



## Veterinary Microbiology

# Duplex nested-PCR for detection of small ruminant lentiviruses



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## ABSTRACT

Small ruminant lentiviruses (SRLV) have high genetic variability which results in different viral strains around the world. This create a challenge to design sensible primers for molecular diagnosis in different regions. This work proposes a protocol of duplex nested-PCR for the precise diagnosis of SRLV. The technique was designed and tested with the control strains CAEV Co and MVV 1514. Then, field strains were submitted to the same protocol of duplex nested-PCR. Blood samples of sheep and goats were tested with AGID and nested PCR with specific primers for *pol*, *gag* and LTR. The AGID results showed low detection capacity of positive animals, while the nested PCR demonstrated a greater capacity of virus detection. Results demonstrated that LTR-PCR was more efficient in detecting positive sheep samples, whereas *gag*-PCR allowed a good detection of samples of positive goats and positive sheep. In addition, *pol*-PCR was more efficient with goat samples than for sheep. Duplex nested PCR performed with standard virus samples and field strains demonstrated that the technique is more efficient for the detection of multiple pro-viral DNA sequences. This study demonstrated a successful duplex nested PCR assay allowing a more accurate diagnosis of SRLV.

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## Introduction

Small ruminant lentiviruses (SRLV) are pathogens that affect sheep and goats causing persistent and progressive infections. These agents are represented by two phylogenetic groups

characterized by the Maedi-Visna virus (MVV) and the caprine arthritis encephalitis virus (CAEV).<sup>1–3</sup> Phylogenetic analysis divides these viruses into five major groups classified as A-E. The MVV and CAEV prototypes of groups A and B, respectively, are widely distributed throughout the world, while C-E groups are geographically restricted.<sup>4,5</sup>

These lentiviruses are closely related and were considered species-specific infecting sheep and goats, respectively, causing progressive lung disease, encephalomyelitis, mastitis and arthritis.<sup>6</sup> However, molecular and epidemiological studies

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conducted in the last decades indicate that these agents are capable of infecting both goats and sheep,<sup>7</sup> transgressing the barrier between species consistently and easily.<sup>4,8,9</sup>

These viruses present high genetic variability related to the viral replication process, resulting in the formation of several viral strains in different geographic regions.<sup>2</sup> Due to the heterogeneity of the SRLV genome it is difficult to use the same set of primers in different geographic regions.<sup>10</sup>

The serological diagnosis of infected animals by the Agarose Gel Immunodiffusion (AGID) technique is widely used and accepted due to its low cost and practicality.<sup>11</sup> In addition, this technique is recommended by OIE.<sup>12</sup> However, some animals may present low antibody titers, late seroconversion or intermittent reactions of seropositivity and seronegativity.<sup>13,14</sup>

Numerous studies have successfully demonstrated the use of the PCR technique in the detection of pro-viral DNA from SRLV<sup>15-18</sup> due to the high sensitivity and specificity this technique presents.<sup>19</sup> A multiplex PCR is a variant of the conventional PCR in which two or more target sequences can be amplified including more than one pair of primers in the same reaction.<sup>20</sup>

Based on these advances of molecular diagnosis, this study aimed to develop a duplex nested PCR protocol for accurate diagnosis of small ruminant lentiviruses, as well as to verify the efficiency of the method in diagnosing sheep and goat field samples.

## Material and methods

This project was approved by the local Ethics Committee for the Use of Animals from the State University of Ceará, protocol number 0784899-15.

### Samples

For testing duplex nested-PCR technique, standard samples of the CAEV-Cork<sup>21</sup> and MVV-K1514<sup>22</sup> strains were used and this virus samples were amplified in cell culture and stored frozen for later use.

These samples were provided by Dr. Roberto Soares de Castro (Federal Rural University of Pernambuco – UFRPE) and originated from the Institut National de la Recherche Agronomique Lyon-France.

To demonstrate the efficiency of the technique in detecting SRLV, samples were collected from animals housed in two farms of northeastern mesoregion from Pará state and two farms in the metropolitan region of Fortaleza, Ceará – Brazil. Herds of Santa Inês breed and undefined breed were used in this study. Similarly, samples of goats were obtained from different properties in metropolitan region of Fortaleza, Ceará – Brazil. Herds of Saanen, Alpina, Murciana breed and crossbred animals were collected. All animals were aged from 1 to 2 years and the findings of clinical symptomatology were described in Table 2.

Blood samples of 20 goats and 15 sheep were collected by venipuncture of the jugular vein using vacutainer<sup>®</sup> tubes with EDTA to perform molecular tests and tubes without anticoagulant for agarose gel immunodiffusion (AGID) assay.

### Agarose gel immunodiffusion assay (AGID)

Before performing duplex nPCR in field strains, all samples were tested by AGID for screening animals and identifying positives and negatives. AGID test was performed using the national commercial kit developed by Biovetech<sup>®</sup>. The above-mentioned AGID kit is produced using supernatant concentrate antigen from MVV and CAEV infected cell cultures and with positive serum harvested from naturally infected animal. This test is indicated for detection of anti-p28 antibodies from goats infected with CAEV and sheep infected with MVV.

### DNA extraction

For DNA extraction from whole blood leukocytes, samples were submitted to the protocol described by Caldas et al.<sup>23</sup>. The samples were centrifuged at 1500 g for 10 min. The leukocyte layer was collected and transferred to 1.5 µL microtubes containing 500 µL TE (10 mM Tris, 1 mM). The material was centrifuged in microfuge at 2700 g for 5 min to obtain the pellet, then the supernatant was discarded and the operation repeated. The pellet was resuspended in K buffer (10 mM Tris HCl pH 8.0, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 0.5% Tween 20, 100 µg/mL proteinase K = 0.1 µg/µL). The samples were vortexed, incubated at 56° C for 45 min and then boiled at 96° C for 10 min. The material was stored at 4° C for 24 h for a better homogenization of sample and then at -20° C until duplex nPCR was performed.

### Primers

For duplex nPCR, a pair of primers was used for each region (*gag*, *pol*, LTR), which are located in more conserved regions (Table 1).

### Nested-PCR

Before performing duplex nPCR in field samples, all samples were tested by nested-PCR (nPCR) for screening the animals and identifying positives and negatives. The detection of pro-viral DNA was performed with nPCR following the methodology described by Barlough et al.<sup>26</sup> with modifications. Modifications were made to the methodology as proposed by Andrioli et al.<sup>27</sup> in which KCl, gelatin and tetramethylammonium chloride were removed from the PCR mix. In addition, modifications were made to the amplification cycles with a decrease in the number from 45 to 35 cycles and an increase in the annealing temperature from 50° C to 63° C.

This was performed to amplify a fragment of the CAEV and MVV pro-viral DNA based on the *gag*, *pol* and LTR regions adjusting the of primer concentration. Individual nPCR was performed for each set of primers, and all samples were tested for the *gag*, *pol* and LTR primers prior to performing the duplex nPCR. The nPCR was performed with *gag* primers were considered as standard to discriminate positive or negative samples for the molecular tests. This choice was made on the basis of findings from the literature confirming that the *gag* region is the most conserved within the SRLV genome.<sup>37,41-43</sup>

**Table 1 – Primers used for duplex nested-PCR standardization.**

Primer	Sequence	Round	Amplicon size expected in second round	Reference
GEX5	5'-CAAGCAGCAGGAGGGAGAAGCTG-3'	First	187 bp	Saltarelli et al. <sup>24</sup>
GEX3	5'-TCCTACCCCATTAATTTGATCCAC-3'	First		Saltarelli et al. <sup>24</sup>
GIN5	5'-GTTCCAGCAACTGCAAACAGTAGCAATG-3'	Second		Saltarelli et al. <sup>24</sup>
GIN3	5'-ACCTTTCTGCTTCTTCATTTAATTTCCG-3'	Second		Saltarelli et al. <sup>24</sup>
LTREX EFW	5'-ACTGTCAGGRCAGAGAACARATGCC-3'	First	203 bp	Ryan et al. <sup>25</sup>
LTREX ERV	5'-CTCTCTTACCTTACTTCAGG-3'	First		Ryan et al. <sup>25</sup>
LTRIN IFW	5'-AAGTCATGTAKCAGCTGATGCTT-3'	Second		Ryan et al. <sup>25</sup>
LTRIN IRV	5'-TTGACGGAATTAGTAACG-3'	Second		Ryan et al. <sup>25</sup>
POLEX P28	5'-CATGAAGAGGGGACAAATCAGCA-3	First	1175 bp	Shah et al. <sup>4</sup>
POLEX P33	5'-CTTCCAVAGTACCTGDGTTGGTC-3'	First		Shah et al. <sup>4</sup>
POLIN P29	5'-GGTGCTGGACATAAAGGGATTTC-3	Second		Shah et al. <sup>4</sup>
POLIN P35	5'-GCCACTCTCCTGRATGCTCTCT-3'	Second		Shah et al. <sup>4</sup>

The reactions was performed in a thermal cycler (Eppendorf®) in a final volume of 50 µL and the solution mix consisted of 10 mM Tris HCl buffer (pH 8.3); 1.5 mM MgCl<sub>2</sub>; 100 µM of each dNTP; 2 U/µL taq DNA polymerase recombinant (Invitrogen®); 3 µL of test sample and DNase-free water to complete a final volume. Different concentrations of the *gag*, *pol* and LTR primers prepared for each reaction were tested with the concentrations of 20 pM, 10 pM, 7.5 pM and 5 pM.

The amplification conditions were as follows: initial denaturing at 94 °C for five min, followed by 35 cycles: denaturation at 94 °C for one min, annealing 63 °C for one min, extension at 72 °C for 45 s, followed by an additional extension phase at 72 °C for seven min. An aliquot of 1.0 µL of this PCR product was transferred directly to a second reaction. The first and second rounds of the PCR were performed under the same previous conditions. All reactions were performed under the same time and temperature conditions.

PCR amplified fragments were analyzed by horizontal cube electrophoresis and the bands were visualized under the ultraviolet light transilluminator (Vilber Lourmat – Transilluminator UV/white light) and photodocumented (Logic 200 PRO Serie). The sizes of the amplified fragments were compared to a 100 bp DNA ladder (Promega) and to the positive control.

#### Duplex nested-PCR using standard SRLV samples

After the serological test and the individual nPCR performed with each set of primers, the duplex nPCR was performed with the same primer concentrations of the previous test. The following concentrations were used 20 pM, 10 pM, 7.5 pM and 5 pM for *gag*, *pol* and LTR primers.

The first step of standardization involved performing the duplex nPCR using the primers for the *gag* and *pol* genes and the second step involved a test using the primers for the LTR and *pol* region.

The combination *gag* and LTR was not evaluated. Primers designed for this combination did not generate satisfactory results in the tests performed, since the amplicons generated have very close sizes, making it impossible to differentiate them.

The reactions were performed in a thermal cycler (Eppendorf®) in a final volume of 50 µL and the solution mix consisted of 10 mM Tris HCl buffer (pH 8.3); 2 U/µL taq DNA

polymerase; 3 µL of test sample and DNase-free water to complete a final volume. The primer concentrations were used according to the results obtained for the conventional nPCR previously performed. To adjust the duplex nPCR technique, different concentrations of dNTPs and magnesium were also tested. Amplification conditions followed the same protocol as previously described for the nPCR.

PCR amplified fragments were analyzed by horizontal cube electrophoresis. Different concentrations of agarose gel were tested to visualize PCR products, in which the following were tested 1%, 1.3%, 1.5% and 2%. The concentration that allowed the best visualization of the different bands formed in the duplex nPCR was verified. The bands were visualized in ultraviolet light transilluminator (Vilber Lourmat – Transilluminator UV/white light) and photodocumented (Logic 200 PRO Serie). The sizes of the amplified fragments were compared to 100 bp DNA ladder (Promega) and to the positive control.

#### Evaluation of technique performance in field samples

After obtaining the best concentration of reagents for duplex nPCR and evaluating effectiveness in detecting the standard virus samples, the developed technique was used to test the samples obtained from animals in the field.

Fifteen sheep and twenty goat samples were tested with duplex nPCR using the *pol* and *gag* primer sets and the *pol* and LTR primer sets as described for the experiment performed with the standard virus samples.

#### DNA sequencing and sequence analysis

Positive samples obtained from Goats 6, 7 and 8 and Sheep 4, 5 and 6 were sent for sequencing.

The partial direct sequencing of the *gag* gene relative to the amplification products obtained in the second round of nested-PCR was performed. Were used the primers internals GIN5 and GIN3.

The samples were purified on 1% agarose gel using commercial kit (BigDye Terminator v3.1 Cycle Sequencing) and sequenced on ABI 3500 platform (Genetic Analyzer). The gene sequences were compared with MEGA7 analysis software, and Basic Local Alignment Search Tool – Blast. All positions with

ambiguous codes or alignment gaps were excluded from the analyses.

The SRLV strains were compared to K1514-MVV,<sup>28</sup> CAEV,<sup>24</sup> 7-29Ont,<sup>29</sup> Br/UFRGS-2/V27,<sup>30</sup> 21G-MG/Br,<sup>31</sup> 39QPI/Br,<sup>31</sup> 19F-MG/Br<sup>31</sup> and BR/CNPC-G4<sup>32</sup> with GenBank accession n°. M60609, M33677, KC155690.1, AJ305039.1, KF861573.1, KF861553.1, EU300979.1 and KF861559.1 respectively.

Through the analysis by BLASTN 2.8.0 tool it was possible to determine the similarity of the strains found with the other strains deposited in the GenBank.<sup>33,34</sup>

## Results

### AGID and nested PCR

All field samples were tested with AGID and nested-PCR techniques, in which the primers for the *gag* gene were used and considered the diagnostic control standards for comparison with the developed duplex nested-PCR technique. In a total of 20 goats evaluated by AGID, only two animals (10%) were positive, while nine animals had inconclusive. Inconclusive results consisting in inadequate formation of the expected precipitation line.

Same samples were tested with nPCR with *gag* primers, in which 17 goats (85%) tested positive. Only three animals that were negative in AGID test continued to demonstrate negative results with nPCR performed with *gag* primers.

In a total of 15 sheep evaluated by AGID, three presented positive results and 12 were negative. In the nPCR results using *gag* primers, 9 (60%) sheep were positive and 6 sheep with negative results in AGID were also negative in nPCR.

As for the technique performed with the *pol* primers, results demonstrated that primers were also efficient for detecting pro-viral DNA from goat samples, but not as efficient as primers for *gag* region. Among the 17 animals positive in nPCR performed with *gag*, 15 animals (75%) were also positive for the *pol* primers, showing an agreement of 88%. However, the nPCR results of sheep samples using *pol* primers demonstrated that these were not as efficient in detecting the positive animals. From the total of fifteen tested animals, only 3 were positive for the technique using the *pol* primers (Table 2).

The nPCR performed with the LTR primers demonstrated that these primers were not efficient for the detection of pro-viral DNA in goat samples of this study, since none of the tested animals presented a positive result. Among the 15 sheep samples tested for this set of primers, 10 were positive. One sample that had been considered negative for the *pol* and *gag* primers showed positivity for the LTR primers.

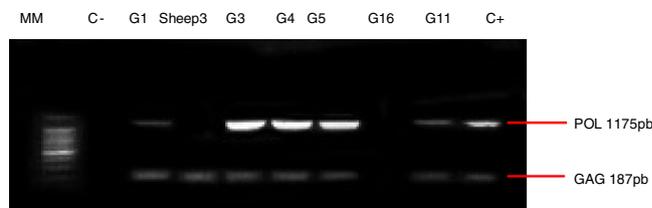
The best primer concentrations for nPCR in this study were 10 pM for *gag* primers and 20 pM for LTR. For *pol* primers the best concentration was 7.5 pM per reaction. For this test, there was no need to modify the concentration of any other reagent or adjust the thermal cyclers.

### Duplex nested-PCR standardization using standard SRLV samples

For standardization of the duplex nPCR technique, tests were elaborated with standard samples of CAEV-Cork and



**Fig. 1 – Duplex nested-PCR. 1.3% agarose gel stained with ethidium bromide, presenting products of amplification of the pro-viral DNA target fragments of small ruminant lentiviruses samples. Products are equivalent to 1175 bp for amplification with POL primers, 203 bp for amplification with primers LTR and 187 bp for amplification with primers GAG. MM = molecular marker (DNA ladder marker 100 pb), C- = negative Control, CA = CAEV standard sample, MV = standard MVV sample.**



**Fig. 2 – Duplex nested-PCR. Agarose gel (1.3%) stained with ethidium bromide, presenting products from the amplification of pro-viral DNA target fragments from small ruminant lentivirus samples. Products equivalent to 1175 bp for amplification with pol primers and.**

MVV-K1514 viruses. This step was performed in order to demonstrate that the primers prepared and used for performing the technique are effective in detecting the SRLV.

Standardization of the duplex nPCR technique demonstrated that the optimal concentrations for the reaction using the *gag* and *pol* primers was 5 pM for *pol* and 10 pM for the *gag*, which generated two satisfactory bands both allowing a conclusive and reliable diagnosis.

For the protocol of duplex nPCR performed with the primers *pol* and LTR, the best primer concentrations were 7.5 pM for the primer *pol* and 20 pM for the LTR primers for both first and second rounds. In this step, there was a better result of duplex nPCR with an increase of dNTP concentration used to 200  $\mu$ M of each (Fig. 1).

To perform the agarose gel electrophoresis, different concentrations of agarose were tested. Among all the values tested, the 1.3% concentration allowed the best visualization of the bands. This concentration was adopted as the standard for the electrophoresis of samples obtained by duplex nPCR.

### Evaluation technique performance in field samples

The results obtained with the first duplex nPCR test with *gag* and *pol* primers using goat and sheep samples showed that *pol* primers were efficient for detection of large number of the positive samples of goats, but was not as efficient as the *gag* primers. From a total of 17 samples positive for *gag*, 15 showed positivity for *pol* and continued to demonstrate this expected result with the duplex technique (Fig. 2). In the evaluated sheep samples, only three were positive for the *pol* primers, demonstrating that the duplex technique performed with *gag* and *pol* primers is effective for more accurate detection of goat

**Table 2 – Description of the clinical findings and the results obtained in the AGID and Nested-PCR tests.**

Samples	Clinical symptoms	AGID	<i>gag</i>	<i>pol</i>	LTR
CAEV Co	–	+	+	+	+
MVV K1514	–	+	+	+	+
Goat 1	Asymptomatic	Inconclusive	+	+	–
Goat 2	Asymptomatic	Negative	+	+	–
Goat 3	Arthritis	Inconclusive	+	+	–
	Productivity decrease				
Goat 4	Arthritis	Inconclusive	+	+	–
Goat 5	Asymptomatic	Inconclusive	+	+	–
Goat 6	Asymptomatic	Inconclusive	+	+	–
Goat 7	Asymptomatic	Inconclusive	+	+	–
Goat 8	Arthritis	Inconclusive	+	+	–
	Productivity decrease				
Goat 9	Asymptomatic	Inconclusive	+	+	–
Goat 10	Asymptomatic	Positive	+	+	–
Goat 11	Asymptomatic	Inconclusive	+	+	–
Goat 12	Asymptomatic	Negative	+	+	–
Goat 13	Asymptomatic	Negative	+	+	–
Goat 14	Asymptomatic	Positive	+	+	–
Goat 15	Arthritis	Negative	+	–	–
Goat 16	Asymptomatic	Negative	–	–	–
Goat 17	Muscular atrophy	Negative	+	+	–
	Arthritis				
Goat 18	Asymptomatic	Negative	–	–	–
Goat 19	Asymptomatic	Negative	+	–	–
Goat 20	Asymptomatic	Negative	–	–	–
Sheep 1	Asymptomatic	Negative	–	–	–
Sheep 2	Asymptomatic	Negative	–	–	–
Sheep 3	Asymptomatic	Negative	+	–	+
Sheep 4	Neurological symptomatology, weight loss, pneumonia and prostration	Positive	+	+	+
Sheep 5	Asymptomatic	Positive	+	+	+
Sheep 6	Asymptomatic	Positive	+	+	+
Sheep 7	Asymptomatic	Negative	–	–	+
Sheep 8	Asymptomatic	Negative	+	–	–
Sheep 9	Asymptomatic	Negative	–	–	–
Sheep 10	Asymptomatic	Negative	+	–	+
Sheep 11	Asymptomatic	Negative	–	–	+
Sheep 12	Asymptomatic	Negative	–	–	–
Sheep 13	Asymptomatic	Negative	+	–	+
Sheep 14	Asymptomatic	Negative	+	–	+
Sheep 15	Asymptomatic	Negative	+	–	+

samples. All samples that were positive in the tests of individual nPCR for each set of primers also demonstrated positivity when tested with duplex nested PCR.

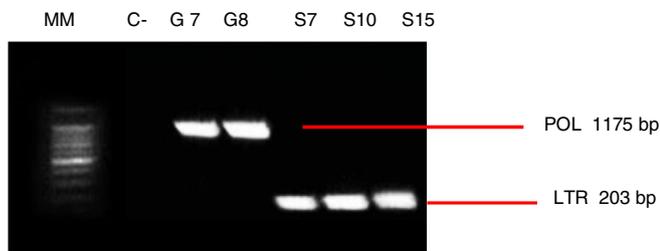
The second step of standardization using the LTR and *pol* primers in field samples demonstrated that these primers are ideal for duplex nested-PCR, considering that the size of the bands formed is very different, allowing a good visualization of the results when analyzed on the agarose gel (Fig. 3). In addition, results demonstrated that LTR primers were more efficient at detecting sheep samples, failing to detect in samples from goats. The results demonstrated that *pol* primers were more specific for SRLV present in goats and LTR primers for SRLV obtained from sheep samples. These results also showed that the duplex nested-PCR technique is more

sensitive to generate more reliable results for detection of lentivirus in sheep and goats.

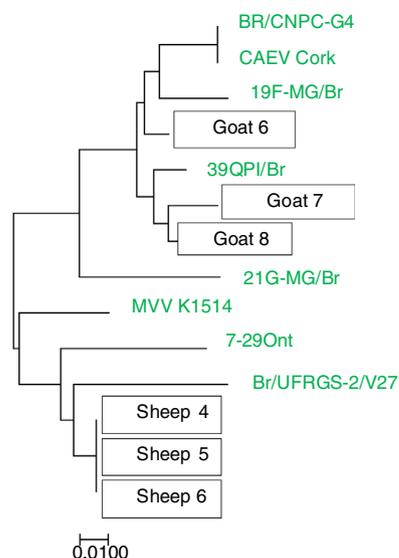
#### DNA sequencing and sequence analysis

The analyses revealed that sequences from sheep and goats described here are related to genotype B1 (CAEV-Co) and genotype A (MVV K514) of SRLV, respectively. In addition, some genetic relationship was observed with other Brazilian strains described and the samples of sheep showed relation with Canadian strain (7-29Ont).

A phylogenetic tree (Fig. 4) was obtained using the NJ method with the *gag* nucleotide sequences, and this tree showed the relationship between the strains detected by



**Fig. 3 – Duplex nested-PCR. Agarose gel (1.3%) stained with ethidium bromide presenting products of the amplification of the pro-viral DNA target fragments of small ruminant lentivirus samples. Bands equivalent to 1175 pb for amplification with POL primers and 203 pb for amplification with primers LTR were observed. MM = molecular marker (DNA ladder marker 100 pb), C- = negative control. Positive samples for pol –Goat 7 and Goat 8. Positive samples for LTR –Sheep 7, 10 and 15.**



**Fig. 4 – Molecular phylogenetic analysis by evolutionary relationships of taxa using the Neighbor-Joining method.**

duplex nPCR and the other known SRLV strains. The phylogenetic tree was determined using the neighbor joining (NJ) method<sup>35</sup> implemented in MEGA, the Tamura-Nei gamma distance.<sup>36</sup>

When comparing the sequences obtained with the samples deposited in the GenBank it was possible to verify the degree of identity between the strains. In Table 3 it is possible to verify the convergence and identity between the analyzed strains.

After analysis of the partial sequences of the *gag* gene from ovine and caprine samples the samples were submitted to the GenBank and are available for access with the following access numbers: MH251628 (Goat 6), MH251627 (Goat 7), MH251626 (Goat 8), MH251631 (Sheep 4), MH251630 (Sheep 5) and MH251629 (Sheep 6).

After the alignment of the samples under study with the standard samples, it is possible to identify the sites where differences in pro-viral DNA occur. These differences can be seen in Fig. 5.

## Discussion

Several studies have shown that the molecular diagnosis of lentiviruses are increasingly sensitive,<sup>37-39</sup> being a suitable technique for monitoring animals, particularly those of high zootechnical value. In this study, the developed technique proved to be efficient for the simultaneous detection of sheep and goat samples, allowing a faster and more sensitive diagnosis of lentiviruses compared to AGID. Considering that duplex nPCR allows the simultaneous diagnosis of different regions of the viral genome in a single assay, it is possible by means of a single assay to perform a more accurate diagnosis of the affected animals. In addition, costs of laboratory material and time are reduced when compared to the conventional nPCR technique.

The AGID technique is recommended by OIE<sup>12</sup> and is widely used for the serological diagnosis of animals infected by SRLV. However, individual variations in immune response are observed and some animals may present low antibody titers, late seroconversion. In this study, among the 35 evaluated animals, comprised by sheep and goats, only five animals positive for SRLV infection in the AGID. Nevertheless, the proviral DNA of SRLV was detected in 27 animals with the nested-PCR. These findings demonstrate that the molecular technique was superior in detection capacity when compared to the AGID technique.

The results obtained corroborate the study carried out in breeding herds, in which animals known to be positive had negative results for AGID and positive for nPCR.<sup>40</sup> In this context, several other studies have successfully demonstrated the use of PCR technique to detect pro-viral DNA of SRLV,<sup>15-18,37,39</sup> considering that this technique presents high sensitivity collaborating for an early diagnosis.<sup>19</sup>

Barquero et al.<sup>10</sup> consider that there is no “gold standard” test for the diagnosis of lentiviruses and with the advances in molecular biology several PCR protocols have been developed. Therefore, it is difficult to use the same primers in different geographic regions due to the SRLV genome heterogeneity.

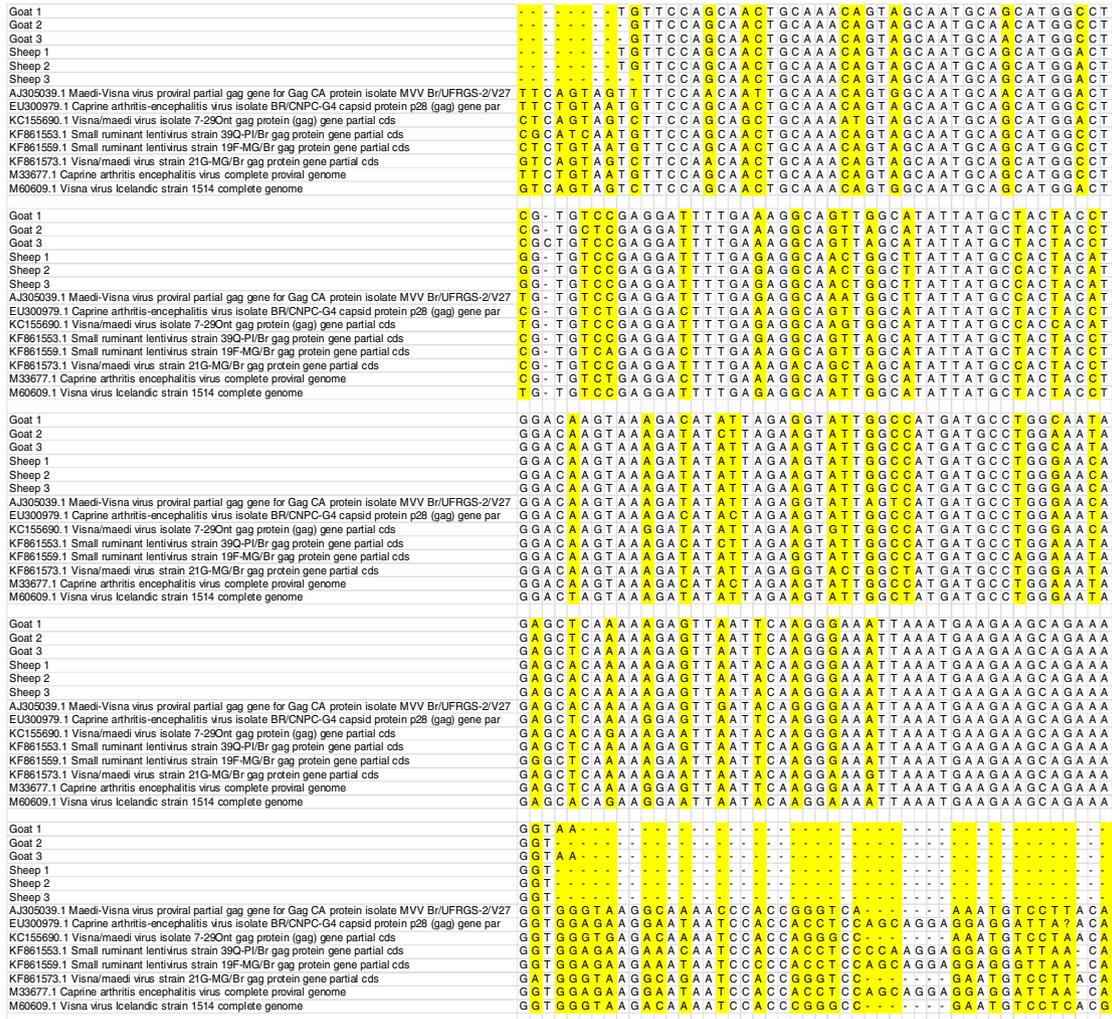
In most studies involving CAEV<sup>41,42</sup> and MVV<sup>37,43</sup>, this detection is performed using primers designed for the *gag* gene, since it has conserved sequences in different SRLV samples. However other regions are described that still have some degree of conservation and can be used to improve the sensitivity of the PCR technique, such as *pol* and *LTR*, allowing detection of a wide spectrum of CAEV and MVV field strains.<sup>10,44-46</sup>

The results of DNA sequence analysis demonstrated that the samples obtained from goats are close to the CAEV group, while the sheep samples are similar to those of MVV. In addition, through the analysis performed by the alignment of the sequenced samples, it was possible to verify the differences that occur in the viral genome, demonstrating the heterogeneity of the samples. Reinforcing the need for a more sensitive and specific test to detect a larger number of samples.

With the molecular tests, this study verified that the primers designed for the *gag* and *pol* regions presented high detection capacity for the circulating strains in the studied

**Table 3 – Similarity of the field strains with the strains used in the multiple sequencing and construction of the phylogenetic tree.**

		Goat 6	Goat 7	Goat 8
Identity	CAEV-Co39Q-PI/BrBR-CNPC-G4KF861559.1	95%97%95%95%	97%97%97%96%	95%97%95%95%
		Sheep 4	Sheep 5	Sheep 6
Identity	MVV-K15147-29OntBr/UFRGS-2/V2721G-MG/Br	93%94%94%91%	93%94%94%91%	93%94%94%91%



**Fig. 5 – Alignment of nucleotide sequences from the partial gag region of SRLV strains. The markings identify the regions where there are differences.**

region with samples from goats. However, the primers of the gag region are more efficient. Gregory et al.,<sup>45</sup> used *pol* primers to detect DNA pro-viral of CAEV in samples from lung, mammary gland, brain and synovial fluid, obtaining promising results.

For sheep samples in this study, *gag* and LTR primers were efficient in detecting positive animals. Several studies have described the use of PCR with primers LTR obtained good results.<sup>15,47</sup> However, other researchers have shown that *gag*-PCR was more sensitive than LTR-PCR for detection of SRLV in sheep and goats.<sup>16,43,48</sup>

In our study, a similar detection was observed between the detection values of positive sheep samples using *gag* and LTR primers. However, one sample was positive for LTR and was negative for *gag*. This demonstrates that the primers for the LTR region are more specific for viral detection of sheep. This finding corroborates with the results obtained by Glaria et al.<sup>46</sup> in which different tissue samples evaluated by *gag*-PCR and LTR-PCR were positive for LTR primers and negative for *gag* primers.

Similar results were obtained by Marinho et al.<sup>38</sup> which reported that the detection of sheep lentiviruses was much

more sensitive when performing nested-PCR with LTR primers than when using *gag* primers.

MVV infection was investigated in the same samples using two PCR tests directed to the LTR and *pol* sequences of the MVV gene. LTR-PCR was more sensitive and allowed to detect infection earlier than the other tests.<sup>49</sup> Similar results were observed in the present study in which the samples of sheep were better detected with LTR-PCR than when the same technique was done using *pol* primers.

From these data, we can conclude that nested-PCR performed with primers from the *pol* region was not efficient for the detection of sheep samples in this study. However, when used for the detection of goat samples, it demonstrated high detection capacity. The nested-PCR performed with the *gag* primers was able to detect some sheep samples, but not all of them, which could lead to a false negative result when the PCR is performed only with the *gag* primers. Concerning primers for the LTR region, these have been shown to be the most efficient for the detection of sheep samples.

Considering the importance of diagnosing etiological agents involved in diseases to avoid the spread among animals, it is necessary to establish a system of rapid and highly specific diagnosis for these pathogens. A method that is sensitive, specific and capable of detecting all the variants of the virus even if present in low concentration are fundamental prerequisites for the success of the control and eradication of lentiviruses.<sup>3</sup>

In this sense, duplex nested-PCR technique for the diagnosis of lentiviruses of small ruminants allowed for a more accurate diagnosis, considering that it is an amplification reaction designed to detect multiple target sequences in the same sample. This promotes the detection of lentiviruses both in sheep and goats, even in the case of cross-infection. Through phylogenetic analysis, it can be concluded that the duplex nested-PCR technique was able to detect CAEV and MVV samples in a single assay.

In veterinary medicine, multiplex PCR has been developed and applied for the simultaneous detection of different diseases in pigs,<sup>50</sup> cattle,<sup>51</sup> birds,<sup>52</sup> dogs<sup>53</sup> and equines<sup>54</sup> in a single assay.

As well as SRLV, equine herpesviruses are considered genetically and antigenically related, which promotes a difficulty in differentiating these pathogens through conventional serological tests.<sup>55</sup> A multiplex nested-PCR technique was developed and successfully applied for the simultaneous detection of equine herpesviruses in different clinical samples. The same result could be obtained in this study with the application of a duplex nested-PCR technique.

The critical factors of PCR reaction are the selection of primers, the concentration of magnesium chloride and the annealing temperature.<sup>56</sup> It was not necessary to change the magnesium concentration or the annealing temperature for performing the technique. However, for the duplex nested-PCR using *pol* and LTR primers, it was necessary adjustments in the mix to optimize the reaction. These alterations of the PCR mix for performing the duplex technique were also necessary in the study by Silva et al.<sup>57</sup>

The results obtained allowed us to conclude that the duplex nested-PCR is an important tool for a more accurate, sensitive and specific diagnosis of the different strains of SRLV that are

circulating in Brazil, allowing a decrease of false negatives and increasing the capacity of viral detection.

## Conflicts of interest

The authors declare no conflicts of interest.

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## REFERENCES

- Narayan O, Clements JE, Strandberg JD, Cork LC, Griffin DE. Biological characterization of the virus causing leukoencephalitis and arthritis in goats. *J Gen Virol*. 1980;50:69–79.
- Pasick J. Maedi-Visna virus and caprine arthritis-encephalitis virus: distinct species or quasiespecies and its implications for laboratory diagnosis. *Can J Vet Res*. 1998;62:241–244.
- Pepin M, Vitu C, Russo P, Mornex JF, Peterhans E. Maedi-Visna virus infection in sheep: a review. *Vet Res*. 1998;29:341–367.
- Shah C, Boni J, Huder JB, et al. Phylogenetic analysis and reclassification of caprine and ovine lentiviruses based on 104 new isolates: evidence for regular sheep-to-goat transmission and world-wide propagation through livestock trade. *Virology*. 2004;319:12–26.
- Reina R, Bertolotti L, Dei Giudici S, et al. Small ruminant lentivirus genotype E is widespread in Sarda goat. *Vet Microbiol*. 2010;144:24–31.
- Franke CR. *Controle sanitário da artrite-encefalite caprina*. Salvador: EDUFBA; 1998:70.
- Berton G, Blacklaws B. Small ruminant lentiviruses and crossspecies transmission. In: Desport M, ed. *Lentiviruses and Macrophages, Molecular and Cellular Interactions*. Norfolk, UK: Caister Academic Press; 2010:277–306.
- Valas S, Benoit C, Baudry C, Perrin G, Mamoun RZ. Variability and immunogenicity of caprine arthritis-encephalitis virus surface glycoprotein. *J Virol*. 2000;74:6178–6185.
- Leroux C, Cruz JCM, Mornex JF. SRLVs: a genetic continuum of lentiviral species in sheep and goats with cumulative evidence of cross species transmission. *Curr HIV Res*. 2010;8:94–100.
- Barquero N, Domenech A, Arjona A, Fernández-Garayzabal JF, Ruiz-Santa-Quiteria JA, Gomez-Lucia E. Comparison of two PCR and one ELISA techniques for the detection of small ruminant lentiviruses (SRLVs) in milk of sheep and goats. *Res Vet Sci*. 2013;94:817–819.

11. Tortora GJ, Funke BR, Case CL. *Microbiologia*. 5th ed. Porto Alegre: ArtMed; 2000:827.
12. OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Seventh Edition*; 2012.
13. Clavijo A, Thorsen J. Application of polymerase chain reaction for the diagnosis of caprine arthritis-encephalitis. *Small Rumin Res*. 1996;22:69-77.
14. Preziuso S, Taccini E, Rossi G, Renzoni G, Braga G. Experimental Maedi Visna virus infection in sheep: a morphological, immunohistochemical and PCR study after three years of infection. *Eur J Histochem*. 2003;47:373-378.
15. Zanoni RG, Nauta IM, Kuhnert P, Pauli U, Pohl B, Peterhans E. Genomic heterogeneity of small ruminant lentiviruses detected by PCR. *Vet Microbiol*. 1992;33(1-4):341-351.
16. Reddy PG, Sapp WJ, Heneine W. Detection of caprine arthritis encephalitis virus by polymerase chain reaction. *J Clin Microbiol*. 1993;31:3042-3043.
17. Rimstad E, East NE, Torten M, Higgins J, Derock E, Pedersen NC. Delayed seroconversion following naturally acquired caprine arthritis-encephalitis virus infection in goats. *Am J Vet Res*. 1993;54:1858-1862.
18. Wagter LH, Jansen A, Bleumink-Pluym NM, Lenstra JA, Houwers DJ. PCR detection of lentiviral gag segment DNA in the white blood cells of sheep and goats. *Vet Res Commun*. 1998;22:355-362.
19. Moojen V. Maedi-Visna dos ovinos. In: Riet-Corrêa F, ed. *Doenças de Ruminantes e Equinos*. vol. 1. São Paulo: Varela; 2001:138-144.
20. Reck C, Menin A, Canever MF, Miletta LC. Rapid detection of *Mycoplasma synoviae* and avian reovirus in clinical samples of poultry using multiplex PCR. *Avian Dis*. 2013;57:220-224.
21. Cork LC, Hadlow WJ, Crawford TB, Gorham JR, Piper RC. Infectious leukoencephalomyelitis of young goats. *J Infect Dis*. 1974;129(2):134-141.
22. Sigurdsson B. Maedi, a slow progressive pneumonia of sheep: an epizootological and pathological study. *Br Vet J*. 1954;110:255-270.
23. Caldas APF, Leal ES, Avila EF, Ravazzolo AP. Detection of feline immunodeficiency provirus in domestic cats by polymerase chain reaction. *Pesq Vet Bras*. 2000;20:20-25.
24. Saltarelli M, Querat G, Konings DA, Vigne R, Clements JE. Nucleotide sequence and transcriptional analysis of molecular clones of CAEV which generate infectious virus. *Virology*. 1990;179:347-364.
25. Ryan S, Tiley L, McConnell I, Blacklaws B. Infection of dendritic cells by the Maedi-Visna lentivirus. *J Virol*. 2000;74:10096-10103.
26. Barlough J, East N, Rowe JD, et al. Double-nested polymerase chain reaction for detection of caprine arthritis encephalitis virus proviral DNA in blood, milk and tissues of infected goats. *J Virol Methods*. 1994;50:101-114.
27. Andrioli A, Gouveia AMG, Martins AS, Pinheiro RR, Santos DO. Risk factors in caprine lentivirus transmission through semen. *Pesq Agropec Bras*. 2006;41:1313-1319.
28. Sonigo P, Alizon M, Staskus K, et al. Nucleotide sequence of the Visna lentivirus: relationship to the AIDS virus. *Cell*. 1985;42:369-382.
29. Santry LA, de Jong J, Gold AC, Walsh SR, Menzies PI, Wootton SK. Genetic characterization of small ruminant lentiviruses circulating in naturally infected sheep and goats in Ontario, Canada. *Virus Res*. 2013;175:30-44.
30. Ravazzolo AP, Reischak D, Peterhans E, Zanoni R. Phylogenetic analysis of small ruminant lentiviruses from Southern Brazil. *Virus Res*. 2001;79:117-123.
31. Braz GF, Valas S, Heinemann MB, Pimenta dos Reis JK, Leite RC, Castro RS. *Plylogenetic analyses of Brazilian SRLV*. Unpublished. Submitted. Av. Antonio Carlos, 6627, Belo Horizonte, Minas Gerais 30161970, Brasil: Medicina Veterinaria Preventiva, Escola de Veterinaria – Universidade Federal de Minas Gerais; 16 Nov 2013.
32. Feitosa ALVL, Teixeira MFS, Pinheiro RR, et al. Phylogenetic analysis of small ruminant lentiviruses from Northern Brazil. *Small Rumin Res*. 2010;94:205-209.
33. Zhang Z, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences. *J Comput Biol*. 2000;7:203-214.
34. Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schäffer AA. Database indexing for production MegaBLAST searches. *Bioinformatics*. 2008;24:1757-1764.
35. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*. 1987;4:406-425.
36. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*. 1993;10:512-526.
37. Oguma K, Tanaka C, Harasawa R, et al. Isolation of Maedi/Visna virus from a sheep in Japan. *J Vet Med Sci*. 2013;76:211-218.
38. Marinho RC, Martins GR, Souza KC, Bezerra Júnior RQ, Teixeira MFS. Detection of Maedi-Visna Virus from sheep bronchoalveolar lavage by nested PCR evaluation of different primers pairs. *Acta Sci Vet*. 2016;44:1368-1373.
39. Sánchez JH, Martínez HA, García MM, et al. The presence of small ruminant lentiviruses in Mexican Pelibuey sheep. *Theriogenology*. 2016;86:1953-1957.
40. Cruz JCM. *Monitoramento sorológico e da presença do DNA pró-viral do lentivirus caprino (CAEV) no sangue e sêmen de reprodutores infectados*. 34fls. Tese – Universidade Federal de Minas Gerais, Escola de Veterinária; 2009.
41. Zanoni R, Pauli U, Peterhans E. Caprine arthritis-encephalitis (CAE) and Maedi-Visna viruses detected by the polymerase chain reaction (PCR). *Vet Microbiol*. 1990;23:329-330.
42. Souza KC, Andrioli A, Sider LH, et al. Detection of proviral sequences of the caprine arthritis encephalitis virus in saliva. *Acta Sci Vet*. 2015;43:1266-1271.
43. Martins GR, Teixeira MFS, Barroso IC, Souza KC, Marinho RC, Beserra RQ. Detection of Maedi Visna virus in bronchoalveolar lavage of sheep lungs. *Rev Bras Med Vet*. 2014;36:312-316.
44. Zanoni RG. Phylogenetic analysis of small ruminant lentiviruses. *J Gen Virol*. 1998;79:1951-1961.
45. Gregory L, Lara MCCSH, Hasegawa MY, et al. Caprine arthritis encephalitis virus detected by PCR and nested-PCR in milk. *Med Vet*. 2011;5:7-11.
46. Glaria IR, Reina H, Ramirez X, et al. Visna/Maedi virus genetic characterization and serological diagnosis of infection in sheep from a neurological outbreak. *Vet Microbiol*. 2012;155:137-146.
47. Extramiana AB, Gonzalez L, Cortabarría N, Garcia M, Juste RA. Evaluation of a PCR technique for the detection of Maedi-Visna proviral DNA in blood, milk and tissue samples of naturally infected sheep. *Small Rumin Res*. 2002;44:109-118.
48. Brinkhof JM, van Maanen C, Wigger R, Peterson K, Houwers DJ. 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. *J Virol Methods*. 2008;147:338-344.
49. A'lvarez V, Daltabuit-Test M, Arranz J, et al. PCR detection of colostrum-associated Maedi-Visna virus (MVV) infection and relationship with ELISA-antibody status in lambs. *Res Vet Sci*. 2006;80:226-234.
50. Hu L, Lin XY, Yang ZX, et al. A multiplex PCR for simultaneous detection of classical swine fever virus African swine fever virus, highly pathogenic porcine reproductive and respiratory syndrome virus, porcine reproductive and respiratory syndrome virus and pseudorabies in swines. *Pol J Vet Sci*. 2015;18:715-723.

51. Mari V, Losurdo M, Lucente MS, et al. Multiplex real-time RT-PCR assay for bovine viral diarrhea virus type 1, type 2 and HoBi-like pestivirus. *J Virol Methods*. 2016;229:1-7.
52. Zeng T, Xie Z, Xie L, et al. Simultaneous detection of eight immunosuppressive chicken viruses using a GeXP analyser-based multiplex PCR assay. *Virology*. 2015;12:226-237.
53. Han JI, Chang DW, Na KJ. A multiplex quantitative real-time polymerase chain reaction panel for detecting neurologic pathogens in dogs with meningoencephalitis. *J Vet Sci*. 2015;16:341-347.
54. Wang L, Raidal SL, Pizzirani A, Wilcox GE. Detection of respiratory herpesviruses in foals and adult horses determined by nested multiplex PCR. *Vet Microbiol*. 2007;121:18-28.
55. Carvalho R, Passos LMF, Martins AS. Development of a differential multiplex PCR assay for equine herpesvirus 1 and 4 as a diagnostic tool. *J Vet Med B*. 2000;47: 351-359.
56. Brent R, Kingston RE, Seidman JG, et al. *Current protocols in molecular biology*. New York: John Wiley & Sons; 1994:1600.
57. Silva MA. *Utilização de PCR multiplex para o diagnóstico etiológico da mastite bovina*. Dissertação (mestrado) – Universidade Federal de Minas Gerais, Escola de Veterinária; 2008, 32 p.