Development of a semi-nested PCR using degenerate primers for the generic detection of small ruminant lentivirus proviral DNA

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Abstract

A PCR assay was developed for the reliable detection of small ruminant lentivirus (SRLV) proviral DNA. The method involved the use of degenerate deoxynosine-substituted primers and a second semi-nested PCR step that increased the polyvalency and sensitivity of the detection, respectively. Primers were designed from the \( pol \) gene conserved motifs of 85 SRLV isolates and were evaluated using different SRLV isolates together with Maedi-Visna virus (MVV) and caprine arthritis–encephalitis virus (CAEV) reference strains. The method successfully detected SRLV proviral DNA in total DNA extracts originating from whole blood samples, separated peripheral blood mononuclear cells (PBMCs) and tissue cultures. The semi-nested PCR was compared with the agar gel immunodiffusion test and proved to be highly sensitive, specific and capable of detecting many SRLV variants in infected or suspect animals. Therefore, it would be useful in the diagnosis of natural SRLV infections, in eradication programs and epidemiological studies. Whole blood samples can be used directly, thus alleviating the need for PBMC separation, and thereby enables a simple, fast and cost-effective analysis of a large number of samples.

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Keywords: Small ruminant lentiviruses; Generic PCR detection; Degenerate primers

1. Introduction

Small ruminant lentiviruses (SRLVs) include Maedi-Visna virus (MVV) of sheep and the caprine arthritis–encephalitis virus (CAEV) of goats (Pasick, 1998). The viruses have a 60–70% nucleotide sequence homology (Saltarelli et al., 1990) and are both members of the genus Lentivirus in the family Retroviridae. SRLVs infection results in progressive interstitial pneumonia and mastitis in sheep, while in goats, approximately 30% of infected animals develop clinical signs, mainly due to arthritis (Kreig and Peterhans, 1990).

The fact that SRLVs are a heterogeneous group of viruses that possess a variable host range and pathological capability, has obvious practical implication on the designation of diagnostic tests and the implementation of control measures (Pasick, 1998).

To date, the routine diagnosis of SRLV infections is based on serology. The agar gel immunodiffusion test (AGIDT) is widely used for the serological diagnosis of SRLV infections (Simard and Briscoe, 1990) and is recommended by the Office International des Epizooties (OIE). However, this test is relatively insensitive with respect to the detection of early seroconversions and low antibody levels (Kwang et al., 1993; Leroux et al., 1997). The use of recombinant proteins (Zanoni et al., 1991) and/or synthetic peptides (Kwang and Torres, 1994) in different enzyme-linked immunosorbent assays (ELISAs) has greatly improved the sensitivity of the serological diagnosis of these infections (Saman et al., 1999). The most sensitive ELISA format requires the use of at least one core capsid (CA) antigen and one envelope transmembrane (TM) antigen. The anti-CA bodies have been shown to be detectable early after natural or experimental infections, while the anti-TM antibodies have been detected 20–33 weeks after naturally acquired infection and are usually associated with the clinical stage of infection (Bertoni et al., 1994; Rosati et al., 1995). Thus, the combination of CA and
TM antigens in a single test format is essential for the identification of seropositive animals at all stages of infection. However, a moderate antigenic heterogeneity of CA has been documented between caprine and ovine lentiviruses and within ovine isolates (Grego et al., 2002). Thus, any single strain-based immunoassay may lack sensitivity if a population is infected with a lentivirus genotype different from that employed in the test (Rosati et al., 2004). Virus isolation from peripheral blood mononuclear cells (PBMCs) in cell cultures has also been used, but it is time consuming and expensive and not practical for routine diagnostic purposes (OIE, 2004).

SRLV infections occur worldwide and cause considerable economic losses. This has lead to control programs in several countries such as The Netherlands, Australia, Switzerland and the USA (Greenwood et al., 1995; Houwers et al., 1987; Rowe et al., 1992; Scher-Czechowski et al., 2000). These programs require annual serological testing of all animals and the removal of newborns from their infected dams. Seropositive animals are culled and the flock in which a seropositive animal is detected is put in strict quarantine, which is maintained until at least three consecutive annual serological assays yield negative results (Shah et al., 2004). However, programs are impeded by delayed seroconversion, observed in both sheep and goats and by the absence of detectable antibodies in infected animals, which lead to delayed diagnosis and hence the re-emergence of the disease (Rimstad et al., 1993). In view of this, the need for an alternative, more rapid method than serological testing is apparent.

Recently, several polymerase chain reaction (PCR) protocols have been designed for the detection of SRLVs in infected sheep and goats. They vary on technical grounds and complexity and have been designed for the detection of SRLVs in infected sheep and goats. Attempts were made to isolate SRLVs using PBMCs from infected cell cultures, separated PBMCs and whole blood. The present study describes the development of a polyvalent and sensitive PCR assay for the reliable detection of SRLV proviral DNA, using degenerate deoxynucleosine-substituted primers and a semi-nested PCR scheme. Its performance was compared with that of the AGID test and was evaluated using different virus isolates and template preparation methods including DNA extracts from infected cell cultures, separated PBMCs and whole blood.

2. Materials and methods

2.1. Animals and blood samples

A total of 218 sheep and goats, 2–4 years of age, belonging to two groups (A and B), were used in this study. Group A consisted of 74 animals (55 sheep and 19 goats) and used to obtain SRLV isolates for the evaluation of the polyvalency of the semi-nested PCR protocol developed here. The animals originated from six mixed flocks in central and northern Greece with a history of SRLV infection and respiratory symptoms. Group B consisted of 144 animals (75 sheep and 69 goats) and used in the comparison of this protocol with the AGID serological test. These animals originated from a mixed flock from Grevena in northwestern Greece, and only goats exhibited clinical signs of infection (respiratory symptoms) at the time of analysis. For the determination of the PCR specificity, eight seronegative animals (four sheep and four goats) were used as negative controls. These were from the Greek National Breeding Center, Diavata, Thessaloniki where animals are screened annually for SRLV infection using serological tests. These animals were also tested by PCR using LTR primers according to Extramiana et al. (2002).

All blood samples were collected by jugular venipuncture. For the AGID test, blood was used from both groups A and B. Serum was separated from the blood clot and stored as aliquots at −20 °C until the test was performed. For the separation of PBMCs, 10 ml of whole blood was collected in vacutainer tubes with EDTA from AGID-seropositive group A animals. It was layered on an equal volume of Histopaque-1077 (Sigma-Aldrich, Germany) and centrifuged at 400 × g for 30 min. PBMCs were aspirated and washed twice, first with 10 ml and then with 5 ml isotonic phosphate buffered saline solution, and stored at −20 °C until DNA extraction or co-cultivation with sheep choroid plexus (SCP) cells. Five ml of whole blood was collected in heparinized vacutainer tubes from group B animals and 500 μl was used for the direct extraction of DNA.

2.2. Agar gel immunodiffusion test (AGIDT)

The AGID test was performed using the Maeditec kit (Maeditec, Veterinary Laboratory Agency, UK). Published procedures were followed throughout (Cutlip et al., 1977). This assay uses the WLC-1 strain of MVV and detects the viral capsid protein (p25) and the envelope glycoprotein (gp135). Readings were made after 24 and 48 h of incubation. Precipitin lines of identity and lines of no-identity were evaluated accordingly.

2.3. Virus isolation in cell cultures

Attempts were made to isolate SRLVs using PBMCs from 11 seropositive animals belonging to group A. Sheep choroid plexus (SCP) cells from tissue explants of MVV free newborn lamb were grown in Dulbecco’s MEM (Gibco, UK) supplemented with 10% fetal bovine serum plus antibiotics [penicillin (100 IU/ml) and streptomycin (100 μg/ml)]. The cultures were incubated at 37 °C to establish primary confluent outgrowth.
These were then trypsinized, subpassaged and used for the isolation of the virus. For co-cultivation with PBMCs, approximately 1 x 10^6 cells were placed on the monolayers of SCP cells. When maximum CPE was observed, cells were harvested and stored at -70°C for DNA extraction. A sample was considered negative if no CPE was seen after three blind passages.

2.4. DNA extraction

DNA was either extracted directly from 500μl whole blood, or separated PBMCs, or infected tissue cultures, using the Flexi Gene DNA Kit (QIAGEN GmbH, Germany) according to the manufacturer’s instructions. DNA was quantified and stored at −20°C until PCR was performed.

2.5. Standard SRLV isolates used in PCR

Fifteen isolates representing different SRLV subtypes were used as standards for evaluating the detection polyvalence of the semi-nested PCR. These were subtypes A3, A4, A5, B1 (two isolates) and B2, originating from Switzerland (EMBL accession Nos.: AY454189, AY454244, AY454246, AY454170, AY454216 and AY454238, respectively, Shah et al., 2004) and six field isolates from The Netherlands, untyped until the time of this study. All were obtained as DNA extracts from PBMCs. Also, the Icelandic K1514 Maedi-Visna virus (Braun et al., 1987), British EV1 (Sargan et al., 1991) and CAEV-Cork (Saltarelli et al., 1990) viruses were used as infected SCP cells. In addition two non-SRLV retrovirus samples, HTLV (human T cell lymphotropic virus) and EIAV (equine infectious anemia virus), were also used to assess the specificity of the PCR reaction for SRLV.

2.6. Oligonucleotide primers

Twenty different degenerate primers were designed after comparative analysis of SRLV sequences corresponding to the most conserved pol polyprotein, containing the RnaseH, integrase and the dUTPase domains. Homologous amino acid and respective nucleotide published pol sequences from 85 SRLV isolates including the K1514, EV1, SA-OMVV (Querat et al., 1990) and CAEV-Cork reference strains, were located and aligned quickly using the WU-Blat2 and the MView tools (Brown et al., 1998), located in the EMBL-EBI (http://www.ebi.ac.uk/blat2/index.html) for the identification of the conserved regions. Degenerate primers were designed based on conserved sequences with minimum codon degeneracy and selection of preferred codon usage for some amino acids (Dovas and Katis, 2003). The selected amino acid regions were checked for possible presence in other non-target organisms by another convenient Blast sequence analysis tool located in the NCBI (http://www.ncbi.nlm.nih.gov/BLAST), to avoid problems in PCR detection specificity. Upstream primers were designed from the “WVPFGMN/KGT” and “QGGID/SGYQQ” motifs located in the RnaseH and dUTPase domains, respectively. Downstream primers were designed from the “HWQVDYTH” and “W(V/I)ETNSG” motifs located in the integrase core domain. Primers were evaluated for their amplification efficiency using 46 SRLVs isolated from group A, along with 15 SRLV standard isolates (Sections 2.1, 2.3 and 2.5). A pair of degenerate primers (LenUp1 and LenDo2) showing highest detection polyvalence and sensitivity was finally selected for use in the initial PCR and a second one (LenNestUp3 and LenNestDo4) for use in the subsequent semi-nested PCR (Table 1).

Primers were modified by introducing deoxycytidine (dI) residues at four-fold degenerate positions, aiming to reduce their degeneracy without decreasing their potential target sequences. During PCR, dI appears to be preferentially recognized as dG by DNA polymerases, resulting in the incorporation of dC opposite to dI, in the PCR products of the second and all subsequent amplification cycles. As a result, “inner” primers LenNestUp3 and LenNestDo4, which have partial nucleotide homology with the “outer” primers LenUp1 and LenDo2, were designed to contain dG instead of dI in the homologous regions (Table 1), exhibiting high amplification efficiency due to the increased stability of the primer–target duplex (Dovas and Katis, 2003; Dovas et al., 2004).

2.7. PCR

PCR reactions were carried out in a T-CY thermocycler (Creacont, The Netherlands). DNA of the WLC-1 Weybridge MVV strain was used as positive control and DNA extracts from a seronegative animal or a non-infected tissue culture were used as negative controls. The 1st PCR was run in a total volume of 25μl. The reaction mixture contained 10mM Tris–HCl (pH

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Location*</th>
<th>Corresponding pol. amino acid sequence</th>
<th>Degeneracy/number of inosines</th>
<th>Assay/product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>LenUp1</td>
<td>5′-CAGGGDGIATMATAAYTUCGG-3′</td>
<td>3960–3982</td>
<td>QGGIDSG</td>
<td>8/3</td>
<td>1st PCR/412 bp</td>
</tr>
<tr>
<td>LenDo2</td>
<td>5′-ARTGIGTIGTACTCCYGGCCG-3′</td>
<td>4371–4349</td>
<td>WQVVQVT</td>
<td>16/2</td>
<td>Nested PCR/404 bp</td>
</tr>
<tr>
<td>LenNestUp3</td>
<td>5′-GOGATMAAYTUCGGREATACGG-3′</td>
<td>3966–3991</td>
<td>GEDGISYGQ</td>
<td>16/30</td>
<td>Nested PCR/404 bp</td>
</tr>
<tr>
<td>LenNestDo4</td>
<td>5′-ARTGIGTIGTACTCCYGGCCG-3′</td>
<td>4589–4347</td>
<td>HWQVDVT</td>
<td>16/30</td>
<td>Nested PCR/404 bp</td>
</tr>
</tbody>
</table>

R=A+G; M=A+C; Y=C+T; T=I=inosine.

* Localization number corresponding to the Visna virus Icelandic strain 1514 (EMBL accession No. M60910).
2.8. Sequence confirmation and analysis of PCR products

Specific semi-nested PCR products obtained from 11 AGID negative animals and one seropositive animal were isolated from the agarose gel with the QIAquick Gel Extraction Kit (Qiagen GmbH, Germany). Both strands of DNA were directly sequenced using the dideoxynucleotide termination cycle method with an ABI3730XL sequencer, using BigDye Terminators 3.1 (Macrogen Inc., Seoul, Korea) and the internal primers “LenNestUp3” and “LenNestDo4”. The amplification and sequencing procedure was repeated for all 12 isolates for verification purposes. Nucleotide sequences were compared with the homologous sequences from reference strains K1514, SA-OMVV, EV1 and CAEV-Cork and analyzed using the ClustalX program (Thompson et al., 1997). A phylogenetic tree was produced using the neighbour joining method. To verify the statistical validity of the clusters obtained on the neighbour joining tree, a bootstrap value was calculated on 1000 trials.

3. Results

3.1. Serology

The results of the AGID test are presented in Table 2. In group A, 46 animals (11 goats and 35 sheep) out of a total of 74 (62.2%), were found to be AGID positive for SLRVs. In group B, 44 animals (29 goats and 15 sheep) out of a total of 144 (30.5%) were found to be AGID positive for SLRVs.

3.2. Isolation of SRLVs from tissue cultures

Viruses were isolated after the second passage, from 11 SCP cell cultures infected with PBMCs from seropositive animals (seven sheep and four goats) of group A. In the case of sheep, viral presence was confirmed by the appearance of the characteristic CPE, consisting of refractile, stellate cells and syncytia, 14–21 days post-infection. With goats however, there was a persistent infection of tissue culture without obvious CPE, in which the case the presence of the virus was confirmed using semi-nested PCR as described below.

3.3. Semi-nested PCR performance

In group A, PBMCs from all of the 46 AGID positive animals, as well as the cell cultures established from 11 of these, were SRLV positive in semi-nested PCR assays and gave the expected amplification signals with HTLV, EIAV and the negative controls. In group B, whole blood samples from the 44 AGID positive animals were also SRLV positive in semi-nested PCR assays (Fig. 1). In addition, 11 more samples which had tested AGID negative were found to be PCR positive for SLRVs (Table 2), thus bringing the total number of SRLV positive animals in this

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Number of positive and negative samples according to AGID test and semi-nested PCR, performed for the detection of SRLVs in different samples from sheep and goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal groups</td>
<td>Sample</td>
</tr>
<tr>
<td>A</td>
<td>Serum</td>
</tr>
<tr>
<td></td>
<td>PBMCs</td>
</tr>
<tr>
<td></td>
<td>SCP co-cultures</td>
</tr>
<tr>
<td>B</td>
<td>Serum</td>
</tr>
<tr>
<td></td>
<td>Whole blood</td>
</tr>
</tbody>
</table>

a In group A, PCR was carried out with PBMCs isolated only from AGID positive animals.
b Viruses were isolated using PBMCs from 11 of the 46 seropositive animals in group A.
due to the low viral load, but also to the failure of these PCR assays
detection methods described to date are not efficient in detect-
ing all SLRVs (Shah et al., 2004). This could be attributed to
the low viral load, but also to the failure of these PCR assays
to detect all strains of SLRVs, and consequently it has been
proposed that they should not be considered as alternatives to
serology, but as complementary tests for use in selected cases
(Peterhans et al., 2004). The high diversity of SRLV genome
sequences is a considerable limitation for PCR efficiency, since
the primers used are not always perfectly complementary to
the target sequence, influencing both detection polyvalence and
sensitivity. In addition, different regions of the viral genome
comprising LTR, gag, pol and env genes have been selected for
primer design (Barlough et al., 1994; Celer et al., 2000; Zanoni
et al., 1992). Most studies have used PBMCs as target cells for
the PCR amplification and the reported sensitivity varies from
70 to 95% of the seropositive animals (Extramiana et al., 2002;
Reddy et al., 1993; Rimstad et al., 1994; Travassos et al., 1999;
Wagter et al., 1998). Consequently, the design of a primer set
with a wide genetic window, encompassing all diverse variants
while still being group specific, is a prerequisite for the estab-
lishment of molecular techniques and an important step in SRLV
diagnosis.

Detection polyvalence is a bottleneck for the diagnosis of dis-
eases involving viruses that mutate rapidly, such as SLRVs, espe-
cially in eradication programs where reliable detection assays
are extremely important. This high natural virus variability hin-
ders the selection of conserved nucleotide regions for primer
design. In this case, sequence information from a high number
of diverse isolates is valuable and the amount of sequence data
needed is proportional to the sequence variability of the virus.
Additionally, many different primers have to be tested exten-
sively and selected for their usefulness in detecting a maximum
number of diverse virus isolates. Generic PCR detection meth-
ods which use degenerate primers can alleviate these problems,
allowing the simultaneous detection of different variants or sev-
eral closely related viruses (Dovas and Katis, 2003; Dovas et al.,
2004). These methods are based on the use of degenerate primers
derived from sequence back-translation of consensus amino acid
sequences, with best candidates being the evolutionary con-
served sequences defining functional domains of the expressed
protein (Preston, 1997). These primers represent all possible
coding sequences for the selected peptide regions and hence
theoretically, they accommodate all potential target sequences
of the variants. In this study, we have shown that degenerate
primers designed after comparative analysis of SLRV amino acid
sequences, corresponding to the most conserved pol polypro-
tein and especially those originating from the LTR/RT and/or
integrase regions, can perform with high detection polyvalence. This was
confirmed by specific positive PCR amplification products that
were obtained with the K1514, EV1 and CAEV-Cork reference
strains, in addition to SRLV field isolates of all five subtypes (A3,
A4, A5, B1, and B2) from Switzerland (Shah et al., 2004), six
field isolates from The Netherlands and 46 field isolates from dif-
ferent geographical regions of Greece. Furthermore, the absence
of amplification signals with HTLV and EIA V and the negative
controls indicate the high specificity of these primers for SRLVs.

4. Discussion

Lentiviruses have one of the most rapidly evolving genome
kinds, with considerable variations, and it has been proposed
that they should be viewed as a broad and heterogeneous group or
quasispecies rather than a collection of distinct species (Pasicz,
1998; Leroux et al., 1995). In this study, we have developed a
semi-nested PCR assay that is reliable, sensitive and specific,
which at the same time is capable of detecting many SRLV
variants directly in whole blood, separated PBMCs and infected
SCP co-cultures.

Due to the low viral load in the post-seroconversion phase of
infection, PCR tests for SRLVs are generally less sensitive than
serological detection techniques, although prior to seroconver-
sion PCR is more sensitive in detecting infected animals. As a
result, it has been suggested that a combination of serology
and PCR might afford optimal detection of SLRV infection (de
Andrés et al., 2005). At present, it would appear that the PCR
detection methods described to date are not efficient in detect-
ing all SLRVs (Shah et al., 2004). This could be attributed to
the low viral load, but also to the failure of these PCR assays

Fig. 1. Electrophoretic analysis of semi-nested PCR products (404 bp) for the
detection of SRLVs originating from 15 different animals, using total DNA
extracts from separated peripheral blood mononuclear cells (PBMCs) (lanes
2–4), whole blood samples (lanes 5–8) and tissue cultures (lanes 9–16). Lane
1: positive control (WLC-1 strain), lane 17 negative control (DNA extract from
non infected PBMCs). M: 100 bp DNA marker.

**Table 1**: Summary of the sensitivity and specificity of the PCR assay for
SRLV detection in different animal tissues.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Blood</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>PBMCs</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>Tissue Cultures</td>
<td>96</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 2**: Distribution of SRLV positive samples in different animal groups.

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Number of Positive Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>45</td>
</tr>
<tr>
<td>Cattle</td>
<td>32</td>
</tr>
<tr>
<td>Pigs</td>
<td>15</td>
</tr>
</tbody>
</table>

**Table 3**: Comparative analysis of PCR amplification products using
degenerate primers designed after comparative analysis of SRLV amino
acid sequences.

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Identity (%)</th>
<th>Parity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTR/RT A3</td>
<td>85</td>
<td>90</td>
</tr>
<tr>
<td>LTR/RT A4</td>
<td>78</td>
<td>85</td>
</tr>
<tr>
<td>LTR/RT B1</td>
<td>68</td>
<td>70</td>
</tr>
<tr>
<td>LTR/RT B2</td>
<td>50</td>
<td>55</td>
</tr>
</tbody>
</table>

**Table 4**: Comparative analysis of PCR amplification products using
degenerate primers designed after comparative analysis of SRLV amino
acid sequences.

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Identity (%)</th>
<th>Parity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTR/RT A3</td>
<td>85</td>
<td>90</td>
</tr>
<tr>
<td>LTR/RT A4</td>
<td>78</td>
<td>85</td>
</tr>
<tr>
<td>LTR/RT B1</td>
<td>68</td>
<td>70</td>
</tr>
<tr>
<td>LTR/RT B2</td>
<td>50</td>
<td>55</td>
</tr>
</tbody>
</table>
goats, while in the remaining 11 seronegative animals the PCR amplified DNAs were all identified as typical SRLV sequences.

An important parameter for the selection of primers with high amplification efficiency is the level of their degeneracy. Increased degeneracy is associated with increased non-specific primer annealing (Compton, 1990). Additionally, it reduces the molar concentration of each single oligonucleotide, and subsequently the concentration of those that can prime synthesis during PCR, resulting in low amplicon yield. For this reason, a lower level of primer degeneracy was achieved, by introducing a universal base such as dI at degenerate positions (Barti, 1997) and by selecting preferred codon usage for some amino acids (e.g. serine, Table 1) in the corresponding target genomic regions of SRLVs, after aligning all available nucleic acid sequences. Furthermore, the introduction of a semi-nested format resulted in the PCR methodology being significantly more sensitive ($P \leq 0.005$) than the AGID serological test in detecting SRLVs since, 11 seronegative animals in group B were found to be SRLV positive by PCR. This indicates that SRLV variants could be detected (by PCR) in the absence of detectable antibody levels. Low antibody levels can arise from a number of factors such as, late seroconversion phenomenon observed in SRLV infections (Rimstad et al., 1993; Wager et al., 1998) or the variation in antibody levels in individual hosts (Kraasng and Schuller, 1998). Moreover, the direct detection of the virus in 500 μl of whole blood from all seropositive animals used in this study, suggests that low viral load may not be the major factor for an amplification failure, but rather it is the diversity in the viral sequences that influences primer efficiency.

In conclusion, we have developed a semi-nested PCR assay that is simple to perform, is capable of detecting many SRLV variants and sensitive enough to detect proviral DNA directly from whole blood. By applying this assay time can be saved, since it does not require the separation of PBMCs or co-cultivation of the virus in tissue cultures, and thus it may serve as a quick and reliable tool in the diagnosis of natural SRLVs infections in the field. However, it should be noted that the sensitivity of this method will be at its highest when DNA preparations from isolated PBMCs are used as templates. Finally, since the method could detect a wide range of SRLV variants from different geographical regions in Greece and strains from other European countries, we propose that it could be a useful tool for SRLV diagnosis, eradication purposes and epidemiological investigations worldwide.

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