests and PCR are compared, it was observed that AGID detected a lower number of positive animals. The PCR did not detect 8 samples that were positive in all the serologic tests and 2 that were positive in all the ELISA tests. On the other hand, PCR detected BLV proviral DNA in blood from 9 animals that tested negative in all the serologic tests. Those results show, again, a disparity between direct and indirect tests (Camargos et al., 2003).

In some situations, serologic tests may fail to identify infected animals. Some examples of these situations are the birth (Gentile et al., 1985; Ebertus et al., 1987), recently infected animals (it is possible that in some cases antibody detection occurs just only three weeks after infection (De Boer et al., 1987), permanently seronegative animals, transient or permanent low level of antibodies, and animals simultaneously infected with BVDV which show depressed immune response to BLV (Roberts et al. 1989; Klintevall et al., 1994). All possible precautions were taken to prevent PCR cross-contamination. DNA extraction was performed in a place free of amplified products, gloves were changed frequently, and negative controls were employed (water and MDBK cells).

The fail to detect infected bovines by PCR may be due to low number of infected lymphocytes (Eaves et al., 1994; Reichel et al., 1998), low concentration of BLV proviral DNA in infected lymphocytes (Molloy et al., 1994), restricted infection of lymphoid organs (Klintevall et al., 1994), as described in HIV patients (Edginton, 1993) and the presence fo due to Taq DNA polymerase inhibitors in DNA samples (Panaccio and Lew, 1991). The use of nested-PCR may improve PCR sensitivity (OIE, 2004).

The infection stage, host-virus interactions, including the number of infected cells or the number of copies of proviral DNA per cell, the regulation of viral antigen expression, the immune response induction and the lymphocyte proliferation may all, directly or indirectly, influence the BLV detection (Cockerell and Rovnak, 1988).

PCR may be used for BLV detection in young calves fed with colostrums from seropositive cows, in tumor cases, to differentiate sporadic from infectious lymphomas, in tumor tissues from suspected cases collected in slaughterhouses, in recent infections, before the development of antibodies, in doubts reactions or weak positive reactions in ELISA tests, for the monitoring of bovines in progeny tests before the use in artificial insemination centers, and in bovines used for vaccine production (Ballagi-Pordany et al., 1992; OIE, 2004).

In the evaluated samples the rgp51 ELISA gave better results than the others serologic tests used. Our PCR test should be improved by using nested primers or by changing the used primers. Future experiments using p24 ELISA, a new PCR test, with samples from herds free of the disease and the follow up of the ani-
Acknowledgement

We thank Mr. Maurício Baltazar de Carvalho Filho for his critical reading of this manuscript.

Bibliography


