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Specific detection of small ruminant lentiviral nucleic acid sequences located in the proviral long terminal repeat and leader-gag regions using real-time polymerase chain reaction

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Abstract

Real-time polymerase chain reaction (RT-PCR) detection of proviral nucleic acid sequences of small ruminant lentiviruses (SRLV) in blood samples was developed and evaluated.

Priming oligonucleotides were designed on the highly conserved 5′ untranslated leader-gag region while those on the long terminal repeat (LTR) assay were derived from literature. DNA was extracted from the buffycoat interlayer of centrifuged blood samples. Real-time PCR was performed by means of LightCycler technology (Roche Applied Science) using melting temperature analysis (SYBR Green I) for detection. Results were compared with those of serology using samples from Dutch sheep and goat flocks with known SRLV statuses, with sequential samples from a natural transmission experiment and samples from different regions in Norway, France, Spain and Italy.

Real-time PCR testing, especially the application of oligonucleotides for priming the leader-gag region appeared promising in detecting SRLV specific proviral DNA in blood samples from both sheep and goats.

Keywords: Lentiviruses; Sheep; Goats; Real-time PCR; Serology; Diagnosis

1. Introduction

Maedi-Visna virus (MVV) and caprine arthritis encephalitis virus (CAEV) constitute the small ruminant lentiviruses (SRLV), a subfamily belonging to the Retroviridae. They contain two molecules of single stranded RNA (Seventh Report of the International Committee on Taxonomy of Viruses, http://www.ncbi.nlm.nih.gov/ICTV). These (+) sense strands integrate by reverse transcription into the host genome as a provirus from which virus replication proceeds (Baltimore, 1970). Cells of the monocyte/macrophage lineage harbour the viral genome and produce the virus, but it was recently shown that other cell types may also support viral replication (Carrozza et al., 2003; Bolea et al., 2006).

Recent work demonstrated that SRLVs may be divided into four sequence groups, A–D, based on differences in gag and pol sequences. Groups A and B appear to contain several distinct subtypes: to date, subtypes A1 and A2 only originated from sheep while subgroups A5, A7, B1 and groups C and D have only been isolated from goats. Subtypes A3, A4, A6 and B2 were isolated from both goats and sheep (Shah et al., 2004).

Detection of infection is generally based on antibody detection by means of methods such as agar-gel immunodiffusion (Terpstra and De Boer, 1973; Dawson et al., 1996) and enzyme-linked immunosorbent assay (ELISA) (Houwers and Schaake, 1987; Saman et al., 1999). Drawbacks of serological methods may include epitope specific reactivity (Grego et al., 2005; Lacarenza et al., 2006) and the sometimes slow antibody response to infection (Rimstad et al., 1993; Vogt et al., 2000). Theoretically, PCR may overcome these disadvantages by detecting the provirus in an early stage of infection, i.e. before antibody production has been initiated.

Over the last decade several PCR assays for SRLV have been developed and published (Zanoni et al., 1992; Rosati et al., 1995; Wagter et al., 1998; Celer et al., 2000; Extramiana et al., 2002; Carrozza et al., 2003; Kuźmak et al., 2003; Álvarez et al., 2006;

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Eltahir et al., 2006; Gil et al., 2006; Leginagoikoa et al., 2006). The use of PCR in routine settings, however, is still limited because of the low sensitivity attained so far and the difficulties arising from the notorious genomic heterogeneity of the SRLV since lentiviruses are among the most rapidly evolving genomes (Blacklaws et al., 2004; Angelopoulou et al., 2005; De Andrés et al., 2005; Reina et al., 2006), which severely complicates the design of generally applicable oligonucleotides for priming and probing. Another reason is the relatively high cost per sample as consequence of the complexity of the sample preparation and PCR procedures developed so far.

A breakthrough in molecular diagnostic methods was accomplished with the introduction of real-time PCR technology. Both the polymerase chain reaction and the detection of amplicons are combined in this technique, so minimising the risk of contamination on the amplicon level and reducing the costs of analysis (Niesters, 2002; Gunson et al., 2006; Watzinger et al., 2006).

This paper describes the development and evaluation of two real-time PCR assays for the detection of proviral SRLV sequences located in the untranslated leader-gag and LTR regions. For detection, melting temperature analysis was performed using the double stranded DNA specific intercalating fluorescent dye SYBR Green I. DNA isolated from the buffycoat cells was used as the test substrate.

Results obtained with these new assays were compared with those obtained by serology using samples from Dutch field flocks with known SRLV-infection statuses, in addition with samples from sheep and goats from different geographical areas in four other European countries and with sequential samples from sheep with naturally acquired infection during a transmission study.

Particularly the assay employing the leader-gag primer set allowed relatively sensitive and specific detection in sheep and goat samples.

2. Material and methods

2.1. Polymerase chain reaction (PCR)

2.1.1. Oligonucleotides for PCR-priming

Primers were designed based on published MVV and CAEV sequences retrieved from the National Centre for Biotechnology Information (NCBI). For alignments the BioEdit Sequence Alignment Editor, version 5.0.9 was used.

GenBank accession numbers: M14149 (Hess et al., 1986), L06906 (Braun et al., 1987), NC_001511 (Querat et al., 1990), NC_001463 (Saltarelli et al., 1990), S51392 (Sargan et al., 1993), S55323 (Andresson et al., 1993), X64109 (Kalinsky et al., 1994), AF322109 (Gjerset et al., 2000), AF425672 (Extramiana et al., 2001), AF479638 (Barros and Fevereiro, 2002) and AY101611 (Hotzel and Cheevers, 2002).

Primer 1 and primer 2, respectively, positions 161–178 and 283–299 on M51543 (Braun et al., 1987) primed the amplification of a 138 bp PCR product. The sequences (5′–3′) were: primer 1, TGGCGCCCAACGTGGGGC and primer 2, CTTCAGGCGTCCCCGAAG with melting temperatures of 64 and 60 °C, respectively.

Primers used for the LTR PCR, LTR2S and LTR2a, were designed to anneal on positions 8990–9013 and 153–176, respectively (Sonigo et al., 1985; Extramiana et al., 2002).

2.1.2. Real-time SYBR Green I PCR

The QuantiTect SYBR Green I PCR kit (QIAGEN) was used to prepare the reaction mixes. This kit consists of a 2× master mix containing a hot start DNA polymerase, buffer, DNA intercalating dye SYBR Green I, dNTP's, the passive reference dye ROX (for use on Applied Biosystems instruments) and $MgCl_2$. Real-time PCR was performed using a LightCycler 2.0 instrument (Roche Applied Science).

Primer concentration used was 0.5 μM, concentration of $MgCl_2$ was 2.5 mM and DNA input was approximately 150 ng based on 260 nm extinction measurement using NanoDrop spectrophotometry (Wilmington, Delaware, USA).

Samples were prepared from 5 to 8 mL whole EDTA blood. In brief, after centrifugation for 10 min at 1500 × *g* the buffycoat interlayer was collected and mixed with an equal volume of a red cell lysis solution (NH_4Cl 16.6 g L^{-1}, $NaHCO_3$ 2.0 g L^{-1}, EDTA 0.185 g L^{-1}, pH 7.3). After 10 min incubation at room temperature on a rotation shaker, buffycoat cells were collected by centrifugation. The cell pellet was resuspended in 200 μL PBS followed by DNA extraction using the QIAamp DNA Mini Kit (QIAGEN) according to the buffycoat protocol.

Cycling parameters were 15 min at 95 °C followed by 45 cycles of 15 s at 95 °C, 20 s annealing at 63 °C (leader-gag PCR) or 65 °C (LTR PCR) and extension at 72 °C for 20 s.

To obtain melting curves the samples were denatured at 95 °C, cooled to annealing temperature and subsequently slowly heated at a temperature transition rate of 0.2 °C/s up to 95 °C, while the decrease of fluorescence was monitored continuously. For improved visualization of melting temperatures, melting peaks were derived from the data obtained during this melting curve routine by plotting the negative derivative of fluorescence over temperature versus temperature [$-d(F)/dT$ versus T].

Determination of amplicon sizes was performed by means of agarose gel electrophoresis. Prior to this, the contents of the LightCycler capillaries were collected by centrifugation for 1 min at 4000 × *g*.

Fragments derived from seronegative but PCR positive sheep and goats were sequenced using both the primers for the leader-gag assay (BaseClear, Leiden, The Netherlands). Results were analysed using the NCBI BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/).

To determine the dynamic range and the efficiency ($E = 10^{-1/\text{slope PCR curve}}$) of the both the leader-gag and the LTR test, samples were assayed in fourfold dilutions up to 1024×.

2.2. Samples

EDTA-blood samples were collected from 14 infected sheep flocks and 8 infected goat flocks from different geographical regions in The Netherlands from 208 and 141 animals, respectively. For specificity testing 111 sheep and 78 goat samples were collected from animals originating from accredited SRLV-free

flocks. All samples were taken in the framework of the official national SRLV-control program.

For further evaluation of the primers used in this study in relation to the genetic heterogeneity of SRLV, samples originating from serologically positive sheep and goats from different geographical areas in Norway, France, Spain and Italy were investigated. These 37 sheep and 43 goat samples were collected within the framework of EU-project CRAFT 70536.

To estimate the relative precocity of the PCR assays as compared with serology, series of sequential blood samples from nine sheep, infected by natural routes in the course of a transmission study, were used. In brief, seronegative animals as repeatedly tested by ELISA were exposed naturally to seropositive sheep and blood samples were collected periodically.

To determine the serostatus of the animals the highly sensitive and specific (99.4% and 99.3%, respectively) ELITEST-MVV ELISA (HYPHEN BioMed, Andrésy, France, Saman et al., 1999) was applied according to the manufacturer's instructions.

2.3. Statistical analysis

The agreement between the PCR assays on the one hand and ELISA on the other was expressed as κ-value (*Kappa* statistics), an index representing the agreement beyond chance. Sensitivity, specificity and test agreement were calculated using WinEpiscope 2.0 (http://www.clive.ed.ac.uk/winepiscope/).

3. Results

3.1. Polymerase chain reaction

3.1.1. Leader-gag priming and PCR products

BLAST search results confirmed the specificity of the selected sequences used for priming. The determined nucleotide sequences of the fragments generated by the leader-gag PCR from seronegative animals, five sheep and five goats, were found to be matching with SRLV specific sequences.

3.1.2. Analytical test performances

Typical amplification curves are shown for the leader-gag and LTR assays (Figs. 1 and 2). The efficiency of the leader-gag PCR was better (1.98) than that of the LTR PCR (1.86). As shown in the melting profiles (Figs. 3 and 4), LTR PCR amplicons showed more distinct melting peaks than the leader-gag assay.

Melting temperatures (T_m) of amplicons from sheep samples using the leader-gag PCR differed from those of goats (82.5 ± 1.1 and 80.7 ± 0.8 °C (mean ± S.D.) respectively while in the LTR PCR almost no difference was observed (83.1 ± 0.8 and 83.0 ± 0.6 °C, respectively).

On agarose gels the expected fragment sizes were observed.

3.2. Diagnostic sensitivity and specificity in sheep and goats

Table 1 shows that a slightly higher percentage of ELISA positive sheep which were also positive by the leader-gag PCR compared to the LTR PCR (88% versus 83%, respectively) but

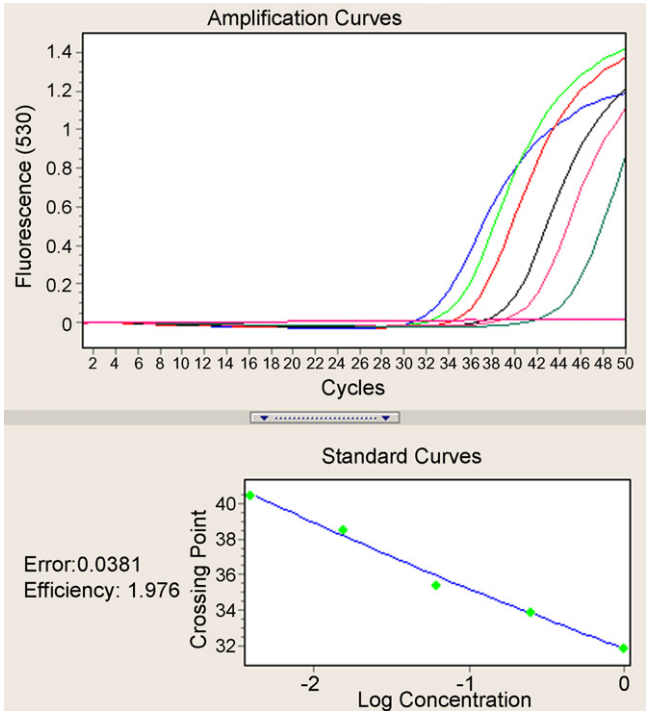

Fig. 1. Amplification profile using the leader-gag PCR. DNA of a sheep sample (# 19) is titrated from undiluted up to 1024×. All sample dilutions show a crossing point combined with the correct melting temperature (Fig. 3), conditionally for a positive sample score. The non-template control does not show a crossing point which is indicative for the absence of primer–dimers.

that a significantly higher percentage of ELISA positive goats were positive by the leader-gag PCR compared to the LTR PCR (82% versus 40%, respectively). Also in seronegative animals from infected flocks a higher percentage were positive by the leader-gag PCR (for sheep 13% versus 6% for LTR PCR and for goats 29% versus 18% for LTR PCR).

Table 2 shows cross-tabulation of the results of testing by means of both the PCR assays of 208 sheep and 141 samples from goats. In the category seropositive sheep ($n = 81$) 67 animals (83%) scored positive in both PCRs while the leader-gag PCR additionally detected another 4 animals to a total score of 88%. In the category seronegative sheep ($n = 127$), 6 animals scored positive in both PCR tests while another 11 animals scored positive in the leader-gag PCR. On the other hand, 2 LTR PCR positive animals were negative in the leader-gag PCR.

In the category seropositive goats ($n = 124$) 46 animals (37%) were positive by both PCRs, while the leader-gag PCR addition-

Table 1
Results of leader-gag, LTR PCR and ELISA of 208 sheep and 141 goat samples originating from infected flocks

Species	ELISA status	Leader-gag+	Leader-gag−	LTR +	LTR −
Sheep	+	71 (88)	10 (12)	67 (83)	14 (17)
	−	17 (13)	110 (87)	8 (6)	119 (94)
Goat	+	102 (82)	22 (18)	50 (40)	74 (60)
	−	5 (29)	12 (71)	3 (18)	14 (82)

Between parentheses the percentage of PCR positive and PCR negative animals.

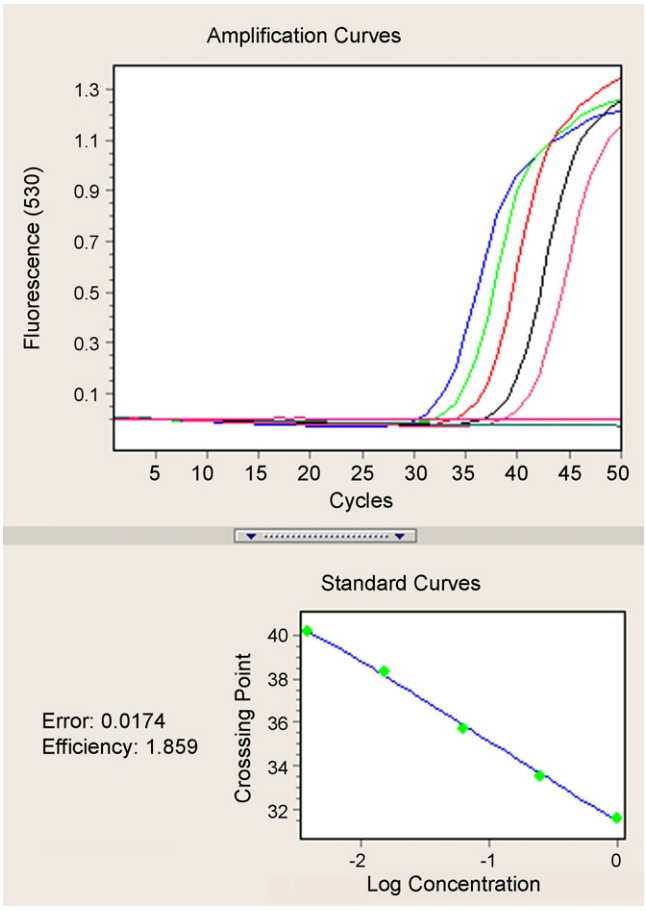

Fig. 2. Amplification profile using the LTR PCR. DNA of a sheep sample (# 19) is titrated from undiluted up to 1024×. Sample dilution 512 still shows a crossing point combined with the correct melting temperature (Fig. 4), conditionally for a positive sample score. Dilution 1024 and the non-template control show no crossing points, indicative for the absence of primer-dimers in this assay.

ally detected another 56 animals to a total score of 82%. On the other hand, four seropositive goats scored positive by the LTR PCR but negative in the leader-gag PCR. In the category seronegative goats ($n = 17$) three animals scored positive in both PCRs with another two animals were positive by the leader-gag PCR.

Table 2
Cross-tabulation of PCR results of 208 sheep samples (bold) and 141 goat samples (italics)

	LTR		
	POS	NEG	Total
Leader-gag			
ELISA+			
POS	**67** (*46*)	**4** (*56*)	**71** (*102*)
NEG	**0** (*4*)	**10** (*18*)	**10** (*22*)
Total	**67** (*50*)	**14** (*74*)	**81** (*124*)
ELISA−			
POS	**6** (*3*)	**11** (*2*)	**17** (*5*)
NEG	**2** (*0*)	**108** (*12*)	**110** (*12*)
Total	**8** (*3*)	**119** (*14*)	**127** (*17*)

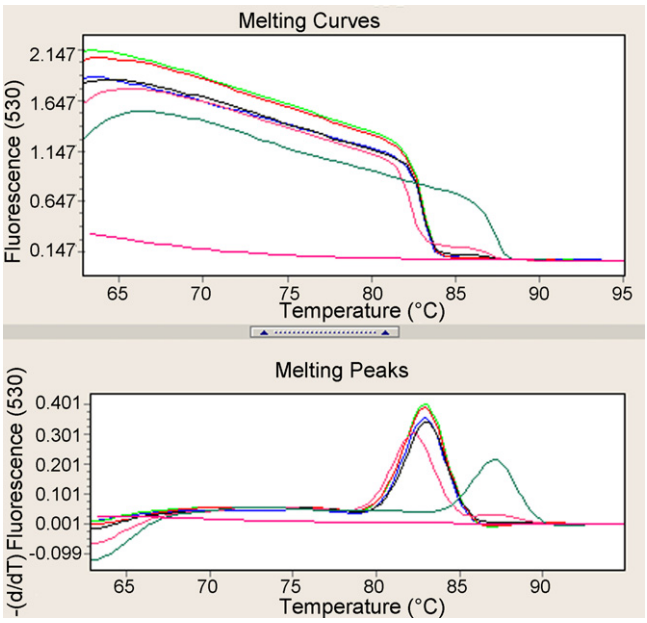

Fig. 3. Melting profile using the leader-gag PCR. For improved visualization of melting temperatures, melting peaks are derived from the data obtained of the melting curve by plotting the negative derivative of fluorescence over temperature vs. temperature [−d(F)/dT vs. T].

Using ELISA as a gold standard, for sheep relative sensitivities of the leader-gag PCR and the LTR PCR were 88% ± 0.035 and 83% ± 0.040 (95% CI), respectively. For goats, relative sensitivities of the leader-gag PCR and the LTR

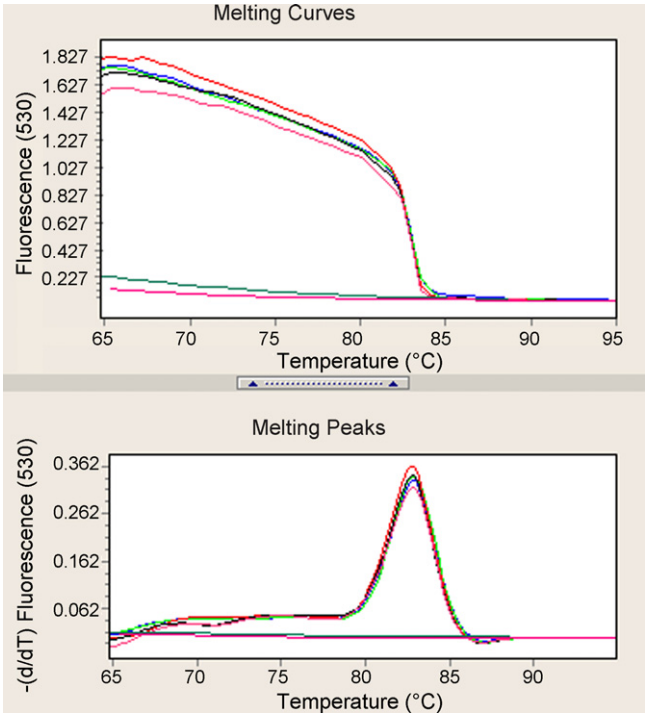

Fig. 4. Melting profile using the LTR PCR. For improved visualization of melting temperatures, melting peaks are derived from the data obtained of the melting curve by plotting the negative derivative of fluorescence over temperature vs. temperature [−d(F)/dT vs. T]. Amplicons generated by this assay show more homogeneous melting profiles.

Table 3
Results of natural transmission experiment (seronegative sheep were kept together with SRLV infected sheep)

Animal	First positive test result in months		
	ELISA	Leader-gag	LTR
3083	10	10	10
610	13	13	13
3054	10	7	10
1490	10	10	10
3015	13	13	13
578	12	7	13
8627	8	8	8
2062	13	9	13
3042	12	10	nt[a]

First positive ELISA, leader-gag and LTR PCR scores of 9 animals are shown in months after introduction of the infected animals.
[a] Not tested.

PCR were 82% ± 0.033 and 40% ± 0.042 (95% CI), respectively.

Among the samples from 47 seropositive animals, 20 sheep and 27 goats, originating from different geographical regions in Norway, France, Spain and Italy no particular differences between countries or regions were observed. In the category seropositive sheep, 85% and 25% were positive byn the leader-gag PCR and the LTR PCR, respectively. In the category seropositive goats, 63% and 23% scored positive by the leader-gag PCR and the LTR PCR, respectively.

ELISA and LTR PCR detected transmission of SRLV in time practically simultaneously, while the leader-gag assay tended to detect SRLV infections earlier (Table 3). In the series of sequential samples from nine sheep which seroconverted after naturally acquired infections, the leader-gag PCR showed a positive score 2 up to 5 months earlier than ELISA and LTR PCR in four sheep. Once an animal was identified as infected by one of the assays the consecutive samplings were consistently positive.

All samples from accredited sheep flocks ($n = 111$) and accredited goat flocks ($n = 78$) were negative in both PCRs, resulting in a diagnostic specificity of 100%.

3.3. Agreement between PCR and ELISA

Comparing PCR test results with those of serology showed a good agreement for sheep samples (Kappa values ±95% confidence intervals (CI) 0.73 ± 0.048 and 0.78 ± 0.045 for leader-gag PCR and LTR PCR, respectively). For goat samples the agreement was fair to poor (Kappa values ±95% confidence intervals (CI) 0.37 ± 0.093 and 0.10 ± 0.041) for leader-gag PCR and LTR PCR, respectively).

4. Discussion

This paper describes the development and evaluation of two real-time PCR assays for the detection of proviral DNA sequences in the LTR and leader-gag regions of the proviral genome of small ruminant lentiviruses. These qualitative methods are based on LightCycler technology using the intercalating dye SYBR Green I for detection.

Leader-gag primer 1 is very similar with the so-called primer binding site (PBS). This retroviral highly conserved sequence consists of 18 nucleotides and is essential for reverse transcription of the viral genome. In spite of its sub-optimal thermodynamics this sequence was chosen as a primer site because of its highly conserved character. LTR PCR primer LTR2a was designed to anneal partly with the PBS.

Based on the negative outcome of the 189 samples from several accredited flocks it was concluded that both assays are highly specific.

The leader-gag PCR appeared to be rather efficient in detecting proviral nucleic acid sequences in seropositive animals. ELISA negative animals from infected flocks were also found positive by both assays but to a larger extent using the leader-gag assay. These, particularly the samples positive in both assays, were not likely false-positives but most probably account for infected animals in which specific antibodies could not (yet) be detected. Several other studies with SRLV-PCRs reported seronegative animals that were found positive in PCR tests (Rimstad et al., 1993; Zanoni et al., 1996; Wagter et al., 1998; Celer et al., 2000). In addition, the results from the series of sequential samples from sheep with naturally acquired infections also clearly demonstrated this tendency. In four out of nine sheep the leader-gag assay detected infections weeks to months before antibodies were detected.

The results with the samples from the other countries strongly suggested that especially the leader-gag primers are directed to a highly conserved region of the proviral DNA. Results of a recent Italian molecular epidemiological study (Pisoni et al., 2006) indicating that their lentivirus strains are closely related to the prototype strain CAEV-CO that was isolated in the US three decades ago also support to the conserved character of the sequences chosen for leader-gag priming. In addition, studies on the human immunodeficiency virus (HIV) indicated that the most conserved part of that genome is not found in one of the open reading frames, but in the 5′ untranslated leader region (Berkhout, 1996; Ooms et al., 2004).

However, using the ELITEST-MVV, a bias may have been introduced for selecting animals mainly reactive to genotype A major proteins. The poor sensitivity of the LTR assay, especially for goat samples may be partly explained by this bias, also because of the fact that the LTR PCR primers were designed mainly based on sheep sequences (Rosati et al., 1995).

As may be concluded from the transmission experiment, the sensitivity of the LTR assay is comparable with that of serology indicating that these tests are functional in the case of productive infections. In this situation, because of efficient activation of the immune system, antibody production proceeds rapidly.

The leader-gag assay showed a clearly better performance than the LTR PCR with respect to the results of the transmission experiment and the detectability of goat samples. No clear differences were noticed with respect to the overall score of goat samples from the different geographical areas, although in Norway predominantly phylogenetic group C sequences were found (Gjerset et al., 2006) and in Spain the more sheep SRLV prototypes related groups A or D are predominant (Reina et al., 2006).

However, the number of samples per country investigated in this study is rather small which prohibits firm conclusions.

The leader-gag PCR seems to be an efficient complementary tool for detection of SRLV infections in seronegative animals from infected flocks and as a confirmatory test for seropositive results. The performance of the ELISA used in this study needs further evaluation because of the observed relatively low detectability for goat samples as indicated by the results of the leader-gag PCR.

5. Conclusions

The leader-gag real-time PCR presents a potentially important test for determining the SRLV infection status of individual seronegative or inconclusive animals and may be a valuable addition to the existing diagnostic tools. Use of PCR as a supplement to serology, so limiting diagnostic test escape, will improve the efficiency of the detection of SRLV infected animals, thus improving the efficacy of control programs. For the monitoring of accredited flocks the leader-gag assay may offer interesting prospects, especially when applied to pooled blood samples or bulk milk samples.

New schemes for eradication and control of SRLV infections may exploit these new real-time PCR developments, with emphasis on the potential of the leader-gag assay.

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