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A rapid and sensitive diagnosis of bovine leukaemia virus infection using the nested shuttle polymerase chain reaction[1]

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ABSTRACT.- González E.T., Norimine J., Valera A.R., Travería G.,Oliva G.A. & Etcheverrigaray M.E. 1999. **A rapid and sensitive diagnosis of bovine leukaemia virus infection using the nested shuttle polymerase chain reaction.** *Pesquisa Veterinária Brasileira 19(2):63-67.* Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, 60 y 118, 1900 La Plata, Argentina.

Bovine leukaemia virus (BLV) is the causative agent of enzootic bovine leukosis (EBL). In Argentina, where a program to eradicate EBL has been introduced, sensitive and reliable diagnosis has attained high priority. Although the importance of the agar gel immunodiffusion test remains unchanged for routine work, an additional diagnostic technique is necessary to confirm cases of sera with equivocal results or of calves carrying maternal antibodies.Utilizing a nested shuttle polymerase chain reaction, the proviral DNA was detected from cows experimentally infected with as little as 5 ml of whole blood from BLV seropositive cows that were nonetheless normal in haematological terms. It proved to be a very sensitive technique, since it rapidly revealed the presence of the provirus, frequently at 2 weeks postinoculation and using a two-round procedure of nested PCR taking only 3 hours. Additionally, the primers used flanked a portion of the viral genome often employed to differentiate BLV type applying BamHI digestion. It is concluded that this method might offer a highly promising diagnostic tool for BLV infection.

INDEX TERMS: Bovine leukaemia virus, diagnosis, Nested–PCR.

RESUMO.- [Diagnóstico rápido e sensível da infecção com o vírus da Leucemia Bovina através de Shuttle Nested Polymerase Chain Reaction.] O Vírus da leucemia bovina (BLV) é o agente causal da Leucose Enzoótica Bovina (EBL). Na Argentina, iniciou-se um programa de erradicação da EBL. Neste estágio, é prioritário possuir uma ferramenta de diagnóstico confiável. Embora seja indiscutível a importância do teste de agar gel imunodifusão, empregado rotineiramente no diagnóstico serológico da EBL, faz-se necessária uma técnica de diagnóstico adicional capaz de confirmar os resultados duvidosos. Foi possivel detectar ADN proviral aplicando Nested-PCR em novilhos experimentalmente infectados com pequenas doses de sangue total (5ml) obtidas de um bovino BLV soropositivo. Esta técnica, cujo procedimento leva 3 horas, demonstrou ser muito sensível, uma vez que foi capaz de detectar a presença do provirus duas semanas após a inoculação. Os primers utilizados são os que detectam uma porção do genoma viral que geralmente é usado para diferenciar os tipos de BLV, utilizando a digestão com BamHI. Sugerimos que este método possa ser um instrumento válido para o diagnóstico precoce da infeção pelo BLV.

TERMOS DE INDEXAÇAO: Leucose bovina, diagnóstico, Nested-PCR.

INTRODUCTION

Bovine leukaemia virus (BLV) is an exogenous retrovirus that is the causative agent of enzootic bovine leukosis (EBL). It primarily infects B cells of cattle leading to a serological response, and in some animals causes persistent lymphocytosis and/or lymphosarcoma (Kettmann et al.1994, Mirsky et al.1996, Schwartz & Levy 1994).

BLV infection is endemic in Argentina, especially in dairy herds of the central and northern areas. In highly infected herds, more than 60% of cows are seropositive to BLV. The economic impact of BLV infection is unknown, but appears to be major due to reduced milk production, slaughterhouse condemnation of tumour bearing carcasses, and replacement

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of sick or dead animals. In the United States, the estimated loss to the dairy industry is reported to be more than $86 million annually. (Da et al.1993).

As we have started an eradication project of BLV infection in Argentina, early diagnosis is important for the implementation of control measures, and is currently assessed by the detection of circulating antibodies against the viral envelope protein gp51. However, this approach has shortcomings in the time taken to detect antibodies and in the failure to detect antibodies in some animals. Furthermore, the possibility of decreased immune responsiveness to BLV in animals persistently infected with bovine viral diarrhoea (BVD) virus must be considered (Roberts et al.1988). Clearly a technique such as the polymerase chain reaction (PCR), which directly detects the presence of proviral DNA, has advantages over methods designed to measure host antibodies, and ought to provide a sensitive diagnostic assay, especially when antibody tests give weakly positive or equivocal results.

The aim of this study was to improve the direct diagnosis of BLV infection by applying a simple detection method based upon a nested PCR. We have used a two temperature protocol to amplify BLV proviral DNA (we call this PCR "shuttle PCR", hereafter), and it proved to be very effective for the rapid and early detection of BLV infection, which we suggest as a practical and superior method for diagnosis. Moreover, BamH1 digestion of the PCR product gives additional information about the BLV genotype.

MATERIALS AND METHODS

Experimental Animals

Two two-year old cows (06/203 and 73/71) served as donors of BLV-infected whole blood. These donor cows had been experimentally infected previously by inoculating 10 ml of whole blood from a tumour-bearing cow and subsequently tested monthly during 3 months, consistently giving BLV antibodies after seroconversion, as determined by the agar gel immunodiffusion (AGID) test.

Six Aberdeen-Angus two-year old cows were obtained from a closed herd consisting of 300 bovines without antecedents of bovine leukosis, and giving seronegative results by the AGID test in three determinations with intervals of two months. These 6 recipient cows were also confirmed seronegative for BLV before inoculation by AGID and PCR, as described later. Whole blood from cows 06/203 and 73/71 were obtained in sterile heparin and administered (5 or 50 ml) subcutaneously to four recipient cows, maintaining two as controls. The scheme of inoculation is shown in Table 1. The recipient and control cows were bled every week thereafter until 3 months after viral inoculation.

Table 1. Inoculants for experimental infection

Donor	Recipient	Route [a]	Blood vol. [b]	Limphocytes Inoc.
06/203	57/40	SC	5	3.6×10^4
	72/72	SC	50	3.6×10^5
73/71	36/34	SC	5	3.0×10^4
	56/64	SC	50	3.0×10^5
Control	54/63			
	92/65			

[a] Inoculation route, SC: subcutaneous.
[b] Microliters.

Haematological examination

Each blood sample was examinated for differential count of white cells by convetional method. Their sera and peripheral blood mononuclear cells (PBMC) were employed for further analysis.

AGID

The AGID test (Miller & Van der Maaten 1976) was performed on all samples at the end of the study with a commercial kit produced in our laboratory (approved by SENASA, National Service for Animal Health in Argentina). Agar plates were placed in humidified trays and incubated at room temperature for 1-3 days.

Extraction of DNA

The DNA extraction were performed essentially as reported by Kelly et al. (1993). Briefly, blood were defrosted, 35 ml of a 10mg/ml stock solution of proteinase K was added and the samples were digested in a 55 °C waterbath overnight. Samples were extracted with phenol, then with 25:24:1 phenol:chloroform:isoamyl alcohol. Fifty microliters of 5M NaCL and 1 ml of 100% ethanol were added, and the samples were centrifuged at 12,000 x g for 30 minutes at 20 to 25 °C. Samples were washed once with 70 % ethanol and suspended in 50 ml of 10 mM Tris-HCl, 1mM EDTA, pH 8.0.

PBMC separation

One ml of heparinised blood was mixed with 1.2 ml of phosphate buffered saline (PBS), layered over Ficoll-Paque (Pharmacia, Uppsala, Sweden) in 5 ml conical tubes and centrifuged at 1350 g for 30-40 min. PBMC were collected from the gradient interphase and washed three times in PBS, centrifuging once at 650 g for 10 min. and then twice 1200 g for 5 min. The total number of PBMC of each sample was determined by conventional method.

PCR amplification

The separated PBMC were resuspended in 50 ml of PBS without calcium and magnesium and boiled for 12 min. Subsequently, the specimens were chilled on ice for 5 min. and 5 ml of each were used as templates in 50 ml of total reaction mixture, providing a final concentration of 50 mM KCl , 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 mM of each deoxynucleotide trisphosphate, 1 U of Taq polymerase (AmpliTaq: Perkin-Elmer Cetus, Norwalk, Conn.) , 0.2 mM of each BLV envelope (env) primer in a final volume of 50 ml . The cycling parameters used in both the first and second amplification were 30 cycles of 1 min. at 94 ° C, 1 min. at 60 ° C. Aliquots (3 ml) of the first-round PCR product were transferred to reach of 47 ml second-round amplification mixtures. The primers used were the same as those designed by Ballagi-Pordany et al.(1992):

External primers:
OBLV-1 5' -GTG CCA AGT CTC CCA GAT ACA-3'
OBLV-6 5'- TAT AGC ACA GTC TGG GAA GGC-3'

Internal primers:
OBLV-3 5'- CTG TAA ATG GCT ATC CTA AGA TCT ACT GGC –3'
OBLV-5 5'- GAC AGA GGG AAC CCA GTC ACT GTT CAA CTG-3'

Products of the second round PCRs were visualised with UV light after electrophoresis in 2 % agarose gels and staining with ethidium bromide. To verify the identity of these products, restriction enzyme digestion was performed using 20 units of *Bam* HI (GIBCO BRL,

Table 2. The time course of detection of BLV infection by indirect (Agar Gel Inmmunodiffusion - AGID) and direct (Polymerase Chain Reaction - PCR) methods

Recipient	Blood vol[a]	Route[b]	ID[c]	PCR	Weeks postinoculation													
					1		2		3		4		5		6		7	
					ID	PCR	ID	PCR	ID	PCR	ID	PCR	ID	PCR	ID	PCR	ID	PCR
36/34	5	SC	-	-	-	-	-	+	-	+	-+	+	-+	+	-+	+	-+	+
56/64	50	SC	-	-	-	-	-	+	-	+	-+	+	+	+	+	+	+	+
57/40	5	SC	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+
72/72	50	SC	-	-	-	-	-	-	-	+	-	+	-	+	-+	+	+	+
Control			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Microliter.
[b] Inoculation route, SC: subcutaneous.
[c] Inmunodiffusion (AGID test).

Gaithersburg, MD) and 10 ml of a second-round product in a final volume of 50 ml. Five ml of the PCR product and 20 ml of digested DNA product were subjected to electrophoresis in a 6 % acrylamide gel to characterise the 2 fragments.

RESULTS

AGID test and haematological examination

As shown in Table 2, the seroconversion was detected in three cases out of 4 experimentally-infected cows at 4 weeks after inoculation using our AGID test kit, and in the remaining case at 6 weeks. One case (36/34) was always weakly positive throughout the study. The time of seroconversion was not related with the amount of inoculant. Haematological findings were unchanged in all cows during this study (data not shown).

Nested shuttle PCR

In three out of four cows, proviral DNA was detected at 2 weeks, and in one at 3 weeks after inoculation using the nested shuttle PCR. This early detection was also independent of the dose of inoculation. We tested 57° C and 60° C as annealing plus extension temperatures, and sensitivities were not different between them (data not shown). By using 60° C as the temperature, the results were consistent with additional experiments using samples from BLV-seropositive field cows. Proviral DNA could be frequently be detected from 5 ml of supernatant of boiled 10^4 PBMC from a cow as early as 2 weeks postinoculation. Supernatants of boiled samples were not different from extracted DNA as a source of template, and may even have given more consistent results.

According to the nucleotide sequence of BLV (Sagata et al.1985), we digested the final PCR products with BamH1 and confirmed the BamH1 site. Interestingly, European BLV strains reportedly do not have this restriction site (Mamoun et al. 1990, Coulston et al. 1990). As shown in Fig.1 and according with Mamoun et al (1990), we may classify BLV strains using BamHI. Argentine strains of BLV seem to belong to the

Table 3. Comparison of the sequence of the BamHI site in the portions flanked by the second primers in BLV env gene among strains isolated in various countries. From the data of Mamoun et al. (1990) and Coulston et al. (1990)

BLV isolates	Origin	Sequences In BamHI site	
		5221	5236
FLK-BLVUSA	USA	ATCAA**GGATCC**TTTTA	
VdM	USA	--------A---------	
1BLV-1	Japan	--------A---------	
pBLV-A1	Australia	-----G--A---------	
LB285	Belgium	--------G---------	
T15-2	Belgium	--------G---------	
LB59	France		
	Argentina	--A---	

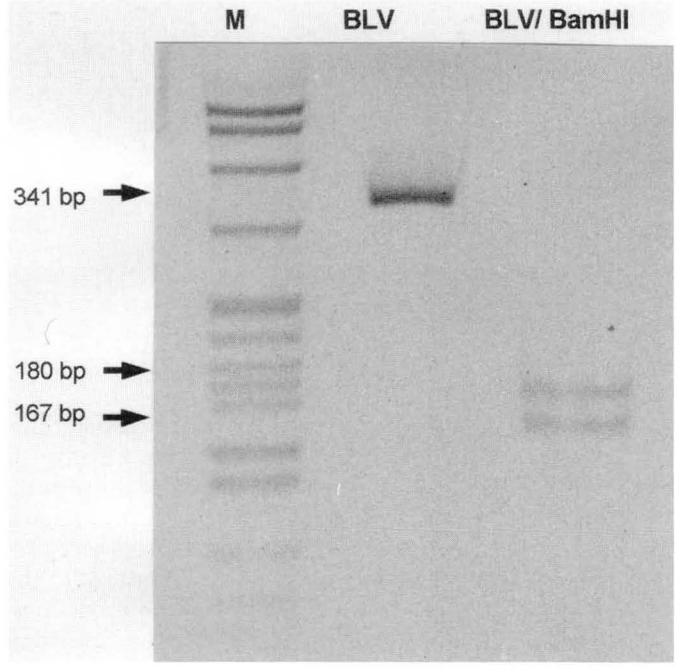

Fig. 1. Detection of BLV proviral DNA by using the nested shuttle PCR and fragments of the product digested with BamHI. Ten ml of the sample were loaded per well, electrophoresed on a 6% acrylamide gel and stained with ethidium bromide. Lane: 1, molecular marker, pBR322 digested with MspI; 2, an amplified product ; 3, fragments of an amplified product digested with BamHI.

Japanese-America subgroup. We also compared with the results reported by Coulston et al. (1990) for Australian strain, that belong to the same subgroup (Table 3), demostrating a high level of amino acid conservation in this biologically importante region.

DISCUSSION

Although a serological screening method, the AGID test is very economic, simple and comparatively reliable for the diagnosis of BLV infection; while its importance remains unchanged, it is not sufficiently sensitive for determining BLV-status in some circumstances, as well as being incapable of discriminating infection from adoptive immunity in young calves born from infected cows. Also it was demonstrated that in cattle persistently infected with BVD virus, a depressed antibody response to BLV occurred and should be considered when formulating regulations governing the testing of animals for freedom from BLV (Roberts et al.1988). Conducting BLV proviral DNA detection using PCR together with the AGID test, we can eradicate these problems, whereupon sensitive and rapid diagnosis of BLV infection becomes possible.

Detection of BLV RNA or proviral DNA using PCR has so far been reported in many laboratories (Abbot et al.1988, Brandon et al.1991, Eaves et al.1994, Jacobs et al.1992, Kelly et al.1993, Klintevall et al.1994, Marsolais et al.1994, Mirsky et al.1993, Naif et al.1990, Poon et al.1993). To attain high sensitivity, a nested PCR or a southern blotting for detecting amplified products is commonly used. Naif et al.(1992) also reported an enzyme-linked assay for PCR-amplified proviral DNA (17).

As our purpose is to establish a practical, sensitive and rapid diagnosis for BLV infection and use it for field survey, we employed a simple nested PCR as BLV provirus detection. Among several reports, the method developed by Ballagi-Pordany et al. (1992) seemed to be one of the more sensitive and practical. We have confirmed that the method is very sensitive and reliable and that a shuttle PCR is appropriate. The shuttle PCR has shortened the time of the 2-round amplification procedure to about 3 hours without reducing the sensitivity. Moreover, this final amplified product has a convenient BamHI site in the middle to confirm BLV proviral DNA specificity. Interestingly, European BLV strains do not have the BamHI site in this portion (Kettmann et al.1981,Rice et al.1984). Mamoun et al. (1990) identified two subgroups of BLV, a Japonese-American subgroup and a European subgroup, and found that genetical differences between the two subgroups are related with differences in biological properties, for example, recognition of the cell receptors (Mamoun et al.1990, Sagata et al.1985). According to this report, the Australian strain may belong to the Japonese American subgroup (Coulston et al.1990), and it has a similar mutation in the sequences associated with biological functions (neutralizable conformational epitopes: F,G and H , also involved in syncytium inhibition). In the present study, although we do not have sequence data, we can predict that the Argentine-type isolate also seems to belong to the Japanese-American subgroup. Dube et al. (1997), have recently reported the phylogeny of BLV using a BLV specific PCR assay, and provided very useful information about the highly conserved BLV sequences in the *env* gene.

There was no striking difference in the time course from infection to seroconversion or in the detection of the proviral DNA among the four inoculated-cows; 4 to 6 weeks for antibodies detected by AGID test and 2 to 3 weeks to detect proviral DNA by nested shuttle PCR.The detection of antibodies to BLV by using our AGID test kit seemed to be comparatively sensitive, because inoculants used were derived from the BLV positive cow with normal white blood cell counts and the amount of inoculants was as low as 5 ml., estimated to be 3×10^4 of PBMC. Naif et al.(1992) reported that they could not detect the BLV antibody from cows experimentally infected with 10^7 lymphocytes of a persistently infected cow up to 4 weeks postinoculation.

Taken together, we consider that the detection of proviral DNA at 2 weeks postinoculation in our experimental infection is sufficiently sensitive to apply during the very early infection period.

In conclusion, we have shown that this protocol of the nested shuttle PCR is very useful in many aspects, and suggest that this method can be very promising and desirable for the diagnosis of BLV infection

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