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NESTED POLYMERASE CHAIN REACTION VALIDATED FOR SENSITIVE DETECTION OF BOVINE LEUKEMIA VIRUS IN BLOOD SAMPLES FROM BRAZILIAN CATTLE HERDS: COMPARISON WITH CONVENTIONAL ELISA AND AGAR GEL IMMUNODIFFUSION METHODS

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ABSTRACT

A sensitive and specific nested polymerase chain reaction (nested PCR) for detection of bovine leukemia virus (BLV) in blood samples was evaluated as a reliable assay for accurate diagnosis of naturally infected animals under field conditions. The standardized nested PCR relies on the identification of BLV DNA fragments of 340 or 444 bp produced by applying 2 different pairs of primers sets to the proviral *env* gene. This protocol was validated by testing 40 bovine blood samples from a BLV-positive dairy herd, and by comparing results with serological diagnosis provided by agar-gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA). The nested PCR protocol was able to discriminate infected animals from that ones serodiagnosed as negative. This method also showed applicability for other body fluids or tissue samples. Investigation of 40 blood samples from a dairy herd showed that 37 animals were positive in ELISA, 36 in nested PCR, and only 25 in AGID. The nested PCR has demonstrated a good agreement in the results with ELISA up to 95%. These results demonstrated that nested PCR could be indicated for an early, sensitive and direct detection of BLV-infection in naturally infected cattle. Compared to the serological tests, this method proved to be the most suitable for identification of BLV-infected cattle with low, transient or without BLV-antibody titers. Moreover, this assay can be used to confirm or exclude BLV-infection in asymptomatic cattle or with doubtful serological results. This nested PCR is a valuable assay for prompt epidemiological investigations of BLV as well as for surveillance of animal health for bovine leukosis.

KEY WORDS: Bovine leukemia virus, diagnosis, nested PCR.

RESUMO

VALIDAÇÃO DE *NESTED* REAÇÃO EM CADEIA DA POLIMERASE PARA DETECÇÃO DO VÍRUS DA LEUCOSE BOVINA EM AMOSTRAS DE SANGUE BOVINO DE REBANHO LEITEIRO: COMPARAÇÃO COM ENSAIO IMUNOENZIMÁTICO E IMUNODIFUSÃO EM GEL DE ÁGAR. A sensibilidade e especificidade de uma *nested* reação em cadeia da polimerase (*nested* PCR) para detecção do vírus da leucose bovina (VLB) foi avaliada em amostras de sangue bovino, como uma metodologia confiável para diagnóstico preciso de animais naturalmente infectados. O protocolo de *nested* PCR padronizado, baseia-se na identificação de fragmentos de 340 pb e 444 pb do DNA de VLB, produzidos pela utilização de 2 diferentes conjuntos de *primers* do gene *env* proviral. Este protocolo foi validado através de testes em 40 amostras de sangue bovino de um rebanho leiteiro VLB positivo e pela comparação dos resultados com o diagnóstico sorológico por imunodifusão em gel de ágar (IDGA) e por ensaio imunoenzimático (ELISA). O protocolo de *nested* PCR foi capaz de discriminar animais infectados dentre aqueles que foram diagnosticados sorologicamente como negativos. Esse ensaio mostrou ter aplicabilidade, também, a outros fluídos corporais ou amostras de tecidos. Investigações feitas em 40 amostras de sangue bovino mostraram que 37 animais foram positivos para VLB com ELISA, 36 com *nested* PCR e somente 25 com IDGA. O *nested* PCR demonstrou uma concordância nos resultados com o ELISA em até 95%. Esses resultados comprovaram

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que o *nested* PCR pode ser indicado para uma detecção precoce, sensível e direta do VLB em bovinos naturalmente infectados. Comparado com os testes sorológicos, esse método mostra-se mais adequado para a identificação de bovinos infectados possuindo títulos de anticorpo anti-VLB baixos, transientes ou ausentes. Além disso, esse ensaio pode ser usado para confirmar os resultados sorológicos duvidosos ou a infecção em bovinos assintomáticos. O *nested* PCR é uma metodologia valiosa para uma rápida investigação epidemiológica do VLB, assim como para a vigilância sanitária da leucose bovina.

PALAVRAS-CHAVE: Vírus da leucose bovina, diagnóstico, *nested* PCR.

INTRODUCTION

Leukemia bovine virus (BLV), a worldwide distributed retrovirus belonging to the *Retroviridae* family, is the causative agent of enzootic bovine leukosis (EBL). In Brazil, this virus is found infecting both beef and dairy cattle, with highest incidence in dairy cattle.

Once infected by BLV, a low viral load persists for 1 to 8 years, leading the animal in a prolonged asymptomatic stage. Thirty percent of infected animals develop a persistent lymphocytosis (PL) with low viral gene expression and persistent antibody titers, and only 0.1 to 10% develop malignant lymphosarcoma (Misky et al., 1996). Most infected animals antibodies are produced against BLV envelope glycoprotein (gp51) and core protein (p24) (Mamoun et al., 1990).

The economic impact of BLV infection in Brazil is unknown, although BLV prevalence data have demonstrated that this virus is highly disseminated in several Brazilian states. The infection rates have reached around 54% in São Paulo, 22% in Mato Grosso do Sul, 13 to 32% in Goiás, 9% in Rio Grande do Sul, 16% in Bahia, 26 a 49% in Pará, 20% in Paraná and 54% in Rio de Janeiro (Oliveira et al., 1990). The meaning of this current picture in Brazil can be inferred through the United States dairy industry reports of losses caused by BLV which reached more than U$ 86 million a year (Da et al., 1993).

Nowaday, BLV infection control programs have considered the extension of virus dissemination, slow evolution of the disease, and the number of animals remaining asymptomatic. These programs are, presently, including prophylactic measures for limiting virus dissemination, and serological surveys for isolating and eliminating of BLV-infected animals from suspected herds.

Generally, the disease control and BLV eradication programs have been performed on the basis of detectable antibody response as an indicator of infection, by quantifying serum antibody titers against BLV proteins.

The method currently employed for BLV-infection diagnosis has been the AGID techinique, due its ease of use, simplicity and rapidity in obtaining results (Johnson & Kaneene 1992). Otherwise, AGID has low sensitivity, failing in the detection of antibodies in the early stages of the infection (Eaves et al., 1994).

Recently, ELISA has been used for detection of anti-BLV gp51 antibodies, which are early on produced by infected cattle. Though a sensitive and a reliable procedure for the identification of infected animals, ELISA as well as other serological methods present some restrictions of application related to the stage of infection and other host-linked factors (Mamerickx et al.,1985; Molloy et al., 1990).

Polymerase chain reaction (PCR) has been described as an alternative method, which directly detects the presence of proviral DNA in BLV infected cattle with low, transient or absent antibody titers (Kittelberger et al., 1996; Klintevall et al., 1994).

The objective of this trial was to validate a sensitive and specific nested PCR for direct detection of BLV in blood samples.

The protocol validated in this work can be used to confirm or exclude BLV-infection in asymptomatic cattle or with doubtful serological results, constituting in a valuable assay for prompt epidemiological investigations of BLV (Beier et al., 2001) as well as for surveillance of animal health.

MATERIALS AND METHODS

Samples

The blood samples were collected from 40 animals of the Nelore strain, 1-2 years old, from BLV-positive dairy herd located in Pirassununga, São Paulo, Brazil. Whole blood samples and sera were kept at -20ºC until performing the tests. A commercial anti-BLV gp51 serum was used as positive standard serum, and the negative control serum was collected in a BLV-free dairy herd.

Extraction of DNA proviral

Proviral DNA was extracted from whole blood using QIAamp® DNA Mini Kit (QIAGE N Inc., Valencia, CA, USA). The DNA used as positive control was extracted by the same way, from fetal lamb kidney (FLK) cells persistently infected with BLV. DNA samples extracted from MDBK and PK-15 cells were used as negative controls.

DNA amplification

The nested PCR was carried out with primers selected from *env* gene region fragments, which encode envelope glycoprotein gp51. Two different pairs of primer sets were tested in this work: 1) the pair *env*5035 / *env*5443 as outer primers (amplification of 428 bp fragment from proviral BLV), and the pair *env*5065 / *env*5377 as inner primers (amplification of 341 bp fragment) (BALLAGI-PORDÁNY *et al.*, 1992)(Board 1); 2) the pair *env*5032 / *env*5099 as outer primers (amplification of 600 bp fragment), and the pair *env*5521 / *env*5608 as inner primers (amplification of a 444 bp fragment from proviral BLV) (BEIER *et al.*, 2001) (Board 2).

Board 1 - BALLAGI-PORDÁNY et al. (1992) primer sets.

Outer primers.
F *env*5035 5'- GTG CCA AGT CTC CCA GAT ACA -3'
R *env*5443 5'- TAT AGC ACA GTC TGG GAA GGC -3'
Inner primers:
F *env*5065 5'- CTG TAA ATG GCT ATC CTA AGA TCT ACT GGC -3'
R *env*5377 5'- GAC AGA GGG AAC CCA GTC ACT GTT CAA CTG -3'

Board 2 - BEIER et al. (2001) primer sets.

Outer primers:
F *env*5032 5'- TCT GTG CCA AGT CTC CCA GAT -3'
R *env*5099 5'- AAC AAC AAC CTC TGG GAA GGG T -3'
Inner primers:
F *env*5521 5'- CCC ACA AGG GCG GCG CCG GTT T-3'
R *env*5608 5'- GCG AGG CCG GGT CCA GAG CTG G -3'

The nested PCR using BALLAGI-PORDÁNY et al. (1992) primers was performed in two rounds, in a total reaction volume of 25 µL for both rounds. Reaction mixture was constituted by: 3.0 mM $MgCl_2$, 0.1 µM of each deoxynucleotide (Invitrogen™ Life Technologies, Brazil); 2.5 U *Taq* DNA polymerase (GibcoBRL®); 0.2 µM of the mixture of outer and inner primers; 3.0 µL DNA in the first round of amplification and 0.5 µL DNA in the second round of amplification. The amplification reactions were carried out in a DNA thermal cycler GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, Conn.) using the parameters of initial incubation and amplification cycles (number, time and temperature conditions) described by BALLAGI-PORDÁNY et al. (1992) and by BEIER et al. (2001). Briefly, initial incubation at 94º C for 2 min, followed by 40 amplification cycles consisting of denaturation at 94º C or 95º C for 30 sec; primer annealing at 62º C for 30sec (outer primer pairs) or at 68º C for 30sec or 72º C for 1 min (inner primer pairs), and primer extension at 72º C for 2 or 1 min followed by a final extension step at 72º C for 5 or 4 min. In order to visualize PCR products, 10 µL of amplified mixture were run on 1.5% agarose gel, followed by ethidium bromide staining. The identity of the PCR product was assessed by restriction enzyme digestion using 5 U *Bam*H I (GibcoBRL®)and 10 µL of a second PCR amplification product, in accordance to the manufacturer instructions. Digested DNA products were run on 12% polyacrylamide gel, followed by ethidium bromide staining.

AGID

For BLV infection survey, as recommended by OIE, all serum samples were submitted to AGID for anti-BLV antibodies detection by employing a commercial gp51 antigen (OIE, 2000).

ELISA

The ELISA of serum samples was performed by using commercial kits (Herd Check, IDEXX Laboratories, USA) that identify antibodies against BLV gp51.

Comparative analyses of sensitivity and specificity of the methods

The sensitivity and the specificity of the AGID and the nested-PCR were compared using ELISA as the standard, according to CHOI et al. (2002). The relative sensitivity (%S) was expressed as the percentage of infected animals detected as positive by the method being compared with the standard. The relative specificity (%P) was expressed as the percentage of uninfected animals correctly identified as negative by the method being compared with the standard. The positive predictive value (%PV) was calculated as the proportion of actual results by the method being compared with the standard in the total of positive results. The negative predictive value (%NP) reflects the proportion of actual results by the method being compared with the standard in the total of negative results. The efficiency of the method (%E) reflects the proportion of the correct results.

RESULTS AND DISCUSSION

In this work, the nested PCR was standardized by analyzing blood samples from animals of a BLV-positive dairy herd.

As expected, applying the primers set 1 (BALLAGI-PORDÁNY et al., 1992) to the proviral *env* gene enabled

amplifications of a 428 bp and a 341 bp fragment produced by the *env*5035 / *env*5443 outer primers and the *env*5065 / *env*5377 inner primers, respectively. In the eletrophoretic evaluation of proviral DNA products obtained from blood samples, a single band was revealed after the second PCR amplification with inner primers, as observed for FLK cells used as positive control (Fig. 1A; lane 4). This 341 bp BLV fragment nucleotide sequence amplified product from the samples was digested by *Bam*H I, producing two fragments of 178 and 163 bp, respectively (Fig. 1A; lanes 1-3). This BLV restriction site was observed in all samples analyzed.

Another primers set, the primer set 2 (Beier et al., 2001), derived from the same region of BLV *env* gene, but with slight difference in the sequence, was tested in this work, in similar experimental conditions. When applied to the proviral *env* gene, the *env*5032 / *env*5099 outer primers enabled the amplification of a 600 bp fragment. In the eletrophoretic evaluation of proviral DNA products a single band was revealed after a second amplification using *env*5521 / *env*5608 inner primers which produced a 444 bp fragment (Fig.1B; lanes 1, 3, 5, 7, and 9).

The 444 bp BLV amplified product was digested by *Bam*H I, producing two fragments, respectively, of 315 and 129 bp (Fig. 1B; lanes 2, 4, 8, 10). We found a sample in which BLV amplified products did not present the *Bam*H I restriction site as showen in at Figure 1B (lane 6).

The detection threshold (expressed in DNA ng / mL) of the PCR assay in blood samples was obtained with proviral DNA extracted from FLK cells, at the initial concentration of 18ng / mL, which was tenfold sequentially diluted and also processed for PCR amplification. Under these conditions, proviral DNA was detected even at 10^{-6} dilution.

The primer sets were chosen because they amplify fragments of the *env* gene that encodes BLV gp51 antigen. This enables a comparison between PCR, which directly detects BLV and the serological assays, which evaluate antibody response against this viral glycoprotein. Moreover, the animal immune response against BLV gp51 antigen is early (*Blankenstein et al.*, 1992).

The sensitivity and specificity of the nested PCR to detect BLV were compared with that observed by conventional methods.

As mentioned before, the AGID and ELISA have been widely used and considered excellent to estimate the prevalence of BLV infection in a herd. These serological methods are accepted worldwide for detection of BLV infection, but they do not always detect an early infection or infection in young calves born from infected cows (Ballagi-Pordány et al., 1992). For practical reasons, current BLV diagnosis is mainly based on such serological methods.

As can be seen in Table 1, the AGID detected only 25 positive samples corresponding to 63% from the total of 40 blood samples analyzed in this work. Among these samples, the ELISA detected BLV infection in 37 samples and the nested PCR in 36, corresponding to 93% and 90%, respectively.

Table 2 shows the results of comparison of the AGID and the PCR assays sensitivity and specificity with ELISA, which was set up, in this work, as the standard method. Compared with ELISA, the AGID showed only 68% sensitivity, 20% specificity, and an agreement in the results (or %E) compared to ELISA of 70%. Indeed, according to data from literature, the AGID sensitivity was not sufficient to identify correctly all the samples from the analyzed group, failing up to 30% in the BLV infection detection (Choi et al., 2002; Da et al., 1993; Johnson & Kanneene, 1992) (Table 2).

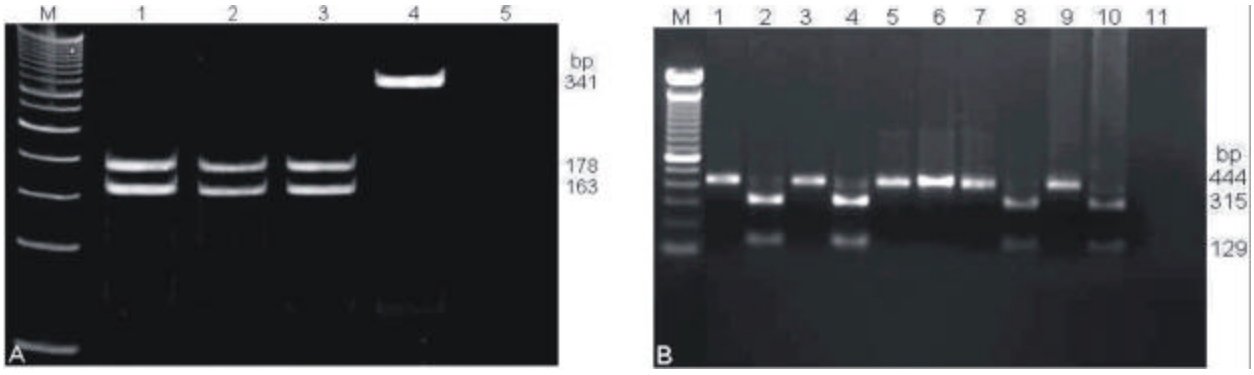

Fig. 1 - Restriction endonuclease profile analysis of the BLV PCR product digested by *Bam*H I. A) *Bam*H I restriction pattern of the PCR products amplified from BLV samples obtained by primers set 1 (lanes 1, 2 and 3); PCR product from BLV positive sample (lane 4); PCR assay reagent control (lane 5); 50 bp DNA molecular weight marker (GibcoBRL®) (lane M). B) PCR products from BLV samples obtained by primers set 2 (lanes 1, 3, 5, 7 and 9); *Bam*H I restriction pattern of the PCR products from BLV positive samples (lanes 2, 4, 6, 8 and 10); PCR assay reagent control (lane 11); 100 bp DNA molecular weight marker (GibcoBRL®) (lane M).

Table 1 - Results of ELISA, AGID and nested PCR of blood samples collected from animals in a BLV-infected dairy herd.

Samples	Assay			
	ELISA	AGID	PCR / primers	
			set 1	set 2
positive	37	25	5	36
negative	3	15	35	4
Total	40	40	40	40
positive / total	93%	63%	13%	90%

Table 2 - Comparison of AGID and nested PCR with sensitivity and specificity, with ELISA as standard method.

ELISA		AGID	PCR / primers	
			set 1	set 2
Positive A (%S)	37 (100%)	25 (68%)	5 (14%)	36 (97%)
Negative (%P)		(20%)	3 (9%)	2(67%)
Total agreement (%E)		28 (70%)	8 (20%)	38 (95%)

%E = method efficiency or agreement with ELISA
%S = relative sensitivity
%P = relative specificity

The nested PCR using primer set 2 showed, in comparison with ELISA, a sensitivity of 97% with 67% specificity and 95% E (Table 2). However, in this work, we also observed that the nested-PCR sensitivity depended on the primer set applied to the proviral *env* gene. The sensitivity observed for this assay by using the primer set 1 was only 14% with 9% P and 20% E.

The differences observed in the results of this assay with ELISA may reflect individual variations, for example, in the number of infected lymphocytes in circulation, at the moment of sample collection.

We attributed the variations observed in the nested PCR sensitivities to feasible heterogeneity of viral population or geographic origin of the infected cattle, which suggested to us the need of more than one pair of primer set for optimizing results, as described by MARSOLAIS et al. (1994) for amplification of *gag* BLV gene segment.

Our data corroborate the use of AGID as a screening assay in high prevalence regions or in initial prevalence surveys. This serological method is able to identify infected cattle from regions where the disease is endemic, the viral load is high, and the animals present detectable antibodies against the agent.

Otherwise, the use of the nested-PCR for proviral DNA direct detection is indicated in cases of asymptomatic cattle, in which the presence of BLV is low, and antibody titers are transient or absent. Moreover, this assay is very suitable in cases with doubtful serological results (KITELBERGER et al., 1996; KLINTEVALL et al., 1994), and showing a good performance even when analyzing other body fluids or tissue samples (data not shown).

CONCLUSIONS

We concluded that the nested PCR using more than one primer set, besides enabling direct and early BLV infection diagnosis, may be also used for assessment of feasible BLV genetic variability in different regions. This investigation is in progress toward gaining knowledge about the Brazilian BLV genetic heterogeneity, which may provide alternative approaches for control of the disease.

This standardized nested PCR has been implemented in our laboratory and had an immediate applicability in the health control of Brazilian bovine production. This assay was recognized by the Brazil Ministry of Agriculture and set forth as a valuable assay for national and international commercial purposes of bovine products.

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