Proviral Detection and Serology in Bovine Leukemia Virus-exposed Normal Cattle and Cattle with Lymphoma

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ABSTRACT

Twenty-seven cattle with lymphoma and 46 cows from a known bovine leukemia virus (BLV)-infected herd were tested for anti-BLV antibody by the agar gel immunodiffusion (AGID) test and an enzyme-linked immunosorbent assay (ELISA). The polymerase chain reaction (PCR) and Southern hybridization were used to detect BLV provirus in the tumor DNA of the 27 cattle with lymphoma. The PCR was used to detect BLV provirus in the peripheral blood mononuclear cell DNA of the 46 normal known-exposed cattle. Two presumed false negative AGID test results compared to ELISA were found. Of ten cattle three years of age or less with "sporadic" forms of lymphoma, four had BLV provirus in tumor DNA, detectable by PCR. In two of these four, BLV provirus was clonally integrated based on digestion of tumor DNA with restriction enzymes followed by Southern hybridization. The BLV provirus was not detected by PCR in 5 of 17 cattle with "enzootic" lymphoma and two of these five were seronegative. Among normal BLV-exposed cows, 6.5% (3 of 46) were serologically positive and PCR negative; serologically negative and PCR positive cows occurred with the same frequency. Serological and PCR test results, when considered in all cattle (n = 73), had a concordance rate of 83.6%. Discordant test results occurred with approximately equal frequency between serologically positive and PCR negative (7 of 73, 9.6%) and serologically negative and PCR positive (5 of 73, 6.8%) groups. These data suggest that the role of BLV in some "sporadic" bovine lymphomas, previously unassociated with BLV, should be reexamined. The BLV provirus was not demonstrable in the tumor DNA from five adult cattle with lymphoma, suggesting that BLV may not be the etiological agent in all adult bovine lymphomas. The findings of persistently seronegative PCR positive and seropositive PCR negative cattle indicate that further work is needed to more fully understand the host-virus interaction. Present serological screening methods may not have sufficient sensitivity for determining BLV status in some circumstances.

RÉSUMÉ

Le matériel d'étude provenait d'un troupeau infecté par le virus de la leucose bovine. Ce groupe d'animaux comprenait 73 vaches dont 27 présentaient un lymphosarcome. Ces animaux ont été vérifiés pour présence d'anticorps contre la leucose par deux méthodes : l'immuno-diffusion sur gélose et la technique ELISA. Deux techniques, une réaction de polymérase et une d'hybridation ont été utilisées pour identifier le provirus de la leucose à partir des mononucléaires du sang chez 46 bovins exposés aux virus de la leucose. Comparés à la technique ELISA, deux animaux se sont révélés faux négatifs par la méthode d'immuno-diffusion sur gélose. Parmi les 10 vaches âgées de moins de 3 ans et affectées de la forme sporadique de la leucose, quatre se sont avérées positives par la méthode de polymérase. Chez cinq animaux du groupe de dix-sept vaches affectées de la leucose enzootique, le provirus n'a pu être détecté par la méthode de polymérase. Parmi les animaux exposés au virus de la leucose, 6,5 % (3 sur 46) étaient séropositifs (ELISA) et négatifs par la méthode de polymérase; on observait aussi, avec la même fréquence, des animaux séro-négatifs et positifs par la méthode de polymérase. On observe une concordance de 83,6 % lorsque l'on compare les deux méthodes de dépistage chez tous les animaux (N = 73). Des résultats contradictoires sont observés à la même fréquence avec les deux méthodes, séro-positifs et polymérase négatifs (7 sur 73, 9,6 %), séro-négatifs et polymérase positifs (5 sur 73, 6,8 %). Ces résultats suggèrent que l'hypothèse du rôle du virus de la leucose bovine mérite d'être réévaluée dans les cas de la forme sporadique de cette pathologie. Le provirus n'ayant pu être démontré dans l'ADN des cellules tumorales de cinq vaches adultes atteintes de lymphosarcome. Cette observation suggère que le virus de la leucose n'est peut-être pas toujours en cause lors de lymphosarcome chez le bovin adulte. Ces résultats positifs et négatifs selon la méthode utilisée, indiquent qu'il serait nécessaire de poursuivre la recherche dans ce domaine afin de bien définir l'interaction hôte-virus. Dans certaines conditions, le dépistage sérologique n'aurait pas la sensibilité nécessaire pour déterminer le statut de la leucose chez le bovin. (*Traduit par Dr Yvon Couture*)

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INTRODUCTION

Lymphomagenicity of bovine leukemia virus (BLV) in sheep and epidemiological data showing the infectious or "enzootic" nature of bovine lymphoma support the role of BLV as the etiological agent of lymphoma in adult cattle (1). The genomic structure of BLV is most similar to the human T-lymphotrophic virus type 1 (HTLV-1) which causes adult T-cell leukemia/lymphoma and a T-cell variant of hairy cell leukemia (2). Integration of BLV provirus in DNA from lymphoid tumors of adult cattle is random, but the integration site in any one tumor is monoclonal or occasionally oligoclonal (1). The monoclonality of integration site is evidence that the tumor arose from a single clone of BLV-infected lymphocytes and indirectly incriminates BLV in lymphomagenesis. Over relatively large areas of genomic DNA there is no preferred integration site which is typical for this group of oncornaviruses (3,4).

Lymphomas in adult cattle are multicentric and occur enzootically. Affected cattle have anti-BLV antibodies and detectable BLV provirus in tumor DNA by Southern hybridization (5,6). In contrast, BLV integration in cattle without lymphoma is polyclonal and Southern hybridization using DNA from peripheral blood mononuclear cells fails to demonstrate provirus in approximately 50% of BLV-infected cattle (7). The lack of sensitivity of Southern hybridization in BLV-infected cattle without lymphoma may be due to the low number of BLV-infected lymphocytes in circulation (8). Lymphomas in immature cattle occur as the juvenile multicentric, thymic, or cutaneous forms. These lymphomas occur sporadically and are not associated with BLV by seroepidemiological and Southern hybridization techniques (1,9,10), with few exceptions (11–14). Initial reports of seroconversion of calves inoculated with tumor homogenates from "sporadic" cases (15,16) may have been due to failure to raise inoculated calves in strict isolation.

Amplification techniques, such as the polymerase chain reaction (PCR), surpass the sensitivity of Southern hybridization, making it possible to detect minute amounts of DNA or RNA associated with numerous congenital, infectious, or malignant diseases (17). The PCR has been used for the detection of the human immunodeficiency (18) and T-cell lymphotrophic viruses in humans (19). Chronic BLV infection in rabbits has been proven using PCR (20). The BLV provirus has been demonstrated by PCR in sheep (21) and cattle (22–24). Seven seronegative cattle had negative PCR test results, however negative PCR test results were also found in 5 of 18 seropositive cattle (23). Serological and PCR test results were found to be in complete agreement in a series of 18 cattle (24). Another study showed that of 20 seronegative cattle all had negative PCR test results except for one individual and 20 seropositive cattle, 16 cattle with persistent lymphocytosis and seven cattle with lymphoma had positive PCR test results (22).

Seronegative BLV-infected cattle could potentially cause persistence of BLV infection in herds or contaminate vaccines produced in cattle or bovine cell cultures. Therefore, it is important to determine with certainty if such individuals exist. Although BLV is considered the causative agent of lymphomas in adult cattle while lymphomas in immature cattle are generally unassociated with BLV, this rigid classification has not been tested using current techniques aimed at detecting small numbers of proviruses. The PCR is suitable for such determinations since it is a sensitive and direct method for demonstrating provirus. To determine if there are seronegative BLV-infected cattle and if there are exceptions to the traditional understanding of the relationship between BLV and the various forms of bovine lymphoma we used PCR, serological, and Southern hybridization tests in nonrandomly selected adult and juvenile cattle with lymphoma and normal BLV-exposed cattle.

MATERIALS AND METHODS

CATTLE AND TISSUES

Tissues from 63 cattle with lymphoma were collected over a three year period. Of these 63 cattle, the 11 that were seronegative by the agar gel immunodiffusion (AGID) test (*vide infra*) were used in the present study. Tissues from 16 cattle out of the remaining 52 seropositive cattle were selected randomly. The lymphomas were diagnosed histopathologically and categorized as adult multicentric (greater than three years of age), juvenile multicentric (three years of age or less), thymic, or cutaneous according to established criteria (1). Tissues from 46 cows, three years of age or greater from a known BLV-infected herd were also used. These cows were vaccinated against bovine virus diarrhea virus (BVDV). Peripheral blood mononuclear cells (PBMCs) from the 46 cows without tumor were obtained by isopycnic centrifugation of anticoagulated peripheral blood. Tumor tissue was obtained at necropsy from affected cattle. Samples for serology and DNA analysis were stored at −20°C.

SEROLOGY

Sera from the 27 cattle with lymphoma were tested once for anti-BLV antibody using the AGID test (Bovi-Leukotest, Institut Armand-Frappier, Laval, Quebec). Sera from the 46 cattle without lymphoma were tested multiple times over two years. All sera were retested for the presence of anti-gp51 antibody in an indirect peroxidase enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody to the gp51 antigen of BLV (Dr. D. Portetelle, Faculty of Agronomy, Unit of Microbiology, Gembloux, Belgium); this ELISA has greater sensitivity and specificity than the AGID test (25). Serological test results were scored as negative or positive.

PROVIRAL DETECTION BY SOUTHERN HYBRIDIZATION

Minced tumor tissues were frozen and pulverized in liquid N_2. Pulverized tumor tissue and PBMCs were suspended in a solution containing 100 mM Tris-HCl (pH 7.4), 10 mM EDTA, 1% SDS, and 1% proteinase K (Boehringer Mannheim Canada Ltd., Laval, Quebec) for 18 hours at 56°C. At the end of this incubation NaCl was added to a final concentration of 0.1 M. DNA was obtained by phenol/chloroform extraction using an automated nucleic acid extractor and protocols supplied by the manufacturer (Applied Biosystems Canada

Inc., Mississauga, Ontario). Approximately 10 μg aliquots of DNA were digested to completion at 37°C using double digests of *Eco*RI and *Hin*dIII according to manufacturer's recommendations (Boehringer Mannheim Canada Ltd.) and electrophoresed on 1.0% agarose gels along with a 1 kilobase ladder size marker (Gibco/BRL Canada, Burlington, Ontario). The electrophoresis buffer consisted of 40 mM Tris-acetate (pH 8.0) and 1 mM EDTA. DNA was transferred to nylon membranes (ZetaProbe, BioRad Laboratories [Canada] Ltd., Mississauga, Ontario) using 0.4 M NaOH as a transfer solvent.

The BLV probe DNA was obtained by digestion of the recombinant plasmid pBLV913 with *Kpn*I as described previously (26). The full length BLV DNA fragment was purified using a silica matrix (Geneclean, Bio/Can Scientific, Mississauga, Ontario) after electrophoresis on 0.8% agarose gels. Probe DNA was labelled with ^{32}P-dCTP (ICN Biomedical Inc., Irvine, California) to a specific activity greater than 1 x 10^8 cpm/μg DNA by a random primer protocol (Boehringer Mannheim Canada Ltd.). Labelled probe was purified by column chromatography on Sephadex G-50 as described elsewhere (27).

Southern blot hybridizations were done according to the manufacturer's protocol (BioRad Laboratories [Canada] Ltd.). Hybridized membranes were sealed in plastic bags and exposed to Kodak XAR-5 film at −70°C with intensifying screens for ten days. The restriction enzymes, *Eco*RI and *Hin*dIII cut the BLV proviral genome near the 3′ end and approximately in half, respectively, and when used together result in a 3.8 kb internal fragment and two variably-sized proviral "tails" (8). Integration was considered clonal if proviral "tails" were present as distinct bands on autoradiograms as reported previously for these enzymes (8). Polyclonal integration results in proviral "tails" highly variable in size and are therefore not seen or are seen as indistinct broad smears. Occasionally, an odd number of proviral "tails" is detected and this is likely due to the superimposition of "tails" of similar size.

PROVIRAL DETECTION BY POLYMERASE CHAIN REACTION (PCR)

Primers targeted to the BLV *pol* gene (28,29) were selected, with the aid of a computerized database (CAN/SND Molecular Biology Database System, National Research Council of Canada), for their priming specificity to the target nucleotide sequences. Unlike other regions of the retroviral genome, the *pol* gene is highly conserved and should be a good target for amplification, even if BLV-strain differences exist. The sequences of BLV *pol* primer 1 and primer 2 were 5′-CTACCTTGCAGATCTCATC (complementing bases 2984 through 3002 of the BLV cDNA sequence) and 5′-GCTTGTCGAAGCTCTGCAATGC (complementing bases 3164

TABLE I. Age, serological test results, and detection of BLV provirus in cattle with lymphoma

Animal identification[a]	Age (years)	Agar gel immunodiffusion test	Enzyme-linked immunodiffusion assay[b]	Southern hybridization of tumor DNA	Polymerase chain reaction of tumor DNA
30	0.1	+	+	−	−
18	0.5	−	+	−	+
24	0.7	−	−	−	−
31	1.0	−	−	−	−
33	1.0	−	−	−	−
22	1.3	−	−	−	+
49	2.0	−	−	−	−
2	2.0	+	+	+	+
28	2.0	−	−	+	+
20	3.0	−	−	−	−
11	3.0	−	−	−	−
15	4.5	+	+	+	+
14	5.0	+	+	+	+
38	5.0	+	+	−	+
32	7.0	+	+	+	+
9	7.0	+	+	+	+
25	8.0	+	+	+	+
29	8.0	+	+	nt[c]	−
51	8.0	−	+	+	+
60	10.0	+	+	+	+
12	10.0	+	+	−	−
19	12.0	−	−	−	−
50	>5.0	+	+	−	−
65	>5.0	+	nt	+	+
66	>5.0	+	nt	+	+
46	>5.0	+	+	+	+
52	>5.0	+	+	+	+

[a]All cattle (n = 27) were female except for cattle numbered 28 and 33. All cattle were Holsteins except for cattle numbered 49 and 28 (Guernsey); 22 (Charolais); and 31 (Simmental). All cattle had multicentric lymphoma with or without leukemia except for cattle numbered 22 and 28 (thymic form); 2, 18, 24, 30, 31, 33 and 49 (juvenile multicentric form); and 20 which had the cutaneous form of lymphoma with leukemia. In every case, DNA was extracted from tumor cells

[b]Sera were tested for anti-gp51 antibody using a monoclonal antibody against BLV gp51 in an indirect peroxidase ELISA (Dr. D. Portetelle, Faculty of Agronomy, Unit of Microbiology, Gembloux, Belgium)

[c]Not tested

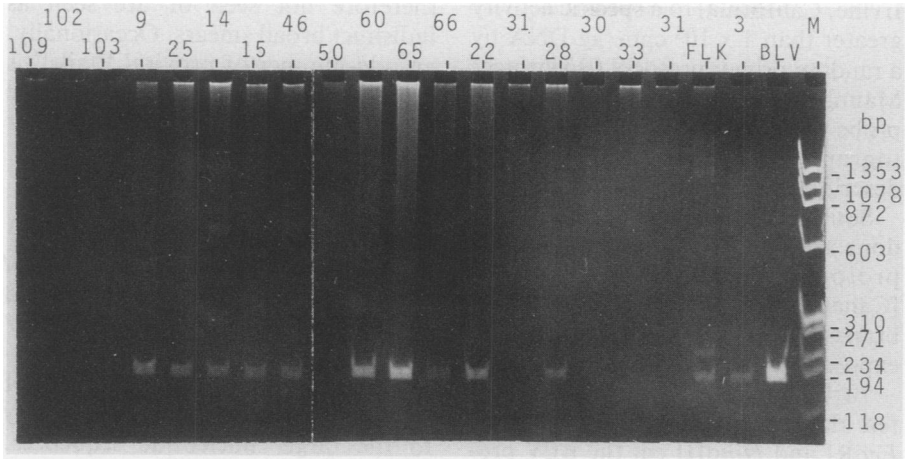

Fig. 1. Ethidium bromide-stained polyacrylamide gel showing examples of 25 cycle PCR amplification of the 202 bp BLV fragment in DNA from peripheral blood lymphocytes of uninfected sheep (lanes 102, 103, and 109), bovine lymphoid tumors (lanes numbered variably from 9 through 66 corresponding to identification number), and BL-3 (lane 3) and FLK (lane FLK) cells (BLV-infected cell lines of bovine lymphocytes and fetal lamb kidney cells, respectively). Plasmid DNA containing the complete BLV genome (pBLV913) was used as the positive control (lane BLV). Lane M is the size marker ϕX 174 RF DNA digested with *Hae*III.

through 3185 of the BLV genomic sequence), respectively. The BLV *pol* primers were synthesized by Connaught Laboratories Ltd., Willowdale, Ontario. The resultant PCR product was a 202 base pair DNA fragment.

The manufacturer's PCR protocol (Perkin Elmer Cetus, Norwalk, Connecticut) for sample preparation was modified slightly. The reaction mixture (100 μL) contained the following: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% w/v gelatin, 200 μM each of 4 dNTPs, 0.2 μM each of BLV *pol* primer 1 and primer 2, 2.5 units Taq DNA polymerase (Amplitaq, Perkin Elmer Cetus) and 1 μg of tumor cell or normal lymphocyte DNA.

For BLV PCR positive controls, 10 ng of pBLV913 and BLV *pol* primers were used. For PCR reaction controls, Lambda DNA (1 ng) and control primers, 1 μM each of PCR01 and PCR02 (Perkin Elmer Cetus) were used. DNAs (1 μg) from three uninfected sheep and two BLV-infected cell lines (FLK, fetal lamb kidney origin; BL-3, bovine lymphocyte origin) were also used as controls. Size standards were generated using a *Hae*III-digest of bacteriophage ϕX 174 RF DNA (BRL Canada, Burlington, Ontario).

The reaction mixtures were covered with 100 μL mineral oil and then cycled 25 times in a thermal cycler (Ericomp Inc., San Diego, California) according to the following protocol: 94°C for 1 min, 45°C for 1 min and 72°C for 2 min. Thirty-five and 50 cycle amplifications were done on those samples that were negative using 25 cycles. All amplifications were repeated at least twice.

Following amplification, 10 μL of each sample mixed with 3 μL of loading solution (0.25% w/v of bromophenol blue in 50% v/v glycerol), along with DNA molecular size marker ϕX 174 RF DNA/*Hae*III fragments were electrophoresed through 6% polyacrylamide minigels in 1X TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.0) for 55 min at 80 volts. Gels were stained with ethidium bromide (0.5 μg/mL) for 10 min and destained with distilled water for 10 min. Results were recorded using Polaroid film and a 302 nm ultraviolet light source. Amplified DNA samples showing a band migrating at 202 base pairs were considered positive. The sensitivity of the 50 cycle PCR test was approximately 17 fg of the targeted BLV proviral DNA, as established by amplifying serial dilutions of the recombinant plasmid, pBLV913, in bovine chromosomal DNA.

The BLV-specificity of the PCR amplified bands was confirmed by Southern blot hybridization using a digoxigenin-labelled probe (random primer, Boehringer Mannheim Canada Ltd.) consisting of a 77 base pair *Nae*I/*Pvu*II BLV fragment from pBLV*pol*, a recombinant plasmid generated by subcloning a 3.1 kb *Bam*HI fragment of the BLV *pol* gene from pBLV913 into pUC19. The recombinant plasmid was amplified in *E. coli* DH_{5a} cells and the probe was isolated by electrophoresis through 2% agarose then purified using a silica matrix (Geneclean, Bio/Can Scientific). DNA fragments were eluted from the silica matrix by boiling. The 77 base pair probe was targeted to the amplified product between the primers and complemented bases 3080 through 3157 of the BLV genomic sequence. DNA samples and controls were electrophoresed through 6% polyacrylamide gels then electroblotted on nylon membranes (ZetaProbe, BioRad Laboratories [Canada] Ltd.). The nylon membranes were baked at 120°C for 30 min and hybridized at 65°C for 12 h. Probe labelling and chemiluminescence detection on Kodak X-OMAT™AR film were done according to the manufacturer's protocols (Boehringer Mannheim Canada Ltd.).

STATISTICAL ANALYSIS

Contingency tables were analyzed by Fisher's exact test. Discordant PCR, Southern hybridization, and serological test results were repeated at least twice using the same samples.

RESULTS

Ten of 27 cattle with lymphoma were three years-of-age or less and by pathological criteria represented "sporadic" lymphomas considered to be unassociated with BLV infection (Table I) (1). The remaining 17 cattle with lymphoma had lesions typical of the "enzootic" or adult multicentric form of lymphoma associated with BLV infection. One three-year-old had cutaneous lymphoma, while the lymphoma in the other three-year-old was considered adult multicentric. Two of 11 cattle with lymphoma that were seronegative by the AGID test (animal numbers 18 and 51) were shown to have anti-BLV antibodies when their sera were retested by ELISA (Table I). There were no instances of cattle having positive AGID and negative ELISA test results, although the sera

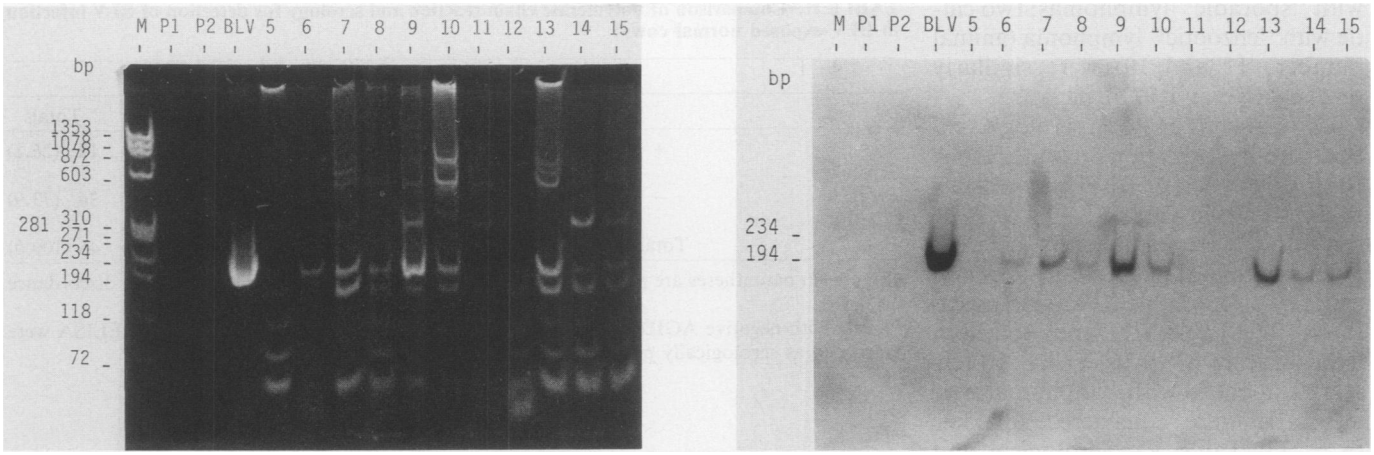

Fig. 2. Ethidium bromide-stained polyacrylamide gel (a) and fluorogram (b) showing Southern blots of PCR amplifications probed with a 77 bp *Nae*I/*Pvu*II DNA fragment derived from the BLV *pol* gene. Lane M is the size marker ϕX 174 RF DNA digested with *Hae*III. Lanes 2 and 3 are Primer 1 (P1) and Primer 2 (P2) (50 pg each), respectively. Lane 4 (BLV) is the PCR amplification (35X) of pBLV913. Lanes 5-11 are 35X PCR amplifications while lanes 12-15 are 50X amplifications. Lanes 6 and 10 are PCR amplifications of tumor DNA from two adult cows with lymphoma (51 and 60); the remaining lanes are amplifications of peripheral blood lymphocyte DNA from normal BLV-exposed adult cattle. DNA samples in lanes 5, 11 and 12 are from seronegative cattle; the 202 bp amplification product is not present in these DNAs.

of two AGID positive cattle were not retested using ELISA.

We found that the 25 cycle PCR yielded distinctive results (Fig. 1) whereas the 35 or 50 cycle PCR created additional bands making interpretation more difficult (Fig. 2a). Southern hybridization on PCR amplified product with the 77 base pair *Nae*I/*Pvu*II BLV fragment verified that the amplified 202 base fragment was BLV in origin (Fig. 2b).

The BLV provirus was found by PCR testing in 16 of 27 cattle with lymphoma. Fourteen of these 16 were seropositive; 12 of which were "enzootic" lymphomas and two were "sporadic" lymphomas (animal numbers 2 and 18). Two cattle with the thymic form of "sporadic" lymphoma and evidence of BLV provirus (animal numbers 22 and 28) were seronegative. The BLV proviruses were clonally integrated in animal numbers 2 and 28. Selected Southern hybridizations on tumor DNAs showing clonal integration are presented in Fig. 3. Digestion of genomic DNA with *Eco*RI and *Hin*dIII results in one internal BLV proviral fragment and two "tails" for each provirus. Occasionally an odd number of "tails" is detected, such as was seen with DNA from animal number 28; this occurrence is likely due to the superimposition of "tails" of similar size.

Eleven of 27 cattle with lymphoma showed no evidence of BLV provirus in tumor DNA by PCR testing. Four of the 11 were seropositive, three of which were in cattle with "enzootic" lymphomas. One calf with "sporadic" lymphoma (animal number 30) was seropositive without evidence of BLV provirus. Seven of the 11 were seronegative, five of which were in cattle

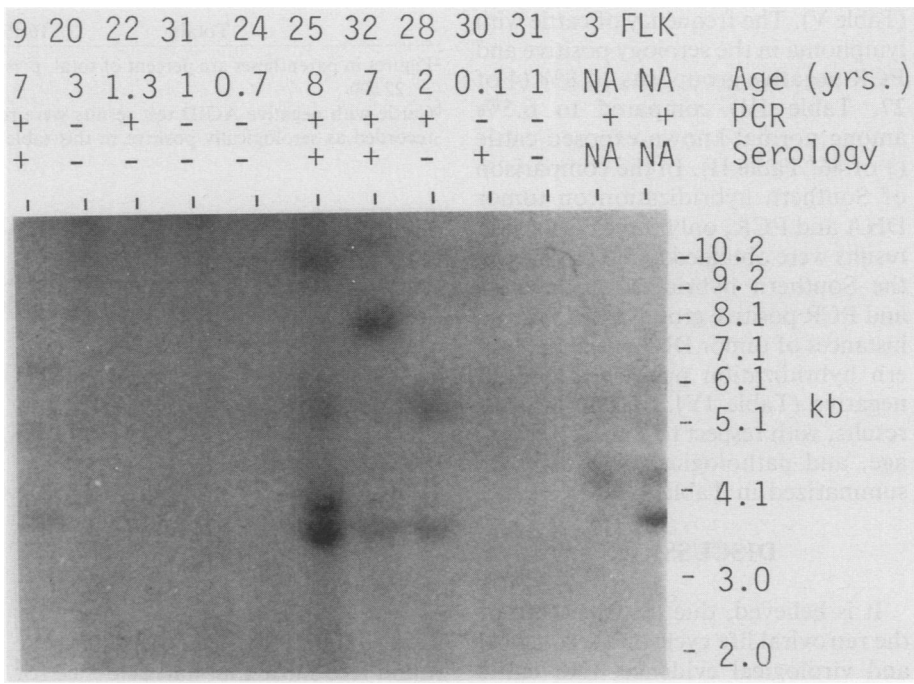

Fig. 3. Autoradiogram of a Southern blot of tumor DNAs probed with the *Kpn*I fragment of pBLV913 (full length BLV). Tumor and control DNAs were digested with *Eco*RI (cuts once near the 3′ end of BLV provirus) and *Hin*dIII (cuts the BLV provirus approximately in half), yielding an internal fragment of about 3.8 kb and two "tails" per provirus. With clonal integration that occurs with lymphoma, the "tails" appear as bands whereas with random integration "tails" are not seen or are seen as broad smudges. Lanes are labelled by animal identification number; age, PCR, and serological status are indicated. Control DNAs were from BL-3 (lane 3) and FLK (lane FLK) cells which are BLV-infected cell lines of bovine lymphocytes and fetal lamb kidney cells, respectively. Size in kilobases are indicated to the right. Virus specific bands were found in lanes with animal identification numbers of 9, 25, 28 (only one "tail" is seen likely due to superimposition), 32, and both control lanes. NA = not applicable.

with "sporadic" lymphomas; two cattle with "enzootic" lymphoma (animal numbers 11 and 19) were similarly seronegative and PCR negative.

Comparisons of PCR, serology, and Southern hybridization on tumor DNA for the detection of BLV-infected cattle are shown in Tables II–V. The PCR test results were significantly different from serological test results in normal cattle (n = 46, Table II) or serological (n = 27, Table III) and Southern hybridization (n = 26, Table IV) test results in cattle with lymphoma or if all cattle were considered together (n = 73, Table V). Serological and PCR tests results, when considered in all cattle (n = 73), had a concordance rate of 83.6% and discordant test results occurred with approximately equal frequency between serology positive and PCR negative and serology negative and PCR positive groups (Table V). The frequency of cattle with lymphoma in the serology positive and PCR negative group was 14.8% (4 of 27, Table III) compared to 6.5% among normal known-exposed cattle (3 of 46, Table II). In the comparison of Southern hybridization on tumor DNA and PCR, only three discordant results were obtained and these were in the Southern hybridization negative and PCR positive group; there were no instances of tumor DNAs being Southern hybridization positive and PCR negative (Table IV). Discordant test results, with respect to serology, PCR, age, and pathological diagnosis, are summarized in Table VI.

TABLE II. Comparison of polymerase chain reaction and serology for detection of BLV infection in BLV-exposed normal cows[a]

		Polymerase chain reaction		
		+	−	Totals
Serology[b]	+	9 (19.6)	3 (6.5)	12 (26.1)
	−	3 (6.5)	31 (67.4)	34 (73.9)
	Totals	12 (26.1)	34 (73.9)	46 (100.0)

[a]Figures in parentheses are percent of total, $p = 3.5 \times 10^{-5}$. Concordance = 87%, discordance = 13%

[b]Cattle with negative AGID test results were retested by ELISA and if positive by ELISA were recorded as serologically positive in this table

TABLE III. Comparison of polymerase chain reaction and serology for detection of BLV infection in cattle with lymphoma[a]

		Polymerase chain reaction		
		+	−	Totals
Serology[b]	+	14 (51.9)	4 (14.8)	18 (66.7)
	−	2 (7.4)	7 (25.9)	9 (33.3)
	Totals	16 (59.3)	11 (40.7)	27 (100.0)

[a]Figures in parentheses are percent of total, $p = 9.0 \times 10^{-3}$. Concordance = 77.8%, discordance = 22.2%

[b]Cattle with negative AGID test results were retested by ELISA and if positive by ELISA were recorded as serologically positive in this table

TABLE IV. Comparison of polymerase chain reaction and Southern hybridization for detection of BLV infection in cattle with lymphoma[a]

		Polymerase chain reaction		
		+	−	Totals
Southern hybridization	+	13 (50.0)	0 (0.0)	13 (50.0)
	−	3 (11.5)	10 (38.5)	13 (50.0)
	Totals	16 (61.5)	10 (38.5)	26 (100.0)

[a]Figures in parentheses are percent of total, $p = 5.4 \times 10^{-5}$. Concordance = 88.5%, discordance = 11.5%

DISCUSSION

It is believed, due to the nature of the retroviral life cycle and serological and virological evidence, that cattle serologically positive for BLV are chronically infected with BLV (1,5,6). All cattle and sheep experimentally inoculated with BLV seroconvert by four months postexposure detected by the AGID test and in as little as two weeks using radioimmunoassay (30). Bovine leukemia virus is considered the etiological agent of lymphomas in adult cattle, while the "sporadic" lymphomas are unassociated with BLV (1). Although the majority of the PCR, serological, and Southern hybridization data conformed to this traditional understanding of BLV infection, there were some important exceptions. We found five cattle that had evidence for BLV infection, provided by positive PCR test results, that were seronegative. The reverse situation (i.e. PCR test result negative but seropositive) was found in seven cattle. The BLV provirus and positive serological test results were found in two cattle with "sporadic" lymphoma while two adult cattle with lymphoma had no evidence of BLV infection based on the failure to demonstrate BLV provirus or anti-BLV antibody.

Southern hybridization on tumor DNA was helpful in confirming the PCR test results, but in several instances we obtained a positive PCR test result, but no viral bands were observed with Southern hybridization. This is likely due to the greater sensitivity of the PCR. There were no instances of negative PCR test results and positive test results with Southern hybridization on tumor DNA supporting the sensitivity of the PCR. False positive PCR test results can occur (31), but the specificity of our PCR protocol was aided by use of purified tissue DNAs, repeated PCR assays, and reagent and negative controls. In addition, the confirmation of a virus-specific PCR product with the 77 bp BLV *NaeI/PvuII* probe in Southern

TABLE V. Comparison of polymerase chain reaction and serology for detection of BLV infection in BLV-exposed normal cows and cattle with lymphoma[a]

		Polymerase chain reaction		
		+	−	Totals
Serology[b]	+	23 (31.5)	7 (9.6)	30 (41.1)
	−	5 (6.8)	38 (52.1)	43 (58.9)
	Totals	28 (38.3)	45 (61.7)	73 (100.0)

[a]Figures in parentheses are percent of total, $p = 6.8 \times 10^{-8}$. Concordance = 83.6%, discordance = 16.4%

[b]Cattle with negative AGID test results were retested by ELISA and if positive by ELISA were recorded as serologically positive in this table

TABLE VI. Summary of discordant test results[a]

Animal identification or number (n)	Age (years)	Physical status[b]	Serology[c]	Polymerase chain reaction[d]
30	0.1	JML	+	−
18	0.5	JML	+	+
22	1.3	TL	−	+
28	2.0	TL	−	+
2	2.0	JML	+	+
11	3.0	AML	−	−
29	8.0	AML	+	−
19	12.0	AML	−	−
12	10.0	AML	+	−
50	>5.0	AML	+	−
n = 3	≥ 3.0	Normal	+	−
n = 3	≥ 3.0	Normal	−	+

[a]Includes cattle in which evidence for BLV infection is inappropriate for age and form of lymphoma according to traditional criteria

[b]AML, JML, and TL represent the adult multicentric, juvenile multicentric, and thymic lymphomas, respectively. Normal refers to 46 normal known-exposed cattle

[c]Cattle with negative AGID test results were retested by ELISA and if positive by ELISA were recorded as serologically positive in this table

[d]In cattle with lymphoma, the clonality of BLV proviral integration was checked using Southern hybridization. Numbers 18 and 22 had positive PCR test results, but viral banding patterns were not present by Southern hybridization. In all other cattle with lymphoma (see Table I) and positive PCR test results, there was clonal BLV integration

blot hybridization showed that there were no nonspecific bands migrating at the 202 bp position. The PCR protocol described here appears to have comparable sensitivity to the PCR protocols previously reported (21–24). In some instances, the sensitivity of the PCR was apparently augmented, beyond that achieved in the present study, by reamplification of PCR amplified products (23) or hybridization of PCR amplified products with BLV-specific probes (21,22).

Of the five serology negative and PCR positive cattle, two had thymic lymphoma and three were normal known-exposed cattle. The BLV-infected cattle are sometimes transiently seronegative under certain circumstances, such as early infections (32), supposed exposure to small inocula, impending parturition or the immediate postparturient state (33–35), prolonged latency, and coinfection with BVDV (36). A previous study identified a cow that was seronegative, in which BLV provirus was demonstrated by PCR, presumably due to early infection (22). *In utero* exposure may be followed by a transient postparturient phase of seropositivity in the calf (37). Tolerance or abnormally delayed immune responsiveness to BLV has been suggested in other studies (6,7,37–41) and could be related to the time of *in utero* exposure, nature of the antigen, or inheritance of immune response genes dictating poor anti-BLV responsiveness. Coinfection with BVDV is an unlikely explanation since the normal known-exposed cattle were vaccinated against this virus. Bovine leukemia virus infection alone is not associated with immunosuppression (42,43) but development of immune compromise or incompetence associated with lymphoma (44–52) and tolerance due to *in utero* exposure may account for the lack of detectable anti-BLV antibody in the two cattle with thymic lymphoma.

Four juvenile cattle with lymphoma and positive PCR test results were found. The "sporadic" forms of lymphoma have been previously unassociated with BLV (1,9,56,57) with some exceptions (11–13). Coincidental infection could account for these four cases however two, both two years-of-age with thymic or juvenile multicentric lymphoma, had clonally-integrated BLV provirus in tumor DNA suggesting a role of BLV in lymphomagenesis. Maternal anti-BLV antibody likely does not account for the positive serological test in the two-year-old calf with juvenile multicentric lymphoma suggesting that there is genuine immune response to the BLV infection. Use of the PCR amplification technique in a relatively large series of "sporadic" lymphomas likely accounts for the findings of the present study compared to previous studies that used less sensitive techniques in smaller populations.

Two seronegative adult cattle with lymphoma were detected; BLV proviral DNA was not found in either case. This is a highly unusual finding (5,6) particularly since the anti-BLV antibody titer often increases with the development of overt lymphoid tumors (53–55) and essentially all adult lymphomas in cattle reportedly have detectable BLV provirus in tumor DNA by Southern hybridization (1). More sensitive serological tests, such as the ELISA or radioimmunoassay, have shown false negative reactions to occur in the less sensitive AGID test (30,32) and in the present study we found two ELISA and PCR positive cattle with apparent false negative AGID test results. False negative ELISA test results in these two adult cattle with lymphoma cannot be eliminated definitively, however, this is unlikely since the normal known-exposed cattle were tested several times over two years with unchanging test

results. The absence of serological and PCR evidence for BLV in these two adult cattle suggest that these lymphomas arose independently of BLV or that BLV may have been required initially, but was not necessary for development or maintenance of the neoplastic state. Restriction endonuclease mapping studies of BLV proviruses in DNA from adult lymphomas sometimes show large deletions in the *pol*, *env*, and *gag* genes; this finding suggests that only a small portion (3′ end) of the BLV genome is required for maintenance of the neoplastic phenotype (1,59). Frequent partial deletions are also found in the HTLV-1 (60), however the 3′ end of the virus is found in all tumor cells of patients with adult T-cell leukemia (61). Our PCR could have missed BLV proviruses that contained *pol* gene deletions since our oligonucleotide primers were *pol* gene specific but, this explanation seems unlikely since viral bands were not visualized by Southern hybridization using a full-length BLV probe although Southern hybridization is less sensitive than PCR. It is conceivable that both techniques, Southern hybridization and PCR, yielded false negative results but for different reasons. Increasing the sensitivity of the PCR as performed here by reamplification or hybridization may be useful in demonstrating provirus in these tumors. Proof of conservation of BLV 3′ sequences in all cases of adult bovine lymphoma will rest upon the demonstration of these sequences by PCR utilizing oligonucleotide primers specific for this region of the BLV genome.

Of the seven cattle that had serological evidence for BLV infection yet had negative PCR test results, three were cattle with adult lymphoma, one was a calf with lymphoma, and three were normal known-exposed cattle. Maternal anti-BLV antibody likely accounts for the positive serological test result in the calf since BLV provirus was not found in tumor DNA. The occurrence of seropositive and PCR negative adult cattle without lymphoma has been reported previously (23). Essentially all cattle with the adult multicentric form of lymphoma are seropositive and hence are considered to be BLV-infected just as all seropositive cattle are assumed to be BLV-infected (1), although contrary data have been reported (39). Despite the lack of evidence of BLV provirus in the three adult cattle with lymphoma and the failure to demonstrate BLV provirus in three seropositive normal cattle, this subgroup of cattle did show a persistent anti-BLV antibody response. This situation could occur only with continuous or periodic exposure to BLV antigens. Unintegrated BLV proviral forms could potentially play a role, but unintegrated forms have not been found *in vivo* (58). The BLV antigen could be produced by virus replicating in a small number of tumor cells (below the limit of proviral detection by PCR), normal lymphocytes, or other tissue compartments not examined here. Strategies aimed at improving upon the sensitivity of the PCR, such as those described above, may be required for the demonstration of BLV provirus in this subgroup of cattle with lymphoma. Alternative explanations include exposure to BLV antigens, or to cross-reacting antigens present in vaccines or the environment or perhaps recent exposure and clearing of BLV-infected cells. The latter explanation seems unlikely considering the retroviral life cycle and the fact that the normal BLV-infected cows had persistent antibody responses over two years.

The potential risk posed by BLV-infected seronegative cattle less than three years of age with lymphoma is difficult to assess, but is likely minimal due to the relatively short time spent in the herd. Potential risk could be much greater in adult asymptomatic BLV-infected cattle or BLV-infected cattle with early lymphoma. The positive correlation of anti-BLV antibody titer and BLV production *in vitro* (62), and presumably infectivity, might indicate that natural transmission of BLV from seronegative cattle could be very inefficient. For routine purposes, such as culling programs aimed at decreasing BLV herd prevalence, the AGID test appears satisfactory and regulatory bodies use this as the "official" test for importation or exportation of cattle. The data presented here suggest that some BLV-infected cattle will be missed using even more sensitive seroassays. In critical situations where the BLV status of an individual or other biological material must be known with certainty, use of the PCR test seems appropriate. One such critical situation may be in vaccine production for human and bovine use.

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