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## DETECTION OF THE BOVINE LEUKAEMIA VIRUS BY THE POLYMERASE CHAIN REACTION

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The polymerase-chain reaction was applied for detection of provirus DNA of the bovine leukaemia virus (BLV). A short fragment of 292 bp including region R and U5 LTR 5' of BLV was amplified, and the optimum parameters of amplification of this fragment were established. Electrophoresis revealed the presence of the 292 bp fragment from the leucocytes of four out of six cows showing a positive serological response to BLV antigens. Application of the polymerase-chain reaction in diagnosis of bovine leukaemia is suggested.

Enzootic bovine leukaemia is an infective disease caused by a retrovirus, bovine leukaemia virus (BLV) [1]. In the infected lymphocytes BLV is integrated with chromosomal DNA in the form of a provirus in a small number (1-3) of copies per diploidal genome [2]). Since transcription of virus proteins *in vivo* is blocked, BLV remains latent and viremia is not observed in the infected animals [3]. However, although replication is undetectable the antibodies appear in the infected animals [4] but are unable to eliminate the virus, and the animals remain carriers till the end of their lives. Therefore, diagnosis of bovine leukaemia is usually based on detection of specific antibodies. However, the lack of serological response is not tantamount to the lack of infection, since only the presence of provirus DNA integrated with the cell genome can serve as an unquestionable criterion.

For determination of provirus DNA we have applied the polymerase-chain reaction which permits rapid and efficient enzymatic amplification ( $10^5 - 10^6$ ) of DNA fragments. In the present work we have used this method for amplification of a fragment of the provirus BLV-DNA from bovine lymphocytes, and for diagnosis of the virus carriers.

### MATERIALS AND METHODS

Six cows infected with BLV, and 2 virus-free cows were used for the experiments. All the infected animals showed positive immunodiffusion (AGID) and immunoenzymatic (ELISA) tests.

Leucocytes, (40 ml of blood) were pelleted in the presence of EDTA by centrifugation for 30 min at 3000 *g* and erythrocytes in the pelled were haemolysed. Leucocytes were then washed twice 10 mM Tris/HCl buffer, pH 8.0, containing 100 mM NaCl and 1 mM EDTA, and suspended in 5 ml of the same buffer.

For isolation of DNA the leucocytes were suspended in 5 ml of 100 M Tris/HCl buffer, pH 8.0 and 10 mM EDTA and were treated overnight at 37°C with 0.5% SDS and 200 µg/ml proteinase K (Boehringer). DNA was extracted with a phenol-chloroform mixture, precipitated with sodium acetate and ethanol, and the DNA sediment was suspended in 1 ml of the above 10 mM Tris/HCl and 1 mM EDTA buffer. The content of DNA was determined spectrophotometrically. DNA prepared in the same way from the cells of the continuous culture of foetal lamb kidney infected with BLV (FLK - BLV) was used as positive control of the reaction.

Two oligonucleotides (18-mers) synthesized with the use of 381A DNA Synthesizer Automatic (Applied Biosystem) were used as primers. The sequence of the primers and position of nucleotides were consistent with the sequence of provirus BLV DNA [5], and was as follows: primer AE11 230-(5'-GACCCCT-CGTGCTCACCTC-3')-248; primer AF29, 504-(5'-GTCTCTCCTGGCC-GCTAG-3')-522. The amplified region included the fragment of 292 bp.

For amplification of DNA 5 µl aliquots of 10 mM solution of each deoxynucleoside triphosphate (Boehringer, Mannheim), 5 µl aliquots of 10 mM solutions of each primer, 5 µl of Taq polymerase (Stratagene) and the isolated DNA (400 µg), were used. The mixture was diluted with water to 50 µl, incubated for 5 min at 95°C, cooled in ice, supplemented with 2.5 units (0.5 µl) of Taq polymerase and covered with a layer of mineral oil (50 µl). Thirty amplification cycles were performed, each consisting of denaturation at 95°C for 1 min, primer hybridization at 68°C for 1 min, and elongation at 72°C for 2 min. The final elongation was prolonged to 5 min.

Oil was extracted with 500 µl of chloroform and isoamyl alcohol, and DNA was analyzed by electrophoresis [4] using the 123 bp DNA Ladder (BRL) as a molecular mass marker.

## RESULTS AND DISCUSSION

The presented work describes application of the polymerase-chain method for determination of provirus DNA in lymphocytes of cattle showing a positive serological response to BLV. This method was chosen because of its numerous advantages over the classical methods used for hybridization of nucleic acids. It is less time-consuming, makes possible simultaneous examination of a larger number of samples, and does not involve the use of isotopes. However, to use it as a screening procedure, it is necessary to determine precisely the optimum conditions for its performance.

The basic observation was that, in cattle, BLV is an exogenous retrovirus and that, using cDNA-BLV as a probe, no homologous sequences can be detected in uninfected animals [6].

It was important to choose for DNA amplification the region showing a highly conserved sequence since this is decisive for the degree of hybridization of primers to DNA matrix. The sequence analysis of various BLV clones demonstrated that the highly conserved regions are located mainly in the flanking regulatory segments of LTR [6, 7]. These observations are in agreement with those of other workers [8, 9] who applied the same technique for diagnosis of infection with HIV.

The amplified BLV fragment of 292 bp included the whole region R and U5 LTR5' of provirus BLV-DNA. This fragment is highly conservative, and its G+C content is high (61%), which assured stability of the duplexes formed but made necessary to rise the denaturation temperature to 95°C.

Another point of crucial importance is the choice of an appropriate temperature for hybridization of the primers, in determining specificity of the reaction. In Fig. 1 (lanes 3 and 4) illustrate DNA from leucocytes of a serologically positive (BLV+) cow, and of DNA from the cells of FLK-BLV line, when hybridization occurred at 65°C. An increase of temperature to 68°C

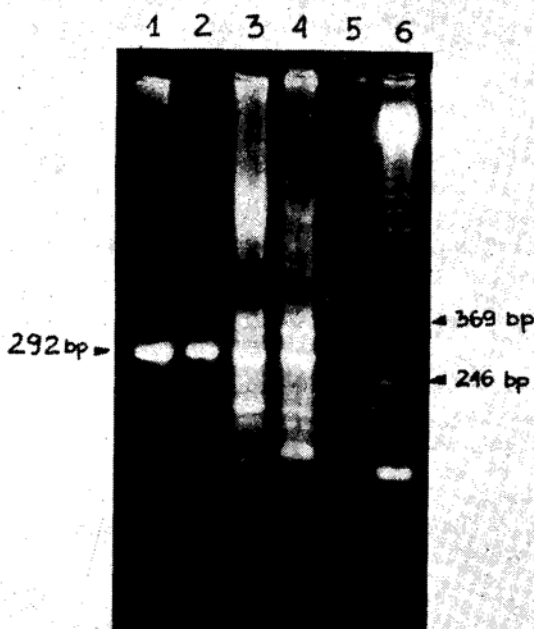


Fig. 1. Electrophoresis of the DNA amplification products (3% agarose NuSieve containing 0.125  $\mu\text{g}/\text{ml}$  ethidium bromide). Lanes 1 and 3, DNA of the BLV+ leucocytes, hybridization temperature 68°C and 65°C, respectively; lanes 2 and 4, DNA of FLK cells, hybridization temperature 68°C and 65°C, respectively; lane 5, DNA of the BLV-leucocytes, hybridization temperature 68°C; lane 6, the 123 bp DNA ladder

eliminated nonspecific hybridizations, resulting in amplification only of the 292 bp fragment (lanes 1, 2). Under the same conditions, DNA from leucocytes of a serologically negative (BLV-) cow was not amplified (lane 5).

Analysis of the amplified DNA from leucocytes of the six cows proved the presence of the 292 bp fragment in four DNA samples. Two samples were negative despite a positive serological response. Similar inconsistencies were, however, observed by Ou *et al.* [9]. They could be due to the insufficient number of the virus copies within the cell, or to small number of infected lymphocytes, as observed by Kettmann *et al.* [10] and Cockerell & Rovnak [11], who found provirus DNA in only 53% of the serologically positive cows. The second possible explanation could be deletion in the amplified region of the virus. An example of such a BLV virus, with a large deletion of LTR5' and *gag* gene, has been described by Ogawa *et al.* [12].

The results presented, although based on a small number of cows, suggest the possibility of using the polymerase-chain technique for diagnosis of bovine leukaemia.

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