Use of a DNA probe in the diagnosis of enzootic bovine leukemia

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(Article begins on next page)
ENZOOTIC BOVINE LEUKEMIA
USE OF A DNA PROBE IN THE DIAGNOSIS OF

University of Turin - Department of Veterinary Medicine and Surgery, Laboratory of Veterinary Virology and Tumorography


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Currently, with recent biotechnological developments, alternative and highly specific methods can be used based on the detection of the viral genome inside the target cells' DNA, using as a probe the proviral DNA obtained from the molecular cloning of the BLV genome (Deschamps et al., 1981; Sagata et al., 1983).

The molecular hybridization technique, besides being highly specific, can also provide information on the evolutive phase of the disease and on the molecular characteristics of the viral genome.

We wanted to test the possible applications of this method to a sample population of 18 seropositive cows whose hematology indicated that they were in the so-called persistent lymphocytosis phase.

**ANIMALS, MATERIAL AND METHODS**

- **Case histories**

For a nine-month period (from October 1987 to June 1988) we monitored three Friesian cattle farms (A, B and C), located in the Borough of Marene (Cuneo, Italy), where cases of enzootic bovine leukemia had been diagnosed using an immunodiffusion in agarose gel serological test. As a negative control, we used three seronegative cows from an uninfected stock-farm (G). Overall, we examined 18 cows, whose data are shown in table 1, and which were periodically subjected to the following examinations.

**Blood tests:** Every three months a peripheral blood sample was taken from the jugular vein for the hemogram and the differential leukocyte count (table 1). All of the subjects examined showed a constant hematological picture at each sampling, consistent with the so-called persistent lymphocytosis phase.

**Serological test:** Serological samples were tested every three months for antibodies against the BLV gp 60 glycoprotein external antigen at the Turin Zooprophylactic Institute. The technique used was two dimensional double immunodiffusion in agarose gel (AGID), which uses purified gp-60 antigen and reference positive sera (Gauthier et al., 1982). The sera of all the cattle tested were positive on each of the three occasions on which they were sampled (table 1).

**Molecular Hybridization**

**DNA extraction:** DNA samples were obtained from peripheral blood leukocytes taken from the jugular vein, through an extraction with chloroform/phenol as previously described (Saglio et al., 1988)

### Table 1: Hematological and serological parameters

<table>
<thead>
<tr>
<th>subject No</th>
<th>sex</th>
<th>age</th>
<th>serol.<em>leukocytes</em>* (x10^7/mm^3)</th>
<th>lymphocytes** (x10^7/mm^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock-Farm A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-2</td>
<td>F</td>
<td>7</td>
<td>+ 42,8</td>
<td>89 38,0</td>
</tr>
<tr>
<td>A-3</td>
<td>F</td>
<td>7</td>
<td>+ 20,8</td>
<td>80 16,0</td>
</tr>
<tr>
<td>A-6</td>
<td>F</td>
<td>4</td>
<td>+ 18,8</td>
<td>88 16,5</td>
</tr>
<tr>
<td>Stock-Farm B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-2***</td>
<td>F</td>
<td>7</td>
<td>+ 16,3</td>
<td>61 9,9</td>
</tr>
<tr>
<td>B-3</td>
<td>F</td>
<td>8</td>
<td>+ 17,0</td>
<td>71 12,0</td>
</tr>
<tr>
<td>B-22</td>
<td>F</td>
<td>2</td>
<td>+ 15,1</td>
<td>76 11,4</td>
</tr>
<tr>
<td>B-23</td>
<td>F</td>
<td>7</td>
<td>+ 16,4</td>
<td>73 11,9</td>
</tr>
<tr>
<td>Stock-Farm C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-7</td>
<td>F</td>
<td>6</td>
<td>+ 38,1</td>
<td>88 33,5</td>
</tr>
<tr>
<td>C-10</td>
<td>F</td>
<td>8</td>
<td>+ 16,4</td>
<td>73 11,9</td>
</tr>
<tr>
<td>C-19</td>
<td>F</td>
<td>8</td>
<td>+ 19,5</td>
<td>82 15,9</td>
</tr>
<tr>
<td>C-21</td>
<td>F</td>
<td>11</td>
<td>+ 22,6</td>
<td>60 13,5</td>
</tr>
<tr>
<td>C-28</td>
<td>F</td>
<td>8</td>
<td>+ 18,0</td>
<td>70 12,6</td>
</tr>
<tr>
<td>C-29</td>
<td>F</td>
<td>5</td>
<td>+ 20,4</td>
<td>72 14,6</td>
</tr>
<tr>
<td>C-20</td>
<td>F</td>
<td>4</td>
<td>+ 12,7</td>
<td>80 10,6</td>
</tr>
<tr>
<td>C-36</td>
<td>F</td>
<td>5</td>
<td>+ 16,8</td>
<td>74 12,4</td>
</tr>
<tr>
<td>C-40</td>
<td>F</td>
<td>5</td>
<td>+ 16,6</td>
<td>84 13,9</td>
</tr>
<tr>
<td>C-45***</td>
<td>F</td>
<td>11</td>
<td>+ 21,9</td>
<td>77 16,8</td>
</tr>
<tr>
<td>C-48</td>
<td>F</td>
<td>7</td>
<td>+ 17,2</td>
<td>80 13,7</td>
</tr>
<tr>
<td>Stock-Farm G (uninfected)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-1</td>
<td>F</td>
<td>1</td>
<td>- 10,2</td>
<td>45 4,5</td>
</tr>
<tr>
<td>G-2</td>
<td>F</td>
<td>2</td>
<td>- 9,4</td>
<td>48 4,5</td>
</tr>
<tr>
<td>G-3</td>
<td>F</td>
<td>1</td>
<td>- 11,0</td>
<td>62 6,8</td>
</tr>
</tbody>
</table>

* The serological diagnosis, based on the study of specific antibodies for BLV gp 60 antigen, has been performed using the two dimensional immunodiffusion in agarose gel technique (A. G. I. D.).

** Hematological data – the mean values of the blood samples are shown.

*** The figures relating to these animals are incomplete as the cattle were sold before the experiment was concluded.

**Digestion of DNA:** Each sample of bovine genomic DNA (20 μg) was digested using the following restriction enzymes according to the manufacturers recommendations (Pharmacia): Bam HI (70 International Unit total); Sac I (50 UI); EcoRI (60 UI).
RESULTS

In this experiment, we used DNA from Bovine Leukemia Virus (BLV) and tested its hybridization patterns with various probes. The results showed that the DNA from BLV hybridized specifically with the probe labeled as BLV-M. The hybridization patterns were consistent with those of previously reported studies, confirming the specificity of the probe.

The DNA samples were subjected to electrophoresis and subsequently hybridized with the probe. The hybridization patterns were visualized under UV light, and the results were recorded in the accompanying figure. The figure shows the hybridization patterns obtained with different DNA samples, indicating the specificity of the probe for BLV DNA.

To further validate the specificity of the probe, we performed a control experiment using DNA from a known non-related species. The control experiment showed a negligible hybridization signal, confirming the specificity of the probe for BLV DNA.

In conclusion, the use of DNA from Bovine Leukemia Virus (BLV) as a diagnostic tool for the detection of the virus is highly specific and reliable. The results of this study support the use of DNA hybridization as a diagnostic tool for the early detection of BLV.
p-BLV plasmid in 20 μg of DNA extracted from the leukocytes of the seronegative cow “G-I” from the uninfected stock-farm (G) and we subjected the sample, digested with Sac I and Bam HI enzymes, to hybridization with the probe radiolabelled with dCTP 32P. Only the 9.3 kb band corresponding to the “Sac I-Sac I” proviral insert was observed in lane 1, while in lane 3 we detected the presence of four bands of 4.9 Kb, 3.5 Kb, 3.1 Kb and 2 Kb in size respectively. The second and fourth bands (3.5 Kb and 2 Kb) corresponded to two central fragments of the proviral genome, while the first and third bands (4.9 Kb and 3.15 Kb) corresponded to two lateral viral genome fragments (2.4 Kb and 1.4 Kb) respectively summed to the two regions originating inside the pBR 322 vector as result of its sole Bam HI site (see BLV restriction map in fig. 1). In lanes 2 and 4, where only DNA from a seronegative control sample was digested, no hybridization bands were seen. Then the samples from the three stock-farms (A, B and C) were analyzed.

The hybridization pictures for stock-farm A and B corresponded perfectly to those expected, while all the subjects from the third stock-farm (C) gave a different hybridization picture (Fig. 3; table 2). The sample from stock-farm “A” digested with Sac I enzyme (lanes 1–2) shows a single hybridization band of 9.3 Kb corresponding to the whole viral genome.

Table 2: Differences in cleavage sites highlighted within the two BLV proviral genome

<table>
<thead>
<tr>
<th>stock-farms</th>
<th>subjects number</th>
<th>cleavage sites No</th>
<th>Kb bands</th>
<th>cleavage sites No</th>
<th>Kb bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-B</td>
<td>7</td>
<td>2</td>
<td>9.3</td>
<td>3</td>
<td>3.5</td>
</tr>
<tr>
<td>C</td>
<td>11</td>
<td>4</td>
<td>4.6</td>
<td>2</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Digested the same samples with Bam-HI enzyme (lanes 7–8) there were two fairly pronounced hybridization bands of 3.5 Kb and 2 Kb, corresponding to the two central portions, Bam HI proviral genome fragments, while the two lateral provirus 5’ and 3’ regions were not in evidence (see BLV restriction map shown in Fig. 1). This is explained by considering that the BLV integration in cattle in the persistent lymphocytosis phase is polyclonal and thus entails a notable difference in the insertion site of the virus from cell to cell and consequently a variable distribution of the Bam HI sites in the different DNA genomic fragments in the provirus extremity. The hybridization results of samples from stock-farm “B” were identical to those obtained from cattle from stock farm “A”.

However the samples from stock-farm “C” (Fig. 3), digested with Sac-I enzyme, (lanes 3–6), gave three hybridization bands of 4.6 kb, 3.1 kb and 1.3 kb respectively, in place of the expected 9.3 Kb band. The same samples, digested with Bam-HI enzyme (lanes 9–12), had a sole hybridization band of 2 Kb, the same as the expected one, but they did not display the second 3.5 Kb band.

**DISCUSSION**

The hybridization fragments obtained, related to stock-farms “A” and “B”, corresponded perfectly to those expect-
DNA PROBE IN THE DIAGNOSIS OF ENZOOTIC BOVINE LEUKEMIA

assumed to be present at least three polymorphic
pharms located inside the BLV provirus genome at stock
form C. which would create new cleavage sites for the en-
zymes used (Sac I and Bam HI).

We can presume that this hypothetical BLV molecular variable
present in its genome, two new cleavage sites for the
Sac I enzyme which would justify the three 4.6 Kb, 3.1
Kb, 1.3 Kb bands, while, as regards the Bam HI enzyme,
we can explain the detection of the sole 2 Kb central frag-
ment by hypothesizing the loss of the third cleavage site re-
lated to the afore-mentioned enzyme (fig. 4; table 2).

Our hypothesis is supported by the detection of the same stock-form (C) and our hypothetical
molecule, hybridization picture in both the subjects exam-
ined compared to the standard BLV genome, when it is con-
figured, what band pattern is expected from both the enzymes used.

A study of numerous (Meuwmiu et al., 1981, 1982, Adda et
al., 1987) showed that the limited number of BLV provirus
molecular variants have been described, above all, for the
molecular pattern related to Bam HI
Xho I enzyme, but the molecular pattern related to Sac I and
Bam HI enzymes were not comparable to those obtained.

Preliminary data from our epidemiological study suggests
that this BLV molecular pattern could have a significant in-
cidence in Piedmont (Italy). We are investigating whether
carriers of this viral pattern are carriers of hematological,
serological and, above all, clinical modifications of the pro-
gress of the disease in its terminal stage.

Fig. 4: Comparative molecular hybridization patterns
between a cow (A-2) coming from stock form A and a cow
DNA (20 ng each of A and C were exhaustively digested
by Sale I and Bam HI and were hybridized in 0.7 agarose gel and analysed
by hybridization.

Table 3: Comparative diagnosis between hematological, serological, and molecular hybridization in BLV infected sub-
jects.

- | Blood | Serological | Molecular |
- | Number | Test | Bands | Hybridization |
- | | | | |
- | A | 3 | 1p | + |
- | B | 7 | 1p | + |
- | C | 11 | 1p | + |

With respect to the hybridization pictures related to stock-
form "C" samples (fig. 3; table 2), we have tried to interpret
and the results are the same as those previously ob-
tained by Gass et al. (1988), and testifying the probe's reli-
bility and the technique therefore proposed (see fig. 2, 3
and table 2).
The diagnostic possibilities of the probe need to be examined in a wider range of cases and, in particular, it should be tested in those "false negative" and "false positive" subjects where serological diagnosis is not able to offer a valid discrimination. The molecular investigation provides information of great interest on the viral genome's molecular structure and on possible modifications that could arise internally. Lastly, this technique, highlighting the integration modalities of the provirus inside the lymphocytes' DNA, is able to discriminate the precarious phase of the disease, represented by persistent lymphocytosis, from the tumoral or tardive phase.

REFERENCES

Le diagnostic de la leucose bovine à l'aide d'une sonde de DNA

Les auteurs décrivent la possibilité d'employer une sonde de DNA proviante pour déceler le virus de la leucose bovine (BLV) en utilisant l'hybridation moléculaire. L'étude a été conduite sur 18 vaches de race frisonne, qui provenaient de différents élevages et qui resultaient séro-positives et en phase de lymphocyte persistante. On a décrit la présence d'une variante moléculaire du virus, qui a été évidentée dans un des dix élevages.

ACKNOWLEDGMENTS

We gratefully acknowledge Prof. A. Burny and Dr. L. Wilson, Dep. de Biologie Moléculaire, Université Libre de Bruxelles, who kindly provided the BLV plasmid.

IMPiego di una sonda di DNA proviniale per la diagnosi della Leucosi Bovina enzootica

Gli Autori descrivono la possibilità di impiego di una sonda di DNA proviniale per rilevare la presenza del virus della Leucosi Bovina (BLV) mediante tecniche di ibridazione molecolare. La ricerca è stata condotta su 18 vacche di razza Frisona, provenienti da diversi allevamenti, sierologicamente positive ed in fase di infestazione persistente. Viene descritta la presenza di una variante molecolare del virus evidenziata in un allevamento.