DETECTION OF BLV PROVIRUS IN DIFFERENT CELLS BY NESTED-PCR

LIDIA MARKIEWICZ, JAN RUŁKA AND STANISŁAW KAMIŃSKI

Department of Animal Genetics,
University of Warmia and Mazury,
10-957 Olsztyn, Poland

Laboratory of Cell Pathology,
National Veterinary Research Institute,
24-100 Pulawy, Poland
e-mail: stachel@uwm.edu.pl

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The usefulness of the nested-PCR technique for the detection of BLV env gene fragment in the cell lines and blood samples from dairy cows and bulls was demonstrated. BLV env fragments were PCR-amplified in two subsequent amplifications: 427 bp by the PCR I and 341 bp by the PCR II (nested-PCR). Generally, among 58 investigated animals, 3 positive and 8 doubtful results by the PCR I and 21 positive by the nested-PCR were found. It is noteworthy that among 14 reproductive and BLV seronegative bulls, 2 were found as positive in nested-PCR. The results indicate the need for improvement of BLV detection by the use of nested-PCR which should enhance the sensitivity and reliability of bovine leukaemia virus detection.

Key words: bovine leukemia BLV, nested-PCR.

Bovine leukaemia virus (BLV), the causative agent of enzootic bovine leukosis is an exogenous, B lymphotropic retrovirus belonging to the Retroviridae family which induces persistence lymphocytosis in cattle and sheep. The virus is mainly transmitted horizontally by direct exposure to biological fluids (blood, milk, saliva and semen) contaminated with infected lymphocytes. After infection, the BLV elicits in immunologically mature cattle a strong immune response against the envelope protein gp51. These antibodies can be detected by ID and also by ELISA tests. Several authors have shown that it is possible to establish BLV free herds by identifying seropositive animals and eliminating them from the herds (6, 8). Others demonstrated that serological control is not sensitive enough to find all BLV infected cattle (4, 7, 11). Problems also arise with animals exhibiting periodically or permanently low titres of BLV antibodies or low serum titres in the periparturient period (2). Furthermore, serological tests cannot discriminate between passive material immunity and active immunity induced by bovine leukaemia infection (1, 4). Analysing animals over a long term, Eaves et al. (6) showed that some naturally infected, BLV provirus carrying animals developed no BLV antibody titres detectable by ID and ELISA during months or years after infection.
In infected cells, BLV is integrated into host DNA in the form of provirus which can be detected by different molecular biology methods. Recently, polymerase chain reaction (PCR) for the detection of BLV proviral DNA has been described (3, 9, 14, 15). Due to some advantages of the PCR method over serological tests many research groups developed this enzymatic reaction for BLV detection. Several authors have also shown that PCR is particularly suitable for controlling herds of cattle with a very low incidence of BLV infection and for clarifying doubtful serological results obtained by ID or ELISA.

The aim of this work was to develop a specific and sensitive nested-PCR method for proviral BLV DNA detection in cell lines and in leukocytes isolated from whole blood of dairy cows and reproductive bulls.

Material and Methods

Cell lines. Three cell lines containing structural virus: BLV/NCR, V-NC and FLK-BLV and recombinant cell line AG were included in the experiment. The lysates from these cells were used as a control material for the verification of PCR I and nested PCR diagnostic value in cattle infected with BLV.

Blood samples. Animals. Thirty Black-and-White breed dairy cows (B, F and G herds), 6 Polish Red breed cows (C), 8 bisons (Bison bonasus) originated from the National Park in Białowieża, Poland (Ż), and 14 Black-and-White of bulls (D) were investigated.

Five ml blood samples were collected from all animals in a good health by the venipuncture. From bisons blood samples were taken by the occasion of an approved hunting. As anticoagulant the versen II (NaCl – 8.0, KCl – 0.2, KH2PO4 – 0.2, Na2HPO4 – 1.15, EDTA – 50.0 i H2O ad 1000 ml) was used in 1:20 dilution.

DNA isolation from the blood. Five hundred μl of the fresh blood were used and followed the DNA isolation by MasterPure Genomic Purification Kit (Epicentre Technologies). The total DNA amount and its quality was estimated by 260 and 280 nm absorbance measurement (mini-spectrophotometer Gene Quant, Pharmacia) and agarose/ethidium bromide gel electrophoresis.

PCR. Two pairs of primers: BI-1: 5’ GTGCCAAGTC TCCCAGATACA 3’ and BI-2: 5’ TATAGCACAGTCTGGGAAGGC 3’ as well as BI-3: 5’ CTTGAAAAATGGCT-ATCCTAAGATCTACGGC 3’ and BI-4: 5’ GAAGAGGGAACCCAGTGTTC-AACTG 3’ which encompassed 427 and 341 bp fragments of env gene, were used respectively. The PCR I was performed in a total reaction volume of 10 μl consisting of 0.6 μl MasterAmp Tfl buffer 20x, with 1.0 M Tris-HCl, pH 9.0 in 25°C and 400 mM NH4SO4, 0.3 U polymerase Tfl (Epicentre Technologies), 3 mM MgCl2, 160 μM of each deoxinucleotide triphosphate - dNTP (Promega), 1 μM of BI-1 and BI-2 primers, 1.0 μl of 10x Enhancer (Epicentre Technologies), and about 100 ng of DNA. The PCR thermal profile was as follows: 94°C for 3 min of initial denaturation, 30 cycles of denaturation at 94°C for 30 s, annealing primers at 60°C for 30 s, and elongation at 72°C for 30 s. After the last cycle the tubes were incubated at 72°C for 5 min.

The nested-PCR was performed in a total reaction volume of 25 μl mixture consisting of 1.25 μl MasterAmp Tfl buffer 20x, with 1.0 M Tris-HCl and 400 mM NH4SO4, 0.8 U polymerase Tfl, 2 mM MgCl2, 40 μM of each deoxinucleotide triphosphate - dNTP, 2 μM of BI-3 and BI-4 primers, and 0.5 μl of PCR I reaction
product. The annealing temperature (62°C) of cycle number (35) of PCR II were different in relation to that in PCR I. Ten μl of each PCR reaction mixture was submitted to 100 V electrophoresis for 30 min on ethidium bromide stained 1.5% agarose gel.

**Immunodiffusion test (ID).** The ID test with the anti-BLV reference serum was carried out according to the obligatory instruction. The set A1120 of BLV commercial antigen (PIWet Pulawy) was used.

**Results**

Fragments of BLV *env* gene were detected in all the cell lines (Table 1) and lymphocytes isolated from cows and bulls (Table 2). Among 30 cows (herds B, F, G) belonging to Polish Black-and-White breed, 5 positive and 5 doubtful results in PCR I and 18 positive results (60%) in PCR II (nested-PCR) were obtained. In the same group of animals tested by immunodiffusion test (ID) only 10 cows were seropositive (33.3%). Among 6 Polish Red breed dairy cows, only one was positive by nested-PCR. In a group of bison one doubtful result was obtained in PCR I (Fig. 1).

The results presented in Fig. 2 show very clearly how nested-PCR enhances the specificity and sensitivity of BLV detection by PCR I and that such enhancement is necessary.

Very interesting results were obtained in group D – reproductive bulls. In PCR I among 14 samples, 2 were doubtful, but in PCR II they were confirmed as positive (Fig. 2). Totally, among 58 animals tested, 3 were positive and 8 doubtful in PCR I, and 21 were positive in nested-PCR (Table 2).

**Discussion**

The detection of BLV infection in cattle is carried out by virological methods - syncytial test (5, 16), serological tests (10) and by molecular biology methods, based on PCR (3, 9, 14, 15). Although ELISA is a sensitive test, it does not detect all BLV infected animals. It is commonly known that in herds approved as free from BLV, seropositive animals still are found. It may be caused by physiological status of the animal or by influence of many immunosuppressive factors (12). The implementation of PCR method to BLV diagnosis could increase the detection number of infected animals.

In the present study, among 58 animals 3 positive and 8 doubtful results in PCR I and 21 positive in nested-PCR were found. Reamplification of PCR I by nested-PCR (PCR II) was shown to be very effective in increasing the detection rate and reliability of BLV testing.

Similar improvement of PCR sensitivity were reported by Marsolais *et al.* (14) who reamplified BLV *gag* gene by nested-PCR and received 31% more positive results than in PCR I. Rulka *et al.* (17) applied nested-PCR found 100% BLV positive results in blood samples from cows, and 80% in milk samples, while 95% samples tested by ELISA were BLV positive in comparing the occurrence of BLV provirus in lymphocytes the blood samples. Kużmak (11) found 11% positive results out of 72 cattle in nested-PCR while in single PCR all the samples were negative.
A special attention should be drawn to the result of BLV detection in bull’s blood. Among 14 seronegative bulls, 2 turned to be doubtful in PCR I and the same two were confirmed as BLV positive by PCR II. These results showed the possibility of BLV spreading through the artificial insemination. Such a route of infection could be possible because BLV was found in the epithelial cells of the testis (13, 17).

Table 1
Occurrence of BLV provirus (env gene fragment) in selected cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Detection of BLV by PCR</th>
<th>PCR I</th>
<th>PCR II (nested)</th>
</tr>
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<tbody>
<tr>
<td>NCR</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>V-NCR</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FLK-BLV</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>Clon AG</td>
<td>+/-</td>
<td>+</td>
<td></td>
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</tbody>
</table>

Table 2
Results of PCR amplification of BLV env gene fragments: 427 bp (PCR I) and 341 bp (PCR II) in DNA from blood of cattle

<table>
<thead>
<tr>
<th>Group of animals</th>
<th>Number of animals tested</th>
<th>PCR</th>
<th>Number of positive results in ID test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+/I</td>
<td>II (nested)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>13</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

nt – not tested
Fig. 1. Electrophoresis of BLV env gene fragment 427 bp (PCR I, upper) and 341 bp (PCR II, down). M – DNA size marker – PhiX 174 Hae III, /K+/ – positive control, cow seropositive by ID and PCR I, /K-/ – negative control (PCR mixture without DNA), K’- negative control of nested-PCR – (PCR mixture without PCR I product), E – dairy cows of Polish Black-and-White breed.

Fig. 2. Electrophoresis of DNA BLV env gene fragment 427 bp (PCR I, above) and 341 bp (PCR II, below). M – DNA size marker – PhiX 174 Hae III, /K+/ – positive control, cow seropositive by ID and PCR I, /K-/ – negative control (PCR mixture without DNA, K’- negative control of PCR II – PCR II mixture without PCR I, D – bulls of Polish Black-and-White breed.
Our results once more indicate the need for improvement of BLV detection by the use of an alternative method, like nested-PCR which should enhance the sensitivity and reliability of BLV infection diagnosis. Further research to evaluate different PCR-based BLV detection procedure and to test bull’s blood and semen as a potential source of BLV infection in intensive dairy cattle breeding is under way.

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References