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Development of a polymerase chain reaction and its comparison with agar gel immunodiffusion test in the detection of bovine leukemia virus infection

Desenvolvimento de uma reação em cadeia pela polimerase e comparação com a imunodifusão em gel de agar na detecção de infecções pelo vírus da leucemia bovina

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Summary

Polymerase chain reaction (PCR) was used for bovine leukemia virus (BLV) detection in the peripheral leukocytes of the infected bovines. The primers used were designed to amplify a part of *env* gene of BLV. PCR products were analyzed by agarose gel electrophoresis stained by ethidium bromide. The analytical specificity of PCR was confirmed by enzymatic restriction analysis of the PCR product with Bam HI and also by nucleotide sequence analysis of three PCR samples. Sixty five animals were tested for anti-BLV antibody, by agar gel-immunodiffusion test (AGID) and for direct BLV detection by PCR. There was a 73.80% concordance rate between the two tests. Four animals positive in AGID were PCR negative, while 13 AGID negative animals were found PCR positive. PCR got a 0.87 diagnosis sensitivity and 0.62 specificity. The developed PCR may be complementary tool in the diagnosis of BLV infection, but should have it diagnosis sensitivity improved.

Key-words
Immunodiffusion.
Bovine leukemia virus.
Gene.
Polymerase chain reaction.

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Introduction

The bovine leukemia virus (BLV), a Deltaretrovirus has a worldwide distribution in cattle herds and causes a disease known as Enzootic Bovine Leukosis (EBL). BLV infected animals may suffer lymphosarcoma, a fatal condition, bovine impairment in milk production and impairment in reproductive efficiency besides commercial restrictions[1,2,3,4,5].

The virus is able to infect, *in vitro*, cells of different species including human cells[6] and it can also infect, *in vivo*,sheep, rabbits and non human primates[6]. It primarily infects bovine B lymphocytes but it is able to infect T lymphocytes and neutrophiles. EBL is considered an experimental model for studies of human leukemias, caused by the Human T-Cell Leukemia virus types 1 and 2 (HTLV-1/HTLV-2, due to HTLV/BLV similarities in genome and

in pathogenesis[7].

Various indirect and direct methods have been used for BLV detection, e.g. AGID, ELISA and PCR[8,9,10,11,12,13]. In some cases the indirect methods fail in the detection of BLV infections[9,14,15,16]. The goal of this work was to develop a PCR for BLV detection in blood samples obtained from cattle, to determine the specificity and sensitivity, and to compare the results of PCR and AGID, this one as the gold standard, applied in a BLV naturally infected dairy herd.

Material and Method

Blood samples

Blood was collected in order to have at least 30 samples from both EBL positive and negative animals. Sixty five samples were collected in tubes with and without EDTA. The farm, where the bovines (Holstein black and white) came from, was localized in the metropolitan region of Belo Horizonte, Minas Gerais State, in Southeast region of Brazil. All tested animals were in good health and their age ranged from 1 to 10 years.

Antibody detection

AGID test was used for anti-BLV antibody detection utilizing p24 and gp51 antigens commercially produced (EMBRAPA/CNPSA, Brazil), as described by Miller and Van der Maaten[17].

DNA extraction

DNA extracted from fetal lamb kidney cells (FLK) persistently infected with BLV were used as PCR positive control[12]. DNA extracted from MDBK non-infected cells (Marvin-Derby Bovine Kidney) was used to verify the occurrence of annealing of the primers with bovine DNA. The controls DNA and the DNA from the infected animals were extracted from whole blood samples by the GFX Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech).

PCR primers

Primers were constructed using Oligo Primer Analysis program[19]. They were selected to amplify 521 base pairs (bp) fragments within the gp51 *env* gene of BLV. They are represented below, the number refers to the sequence accession number K02120:

Forward 5 ' - GGGCCATGGTCACATATGATTG- 3' (genome position 5128-5149)

Reverse 5 ' - CGTTGCCTTGAGAAACATTGAAC- 3' (genome position 5627-5649)

PCR amplification

PCR was prepared in a 20 ml total volume, which contained 10 mM TrisHCL solution, ph 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 1,00% glycerol (v/v); 1,00% (v/v) dimetilsulfoxide (DMSO); 10 pmoles of each primer; 0.2 mM of each deoxinucleotide (Promega, USA); 0.5 Taq DNA polymerase (Gibco-BRL, USA) and 1 ìl of DNA preparation containing approximately 150 ng of DNA. One drop of mineral oil was then added to each tube. Amplification reaction was carried out in a PCR programmed thermocycler (MJ Research, Inc., USA), in the following cycles: initial incubation at 95° C 3 min., followed by repeated 35 cycles: denaturation 95° C 60 sec., annealing 59°C 60 sec. and extension by polymerase at 72°C 60 sec. The last cycle was run at 72°C for 10 min. Amplification products was analyzed in 1,00% agarose gels stained with ethidium bromide.

Estimation of PCR detection limit

One sample of DNA obtained from BLV seropositive bovine was diluted and DNA concentration was estimated spectrophotometrically (UV-160 A – Shimadzu spectrophotometer) at the waves length 260 nm. Tenfold DNA dilutions were prepared and then a PCR was ran to verify at which input DNA could be amplified.

Estimation of PCR analytical specificity

PCR specificity was evaluated by restriction-enzyme analysis and by nucleotide sequence analysis of the amplified fragments of three samples. The PCR products were purified by Wizard PCR Preps DNA Purification System Kit (Promega, USA) according to manufacturer recommendations. The samples were digested with Bam HI restriction enzyme (Promega, USA), as recommended by the manufacturer, by 60 min. incubation at 37° C. The obtained products were analyzed by electrophoreses in 1.00% agarose gels (w/v) stained by ethidium bromide. Sequential analysis was carried out in an automatic sequence analyzer (ALF, Pharmacia, Sweden) at Bioagro-UFV, Viçosa, Minas Gerais, Brazil.

Statistical evaluation

Association between PCR and AGID was statistically estimated by the kappa test (k)[20]. The PCR diagnosis sensitivity and specificity were calculated using AGID as the gold standard[20].

Results

Detection of proviral DNA in whole blood of infected animals

The PCR described here, utilizing primers for BLV-*env*, gene which coding for gp51 env protein, amplified a fragment of approximately 521 bp. The chosen sequence was localized in a rather conserved region of *env* gene and, additionally, primers annealed at quite high temperature which increased the specificity of BLV *env* gene amplification. Fragments lacking specificity were not detected. Figure 1 shows PCR results obtained with DNA extracted from bovine blood samples.

PCR detection limit

One DNA sample had its concentration estimated in 120 ng/ìl. This sample was diluted in deionized water by ten fold dilutions and analyzed by PCR at the same conditions previously described. Visible amplification was seen up to the dilution 10^{-3} (Figure 2) which corresponded to 120 pg of bovine DNA/ìl meaning that this is the minimal concentration of DNA able to give a positive result in our PCR test. However, we have analyzed just one sample and therefore it is not possible to generalize this result.

Determination of PCR analytical specificity

Specificity of the amplified product was confirmed by enzymatic restriction analysis with Bam HI and also by sequence analysis of three samples. The enzymatic restriction produced, as expected, two fragments with 422 bp and 99 bp respectively (Figure 3). Besides, no amplification could be seen when DNA isolated from MDBK was used which proves that the primers did not anneal with bovine genome (Figure 1). Sequence analysis showed that the amplified fragments correspond to the BLV *env* gene. The obtained sequences were deposited in GenBank, USA http://www.ncbi.nlm.nih.gov:80/Genbank/

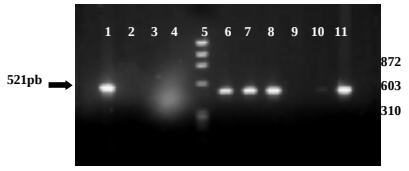

Figure 1
PCR results. The position of 521 bp band is pointed by the arrow. Lane 1: positive control, lane 2: negative control; lane 3: reagents control; lanes 4 and 9: BLV DNA negative samples; lanes 6, 7, 8, 10 and 11: BLV DNA positive samples; lane 5: molecular length markers (fX174, Life/USA). The corresponding molecular length is given on the right side of the figure

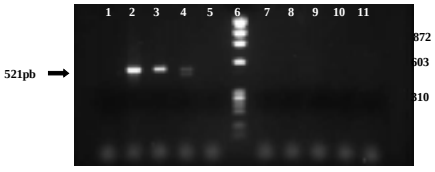

Figure 2
Determination of detection limits of PCR. The position of 521 bp band is showed by the arrow. Lane 1: reagents control; lane 2: 120 ng/ul tube (estimated concentration); lane 3: 120.10^{-2} ng/ul tube; lane 4: 120.10^{-3} ng/ul tube; lane 5: 120.10^{-4} ng/ul tube; lane 6: molecular length marker (fX174); lane 7: 120.10^{-5} ng/ul tube; lane 8: 120.10^{-6} ng/ul tube; lane 9: 120.10^{-7} ng/ul tube; lane 10: 120.10^{-8} ng/ul tube and lane 11: 120.10^{-9} ng/ul tube

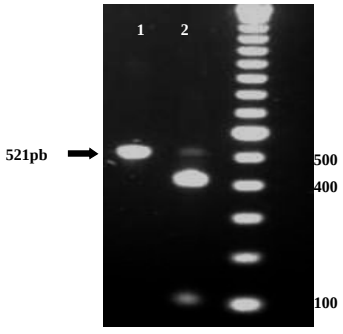

Figure 3
Determination of PCR analytical specificity by restriction enzyme analysis. The position of 521 bp band is pointed out by the arrow. Lane 1: purified BLV *env* gene fragment; lane 2: fragments obtained after digestion with the restriction enzyme BamHI; lane 3: molecular length marker (100 bp DNA Ladder, Gibco-BRL/USA)

index.html) and can be reached by the following access numbers: AF399702, AF399703 and AF399704.These were the first submission of BLV *env* sequences from Brazilian cattle.

Statistical evaluation

The results are shown in Table 1. From 65 tested samples, 31 were positive in AGID test while 4 of these samples were PCR negative. On the other hand, 13 out of 34 negative samples in AGID test were positive by PCR. Showing a 73.80 % agreement between the two tests. The statistical kappa value (k) was 0.48 which reflects a moderate alignment between PCR and AGID. The PCR diagnosis sensitivity was 0.87 and specificity 0.62.

Table 1
Comparison between PCR and AGID tests in BLV detection

	AGID positive	AGID negative	Total
PCR positive	27	13	40
PCR negative	4	21	25
Total	31	34	65

Discussion

The PCR developed for partial amplification of BLV *env* gene able to detect BLV in blood samples of infected cattle. Specific fragments were obtained as confirmed by BamHI enzyme digestion and nucleotide sequence analysis. It was designed as a supplementary test to be used mainly in herds at final phase of a EBL eradication program or when unclear results are present in serologic tests. This is the first study evaluating the usefulness of a PCR for field diagnosis of EBL on a naturally infected Brazilian herd.

So far, the PCRs described in literature, that could detect small quantities of proviral DNA, were obtained by PCR associated with hybridization, reamplification or semi-nested procedures[10,11,21,22,23,24].

We found a moderate 73.80% agreement between PCR and AGID tests, although up to 100.00% association between these two tests has

been described by other authors[7,11,25,26,27]. We believe this high agreement may be due, however, to a small number of the samples utilized or due to the higher sensitivity of those PCRs. The PCR diagnosis sensitivity should be improved, maybe by the use of nested primers and/or improving in proviral DNA purification. The low diagnosis specificity reached it is not because of false positive results in PCR, but it is probably due to detection of recently infected animals by PCR, that AGID could not detect.

Higher BLV detection rate by PCR in blood screening tests is generally due to its higher sensitivity when compared with serologic tests. Early stages of BLV infection with no anti-BLV antibodies production, chronic BLV infections without or with low anti-BLV antibody levels as well as lower AGID sensitivity are further factors regarded as influencing the outcome of the PCR/AGID screening results[9,14,15]. Roberts et al[16] observed that animals persistently infected with the bovine diarrhea virus (BVD), presented an impairment in their immune response to BLV. Genetic variants of the BLV *env* gene can also influence the outcome of PCR/AGID testing by exhibiting atypical forms of BLV infection with decreased immune response[28]. Our lab have been working in cloning and sequencing Brazilian isolates of BLV to figure out the main variation in *env* gene. This will give us the possibility of constructing primers more specifics in conserved regions of the genome besides mapping *env* from different regions.

The findings of PCR negative, AGID positive samples could be explained by the presence of Taq DNA polymerase inhibitors in blood samples[29], by absence of BLV in circulating lymphocytes[9,24,30], small number of provirus copies[31] or restricted infection to lymphoid organs[32].

Some authors showed large variations among PCR results utilizing samples collected from different geographical regions, which was probably due to non annealing of primers caused by genetic variations of the BLV provirus[33,34]. Mamoun et al.[35], compared nucleotide sequences of BLV *env* gene isolated from different geographical regions and found a 3.50% rate of nucleotide variation among those isolates, with the main part being located between nucleotides 4945 and 5111 of BLV *env* gene. For this reason the primers used in this work were chosen from a more conserved *env* gene region as previously described[35,36,37].

On large scale BLV infection screenings, when PCR negative/AGID positive samples might be found. It is recommended to use the PCR as a complementary test. PCR will be also very important to screen AGID or ELISA negative samples as well as to determine BLV infections calves that received colostrum from BLV seropositive cows. Results of PCR are available within one day while for AGID tests three days are usually needed. Recent BLV infections can be detected by PCR into 2–4 weeks earlier than by AGID test[38,39]. PCR should be used in BLV eradication programs, mainly in those herds with a low number of infected animals. Being also a reliable method when checking animals for export or selecting BLV free animals for vaccine preparations (e.g., anaplasmosis, babesiosis).

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Resumo

A reação em cadeia pela polimerase (PCR) foi utilizada para a detecção do vírus da leucemia bovina (VLB) em leucócitos periféricos de bovinos infectados. Os iniciadores utilizados foram construídos para amplificar uma parte do gene *env* do VLB. Os produtos da PCR foram analisados por eletroforese em gel de agarose corados por brometo de etídeo. A especificidade analítica da PCR foi confirmada por restrição enzimática dos produtos da reação com Bam HI e também pela análise da seqüência de três amostras. Sessenta e cinco animais foram testados para a presença de anticorpos anti-VLB, pela imunodifusão em gel de agar (IDGA) e pela PCR, para detecção direta do VLB. Houve 73,80% de concordância entre os dois testes. Quatro animais positivos na IDGA foram PCR negativos, enquanto 13 animais negativos na IDGA foram positivos na PCR. A sensibilidade diagnóstica obtida foi de 0,87 e a especificidade diagnóstica 0,62. A PCR desenvolvida pode ser uma ferramenta complementar no diagnóstico de infecções causadas pelo VLB, mas deve ter sua sensibilidade diagnóstica melhorada.

Palavras-chave
Imunodifusão.
Vírus da leucemia bovina.
Genes.
Reação em cadeia pela polimerase.

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