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Evaluation of Polymerase Chain Reaction (PCR) Application in Diagnosis of Bovine Leukaemia Virus (BLV) Infection in Naturally Infected Cattle

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With 1 figure and 2 tables

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Summary

The practical application of polymerase chain reaction (PCR) for the diagnosis of bovine leukaemia virus (BLV) infections in naturally infected cattle was evaluated. Compared to serological tests the PCR was definitely found to be a more sensitive method, yielding the highest number of positive results (10% more compared to enzyme-linked immunosorbent assay, (ELISA), and 17.7% more compared to agar-gel immunodiffusion, (AGID)). In testing cattle from herds with BLV incidence under 5%, out of 52 provirus positive cattle only 43 were correctly identified by ELISA. When compared to AGID only 37 of the 52 PCR positive animals were correctly identified. Of 18 cattle imported from the Slovak Republic and kept in a quarantine stable, four were found to be BLV provirus positive by PCR, while serological tests indicated one animal positive and three negative. Therefore, it is impossible to prevent the spread of the infection from one country to another by serological testing only. Moreover, it is feasible to identify animals with changing antibody titres correctly by PCR. Using PCR we were also able to distinguish BLV infected from uninfected calves that were serologically positive due to colostral antibodies. Higher sensitivity of BLV provirus detection by PCR was achieved using *env* gene rather than *tax* gene specific primers. Negative results by PCR in cases of positive serological reactions are still possible, as shown in case of one adult animal. These findings indicate that PCR is a highly sensitive method and might be successfully used and economically advantageous for different practical applications in detection of BLV infection in naturally infected cattle.

Introduction

Bovine leukaemia virus (BLV), the causative agent of enzootic bovine leukosis (EBL) is an exogenous, B lymphotropic retrovirus which has been found in many countries throughout the world. The BLV infection is important because of its economic impact, i.e.

shorter life span and decreased dairy production of infected cows (BRENNER et al., 1989; WU et al., 1989). Successful eradication programmes carried out during the past 15–20 years have substantially decreased the incidence of EBL in Europe. Some countries in West and Middle Europe are considered practically leukosis free or report very low incidences of EBL (GACHET-PIGUET, 1990; LORENZ, 1990; KLINTEVALL et al., 1991; MITRO, 1993). Economic losses in these countries result first of all from trade and commercial restrictions and spending for prevention of epizootics.

The epizootic control of EBL is mainly based on agar-gel immunodiffusion tests (AGID) and in recent years also on enzyme-linked immunosorbent assays (ELISA). Several authors have shown that it is possible to establish BLV free herds by identifying seropositive animals and eliminating them (HOFF-JORGENSEN, 1989; KLINTEVALL et al., 1991). Others demonstrated that serological control is not sensitive enough to find all BLV infected cattle (JACOBS et al., 1992; BURKHARDT et al., 1993; EAVES et al., 1994). Problems also arise with animals exhibiting periodically or permanently low titres of BLV antibodies (KAADEN et al., 1982; GENTILE et al., 1985; FLORENT, 1988) or low serum titres in the periparturient period (BURRIDGE et al., 1982). Furthermore, serological tests cannot discriminate between passive maternal immunity and active immunity induced by BLV infection (BALLAGI-PORDANY et al., 1992; AGRESTI et al., 1993; EAVES et al., 1994). In addition, there are problems with the specificity of ELISA (DE BOER et al., 1987; KLINTEVALL et al., 1991). Analysing animals over a long term, BURKHARDT et al. (1993) and EAVES et al. (1994) showed that some naturally infected BLV provirus carrying animals developed no BLV antibody titres detectable by AGID and ELISA months and years after infection.

Different research groups (NAIF et al., 1990; BALLAGI-PORDANY et al., 1992; JACOBS et al., 1992; AGRESTI et al., 1993; EAVES et al., 1994; KLINTEVALL et al., 1994) have demonstrated the advantages of polymerase chain reaction (PCR) over serological tests in a number of different applications.

The aim of this study was to evaluate practical applications of PCR for detection of BLV infection in cattle, especially considering the conditions in countries like Germany, with a very low incidence of BLV infection.

The authors have shown that PCR is particularly suitable for controlling herds with a very low incidence of BLV infection, for clarifying doubtful results obtained by serological tests, for testing imported animals and for ruling out BLV infections in newborn calves. Higher sensitivity of PCR was achieved using primers from the conservative *env* gene compared to primers from the more variable *tax* gene of the BLV provirus. It was impossible to find one ELISA positive adult animal by PCR.

Materials and Methods

Animals

Animals of various origin, serological status and age were studied. All animals (39 cows, 41 heifers and five calves) used in the study were female cattle from different herds in Germany (see Table 1).

Collection of blood

Animals were bled from the jugular vein. For DNA extraction the blood samples were collected either with sodium heparin (5 U/ml) or with sodium citrate (4% final concentration) and stored at −20 °C. For longitudinal study two samples were taken from each of three animals and three samples from a fourth (Table 2). These four animals came from herds with BLV incidence under 5%.

Serology

Agar-gel immunodiffusion tests were performed using Riemser Rinderleukose Testbesteck (RTAM, Insel Riems, Germany) following the manufacturer's instructions. The ELISAs were performed using CHEKIT-Leucotest kit (Bommeli AG, Bern, Switzerland) and Biogen-Rinderleukose

Table 1. Comparison of the results in the AGID, ELISA and PCR and the origin of the cattle

Origin	Number of animals	Positive results			Negative results			Doubtful results	
		AGID	ELISA	PCR[a]	AGID	ELISA	PCR[a]	AGID	ELISA
A	18	1	1	4	17	17	14	0	0
B	59	37	43	52	17	12	7	5	4
C	4	2	3	3	1	0	1	1	1
D	1	0	0	0	1	1	1	0	0
E	3	1	3	3	1	0	0	1	0

A, cattle from the Slovak Republic which were BLV negative as determined by serological methods prior to import and kept in a quarantine stable; B, cattle from herds with an incidence of BLV infection <5%; C, calves which received colostrum from their BLV infected dams; D, calf from a BLV infected mother, whose blood was taken before it received colostrum from the dam; and E, cattle with doubtful results during formerly performed serological tests
[a]Results of *env*- and *tax*-PCR

Table 2. Results of a longitudinal study

Animal no.	Screening time	AGID	ELISA	*tax*-PCR	*env*-PCR
	4/92	–	+	–	+
26	6/92	–	+	–	–
	8/92	–	+	–	–
29	5/92	+/–	+	+	–
	7/92	–	–	+	+
30	5/92	+/–	+	+	+
	7/92	+	+	+	+
32	5/92	+	+	+	n.d.
	6/92	+	+	+	+

n.d., not done

Virus (BLV) Antikörper EIA (Biogen, Rostock, Germany), according to the recommendations of the manufacturers, or catch-ELISA using monoclonal antibodies gp51/22 against epitope B of the BLV gp51, kindly provided by Dr C. PLATZER and Dr H. SIAKKOU (Institute of Virology, Faculty of Medicine (Charité), Humboldt University, Berlin). The catch-ELISA was performed according to SIAKKOU et al. (1990).

DNA preparation

Bovine DNA was obtained directly from frozen whole blood using two different methods. In the first, 10 ml whole blood were mixed with 40 ml of hypertonic buffer (320 mM sucrose, 10 mM Tris-HCl at pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100) to disrupt cell cytoplasmatic membranes. Cell nuclei were pelleted by centrifugation for 10 min at 2500 ***g***, 4 °C. The pellet was resuspended in 2 ml of 0.9% NaCl and 2 ml of 500 mM EDTA at pH 8.0, and 400 µl of 10% SDS and 880 µl of 5 M $NaClO_4$ were added. The DNA was extracted twice with one volume of chloroform/octanol (24:1) and chloroform by centrifugation for 20 min at 4000 ***g***, 4 °C, respectively, and precipitated with one volume of isopropanol. Then the DNA was stirred out with a glass rod and suspended in double-distilled (dd) H_2O. A total of 1 µg of DNA was used for amplification. In the second method, 20 µl of frozen whole blood were lysed with osmotic shock and vortexing after addition of 1 ml of TE buffer (10 mM Tris HCl at pH 7.6, 1 mM EDTA). Cell lysate was pelleted by centrifugation for 30 s at 14000 r.p.m. at

room temperature with Eppendorf 5415 C. The pellet was washed twice with 1 ml of TE buffer (pH 7.6) and resuspended in 37 µl of dd H_2O. The preparations were heat treated at 95 °C for 12 min and were frozen immediately. The whole preparation was used in PCR.

PCR primers

Oligonucleotide primers for PCR were designed according to sequence data published by SAGATA et al. (1985). Primers to the *env* gene were selected because this region is highly conserved among different BLV provirus isolates (RICE et al., 1984; SAGATA et al., 1985; MAMOUN et al., 1990). Alternatively, PCR primers to the *tax* gene shown to be more variable amongst BLV provirus isolates (RICE et al., 1984; SAGATA et al., 1985) were used.

Sequence of the forward primers were:
env_{5032}, 5′-TCTGTGCCAAGTCTCCCAGATA-3′,
env_{5099}, 5′-CCCACAAGGGCGGCGCCGGTTT-3′,
tax_{7719}, 5′-TTCCTTACTCCCCTCCTTCG-3′.
Sequence of the reverse primers were:
env_{5521r}, 5′-GCGAGGCCGGGTCCAGAGCTGG-3′,
env_{5608r}, 5′-AACAACAACCTCTGGGAAGGGT-3′,
tax_{7975r}, 5′-GTTAGGAATAGGTCGATCGC-3′.

Primers env_{5099} and env_{5521r} were identical with the primers used by NAIF et al. (1990, 1992) and BRANDON et al. (1991). All primers used in this study were obtained from MWG Biotech (Ebersberg, Germany).

DNA amplification

Reaction mixture was prepared and distributed into 50 µl aliquots. The mixture contained: 5 µl of 10 × PCR buffer (500 mM KCl, 100 mM Tris-HCl at pH 9.0, 1 % Triton X-100; Promega, Madison, USA), 1.5 mM $MgCl_2$, 75 µM of each deoxynucleotide triphosphate (dNTP) (Boehringer Mannheim, Mannheim, Germany), 0.2 µm of each primer, 1.25 units of *Taq* polymerase (Promega, Madison, USA) and DNA as described above.

The *env*-PCR was carried out with the primer pair env_{5099}/env_{5521r} resulting in amplification of a 440 bp fragment from double PCR or from nested PCR with the primer pair env_{5032}/env_{5608r} (external primers, resulting in amplification of a 600 bp fragment) and env_{5099}/env_{5521r} (internal primers). The *tax*-PCR was carried out with the primer pair tax_{7719}/tax_{7975r} for double PCR. The amplification reactions were performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Inc., Weiterstadt, Germany) or Trio-Thermoblock (Biometra, Göttingen, Germany). First, an initial incubation at 94 °C for 2 min was performed followed by 40 amplification cycles consisting of denaturation at 95 °C for 30 s; primer annealing at 62 °C (primer pair tax_{7719}/tax_{7975r} and env_{5032}/env_{5608r}) or at 70 °C (primer pair env_{5099}/env_{5521r}) for 30 s; and primer extension at 72 °C for 60 s. For the first and last five cycles of the PCR the extension time was increased to 75 s, followed by final extension at 72 °C for 4 min. For the second round of PCR 1 µl of product was taken from the first amplification and reamplified. In order to visualize PCR products, 20 µl of amplified mixture were run on 1.5 % agarose gels followed by ethidium bromide staining.

Restriction analysis

Direct digestion of 20 µl *env*-PCR product with 5 U *Bcl*I (Gibco BRL, Eggenstein, Germany) and 1.3 µl buffer M (Boehringer Mannheim) resp. 20 µl of *tax*-PCR product with *Eco*RI (Boehringer Mannheim) and 1.2 µl buffer H (Boehringer Mannheim) was used to verify the identity of bands.

Nucleic acid hybridization

The specificity of the amplified bands was confirmed by Southern blot hybridization using standard methods (SAMBROOK et al., 1989). A BLV specific probe was obtained using a 440 bp BLV proviral DNA fragment amplified with primer pair env_{5099}/env_{5521r} and plasmid construct pBLV-12, kindly provided by Dr H.-J. WAGNER (Institute of Biochemistry, Faculty of Veterinary Medicine, Free

University Berlin), containing the full length BLV proviral DNA in pBR-322 as the template. The DNA was labelled with DIG-11-dUTP using the DIG DNA Labeling and Detection Kit (Boehringer Mannheim).

Avoiding contamination

Due to the very high sensitivity of PCR, special precautions were adopted. All steps of this assay, including DNA isolation, preparation of amplification mixture components, PCR amplifications and product analysis were carried out in separate rooms. To eliminate carry over of amplified DNA, all pipetting procedures related to PCR were performed under Plexiglas boxes. Rooms were irradiated with UV light after PCR.

Results

In this report three main methods of diagnosing the BLV infection in cattle were evaluated. First we compared the results of 90 samples tested by AGID and ELISA. Of the tested samples 46.7% were positive and 35.5% negative in both tests (correspondence between AGID and ELISA: 82.2%). On the other hand 17.8% of the results did not match in different assays (Fig. 1a).

Then we compared the results obtained by PCR (results of *env*- and/or *tax*-PCR) with both AGID and ELISA. Results obtained by AGID and PCR showed that 45.6% of the 90 samples were positive and 27.8% were negative in both tests (correspondence between AGID and PCR: 73.4%). Different results were seen in 26.6% of the tested samples (Fig. 1b). By ELISA and PCR (*env*- and/or *tax*-PCR), from 90 tested samples 56.7% were found positive and 24.4% were negative (correspondence between ELISA and PCR: 81.1%). Of the tested samples 18.9% gave different results in both tests (Fig. 1c).

To evaluate the correspondence between results obtained by *tax*-PCR and *env*-PCR, 52 samples were tested using appropriate primer pairs. In both analyses 78.9% positive and 11.5% negative samples were found (correspondence between PCR tests with different target sites: 90.4%). A total of 9.6% gave different results in both tests (Fig. 1d).

The results of AGID, ELISA and PCR obtained by testing of 85 cattle from different groups are shown in Table 1. Eighteen BLV negative cattle as determined by serological methods prior to import were tested. Three animals were found positive by PCR while their serological tests were negative and one animal was positive by AGID, ELISA and PCR (Table 1, A).

In cattle from herds with a low incidence of BLV infection 52 of 59 investigated samples were positive by PCR, but only 43 by ELISA and only 37 by AGID as shown in Table 1, B. In addition five resp. four animals yielded doubtful results in either AGID or ELISA.

Of four calves who had received colostrum from their BLV infected dam, three were found positive and one negative by PCR as shown in Table 1, C. The PCR negative calf was positive by AGID and ELISA. The calf tested before it had received colostrum from its BLV infected mother was negative in all tests (Table 1, D).

All three cattle tested because of their changing antibody titres during former investigations were found positive in ELISA and in PCR, but only one was positive in AGID (Table 1, E).

The results of repeated screening experiments are summarized in Table 2. Blood was taken twice from three animals (numbers 29, 30 and 32) and three times from one (number 26). Complete agreement was found between the results of serological tests and PCR in animal 32. Animal 30 also showed complete agreement with the exception of AGID, where first a doubtful and then a positive result was obtained. Animal 29 was positive only in ELISA and *tax*-PCR at the first screening. After the second screening negative results were obtained in all serological tests and positive results by *tax*- and *env*-PCR. Animal 26 was tested three times. The *env*-PCR was positive only after the first test, while later no BLV provirus was found. Specific antibodies were found by ELISA on all sampling occasions. The AGID analysis of this animal was negative on other occasions.

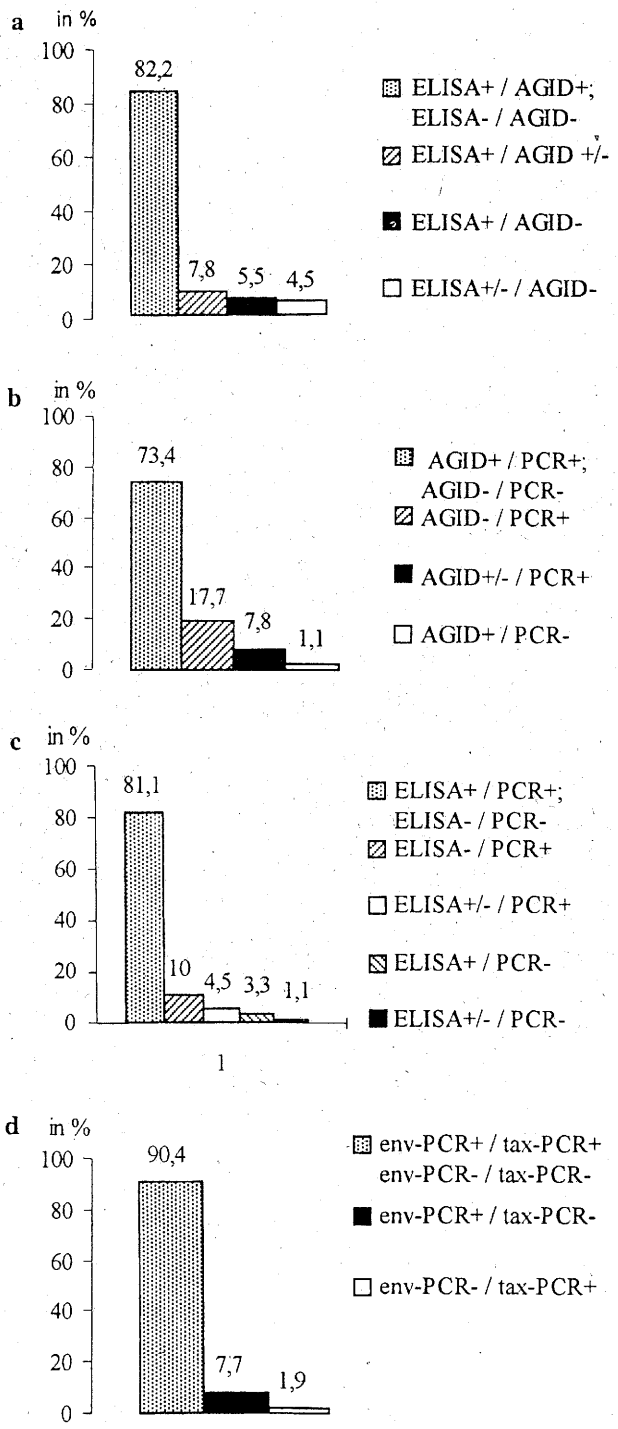

Fig. 1. Comparison of AGID, ELISA and PCR results. (a) Correspondence between ELISA and AGID results; (b) correspondence between AGID and PCR (*tax*- plus *env*-PCR) results; (c) correspondence between ELISA and PCR (*tax*- plus *env*-PCR) results; and (d) correspondence between *tax*- and *env*-PCR results

The specificity of the PCR products was proved by restriction enzyme analysis and Southern blot hybridization. All investigated PCR products (samples from more than 35 animals) yielded BLV specific hybridization bands and restriction pattern.

Discussion

Polymerase chain reaction is a well documented method for the diagnosis of BLV infection (NAIF et al., 1990, 1992; BRANDON et al., 1991; MURTAUGH et al., 1991; BALLAGI-PORDANY et al., 1992; JACOBS et al., 1992; AGRESTI et al., 1993; KLINTEVALL et al., 1993, 1994; EAVES et al., 1994). It has been shown by different research groups that this method has advantages in resolving specific questions when compared to AGID and ELISA, e.g. diagnosis of the BLV infections in calves fed on colostrum from their BLV infected dams (BALLAGI-PORDANY et al., 1992; AGRESTI et al., 1993; EAVES et al., 1994). Nevertheless it was concluded by EAVES et al. (1994), who tested more than 350 cattle in a field study, that serological tests are more practicable for routine diagnosis of BLV infections and have a comparable sensitivity.

The aim of this study was to evaluate application of PCR to diagnose BLV infection in cattle, especially in countries like Germany, where a very low incidence of BLV infection occurs.

Altogether comparison of PCR with other serological tests showed a concordance of 73.4% for PCR and AGID and 81.1% for PCR and ELISA. This results primarily from a higher sensitivity of PCR that identifies 17.7% (compared to AGID) and 10% (compared to ELISA) more positive samples. Moreover all doubtful samples (except one) in serological tests were found positive by PCR (Fig. 1b,c). Contrary to the present study, other groups (NAIF et al., 1990; BALLAGI-PORDANY et al., 1992; EAVES et al., 1994) have found a significantly higher concordance between PCR and serological tests in the diagnosis of BLV infections. In the opinion of the present authors, the main reason for this discrepancy is the principle of selecting animals to be tested (see Table 1).

Almost 70% of tested animals originated from herds with a low incidence (<5%) of BLV infection. It was demonstrated previously by BAUER et al. (1984) and MANZ and BAUER (1985) that in such herds more animals have very low antibody titres than in herds with a high incidence of BLV infection. But there are no studies comparing serological and provirus detecting tests in these herds. We found only 37 AGID positive cattle out of 43 ELISA and 52 PCR positive cattle originated from these herds. In addition, it was impossible to find all BLV provirus carriers by ELISA. These results may be explained by transmission of the virus from BLV infected cattle kept together with these animals that occurred some weeks or days before blood collection, so that serological tests still remained negative. For example KLINTEVALL et al. (1994) demonstrated that BLV infection can be detected more than two to three weeks earlier by PCR than by ELISA or AGID. Alternatively it may be possible that some of these cattle carry BLV provirus but do not develop detectable antibody titres over a long period, as shown by BURKHARDT et al. (1993) and EAVES et al. (1994). Altogether these results demonstrate that in herds with a low incidence of BLV infections it is possible to find considerably more BLV infected cattle by PCR than with serological tests.

By AGID and ELISA it is not always possible to discriminate exactly between positive or negative results. Doubtful samples are usually retested immediately or some weeks or months later. In our study, nearly all doubtful samples (seven by AGID and four out of five by ELISA) were found positive by PCR. Also those animals which had variable results in previously performed serological tests were clearly identified as BLV provirus carriers. Therefore PCR might be a good tool to identify truly positive animals with serologically ambiguous results.

An interesting result was found in four heifers from the group of 18 imported from the Slovak Republic and kept in quarantine. All four animals were found to be BLV provirus positive by PCR. Three of these cattle were negative by AGID and even by

ELISA. These data show that routine serological testing of imported animals does not prevent the spread of BLV infections across the borders.

We tested four calves fed on colostrum from their BLV infected dams. Three calves were found to be positive and one negative by PCR. Thus we were able to differentiate uninfected newborn calves with colostral antibodies from BLV infected calves as described previously by BALLAGI-PORDANY et al. (1992), AGRESTI et al. (1993) and other groups.

In testing of four animals longitudinally we found interesting results for two animals. Animal 29 had unambiguous changing antibody titres. The ELISA positive animal 26 was positive by PCR only at the first investigation. Testing of new blood samples from this animal 2 and 4 months later yielded negative results in PCR. There are several possibilities which might explain these results. There may be a temporary occurrence of BLV infected cells in the circulation. For example, MURTAUGH et al. (1991) could not find BLV provirus in circulating lymphocytes of many serologically positive cattle by PCR and concluded that the virus may be sequestered in cells in lymphoid tissues and thus not be detected in the circulation. Furthermore KLINTEVALL et al. (1994) have found BLV proviral DNA in the spleen but not in peripheral blood lymphocytes in one experimentally infected animal. Therefore, the detection of all BLV infections in naturally infected cattle by PCR using DNA extracted from the peripheral blood cells may be impossible.

We have used primer pairs for PCR with target sequences in the *env* gene or the *tax* gene, in order to evaluate the most sensitive variant. Our results show that amplification of the *env* gene sequences was more efficient than those of the *tax* gene (Fig. 1d), although in both cases we were able to detect as few as 10 BLV provirus copies (results not shown). The results of PORTETELLE et al. (1989) and MAMOUN et al. (1990) demonstrated that in the *env* gene there are only small nucleotide variations between different BLV provirus isolates, while the *tax* gene of different BLV provirus isolates is more variable (RICE et al., 1984; SAGATA et al., 1985; COULSTON et al., 1990). This difference may cause primer mismatches and therefore lower the efficiency of *tax*-PCR. Therefore, using primers from the *env* gene of the BLV provirus is more suitable for the diagnosis of BLV infection.

The BLV specificity of the PCR products found after agarose gel electrophoresis could be confirmed by DNA restriction and/or hybridization for all of the investigated samples. Problems with contamination of samples with PCR amplificates were effectively overcome through the use of a complex system to block the carry over of amplificates.

In summary, our results show that PCR might be successfully used for different practical applications for the diagnosis of BLV infections. The highest sensitivity of PCR may be achieved using primers from the conservative *env* gene of the BLV provirus. However, it seems unlikely that all BLV infected animals will be found by PCR using DNA extracted from peripheral blood cells.

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