Heliyon

Detection of *Sarcocystis aucheniae* in blood of llama using a duplex semi-nested PCR assay and its association with cyst infestation

Cecilia Decker Franco [a,b], **Sandra Romero** [c], **Alejandro Ferrari** [b,d],
Leonhard Schnittger [a,b], **Monica Florin-Christensen** [a,b,*]

[a] *Instituto de Patobiología Veterinaria, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas (CICVyA), Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina*

[b] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina*

[c] *Instituto de Investigación y Desarrollo Tecnológico para la Agricultura Familiar Región NOA, Argentina*

[d] *Instituto de Estudios de la Inmunidad Humoral (IDEHU, CONICET-Universidad de Buenos Aires, UBA), Argentina*

[*] Corresponding author.
E-mail address: jacobsen.monica@inta.gob.ar (M. Florin-Christensen).

Abstract

The protozoon *Sarcocystis aucheniae* is the causative agent of South American camelid (SAC) sarcocystosis. Infections are characterized by the presence of cysts in muscles which are in size and appearance similar to rice grains. As consumption of insufficiently cooked infected meat produces gastroenteritis, cyst-containing SAC meat is confiscated by sanitary authorities or depreciated with serious economic consequences for SAC breeders. In this work, a duplex semi-nested PCR was designed to simultaneously detect parasite and llama DNA in host blood samples. Species-specific regions of *S. aucheniae* 18S rRNA gene and *Lama glama* 16S mitochondrial gene were amplified, yielding bands of 583 and 257 bp, respectively, and separated by gel electrophoresis. The method proved to be highly sensitive, with a detection limit lower than one parasite per milliliter blood, and the inclusion of primers to detect llama-specific DNA resulted useful as a methodological control. Blood samples collected from llamas of Argentina and Bolivia (n = 225) were

analyzed using this method, and 18.7 % resulted positive for *S. aucheniae*. No correlation was found between PCR results and llama age, sex or the finding of macroscopic cysts in meat after slaughter. Lack of molecular detection in the blood of some llamas harboring macrocysts suggests that parasite circulation in the bloodstream after encystment is under the detection threshold of the test or even absent, while PCR positive results in cyst-infested animals suggests that prior exposure to the parasite does not impede subsequent infections. The described method can be useful to detect active foci of infection, to assess the effectiveness of parasiticide treatments, and for the surveillance and tracing of definitive hosts.

Keywords: Microbiology, Molecular biology, Bioinformatics

1. Introduction

The domestic South American camelids (SACs) llama and alpaca have adapted to the extreme environmental conditions, such as high altitude, lack of water, steep slopes, and frost, present in the Andean regions of Argentina, Bolivia, Peru, Chile, and Ecuador (Wheeler, 1991). Domestic SACs are part of the life strategy of Andean rural communities, since they are used for transportation, and are a source of manure as fertilizer and fuel, wool for the manufacturing of clothing, and meat as a major protein source (Leguía, 1991; Vilca, 1991). Due to its reduced cholesterol content in comparison with beef, SAC meat is particularly attractive for the local and international gourmet cuisine, and constitutes a source of income, with high growth potential, for small and medium-scale producers (Leguía and Casas, 1999; Vilca, 1991; Mamani-Linares and Gallo, 2014).

In general, SAC breeding is carried out under traditional management procedures and, often, poor sanitary conditions. One of the limitations for the production and commercialization of SAC meat is the frequent finding of macroscopic cysts (1−5 mm) resembling rice grains in skeletal muscles (Decker Franco et al., 2018; Moré et al., 2016; Saeed et al., 2018). Cysts essentially consist of encapsulated conglomerates of up to 2×10^7 protozoan parasites of the *Sarcocystis aucheniae* species, and have a brain-like structure when examined under electron microscopy (Carletti et al., 2013; Decker Franco et al., 2018; Moré et al., 2016). Cysts are usually very abundant and give an unpleasant aspect to SAC meat, which leads to confiscation of carcasses by sanitary authorities or devaluation of their commercial value. Importantly, human consumption of infected raw or undercooked meat can produce gastroenteritis with nausea, diarrhea, cramps, and chills (Leguía, 1991; Saeed et al., 2018).

Most aspects of *S. aucheniae* cycle are yet to be elucidated, but can be inferred from studies carried out in other *Sarcocystis* species (Dubey et al., 2015; Decker Franco et al., 2018; Saeed et al., 2018). These coccidians complete their life cycle in an

intermediate herbivore and a carnivore definitive host, in which, respectively, the asexual and sexual reproduction phases take place. The intermediate host — a SAC in the case of *S. aucheniae*— becomes infected by ingesting sporulated oocysts or sporocysts from the environment. After uptake, spororozoites are liberated in the host intestine, penetrate endothelial cells of blood vessels and undergo an unknown number of schizogony cycles. Eventually, merozoites bud out of schizonts and migrate through the blood. Merozoites use apicomplexan-specific invasion mechanisms to penetrate myocytes with the formation of a parasitophorous vacuole. Several cycles of asexual multiplication produce millions of bradyzoites that become surrounded by a proteinaceous wall forming a cyst. The definitive host becomes infected when consuming raw cyst-infested meat. In its digestive system, parasites are released from the cysts, invade intestinal epithelial cells and, through gametogony, develop into gametes that fuse to form a zygote. Zygotes develop into sporocyst-containing oocysts which are released with the feces into the environment, contaminating food and water. Dogs are the most prominent potential definitive hosts of *S. aucheniae*, as based on some experimental infection studies (Cornejo et al., 2007; Choque et al., 2007). However, further investigations using molecular tools are needed to substantiate this notion. Additionally, other carnivores might participate in the life cycle of *S. aucheniae*, and play a significant role in its epidemiology.

In a previous study, we showed for the first time that the transit of *S. aucheniae* through the SAC bloodstream can be detected by molecular methods (Martín et al., 2016). The present work was aimed at improving the molecular detection of *S. aucheniae* and applying it to llama samples of Argentina and Bolivia to estimate the field prevalence and risk factors, as well as studying the possible usefulness of this test for pre-slaughter diagnosis to predict the presence of cysts in llama meat.

2. Materials and methods

2.1. Isolation of cysts and quantification of parasites

Macroscopic cysts were isolated by visual examination of cervical muscles of slaughtered llamas from the Andean flatlands of Argentina and Bolivia. Cysts measuring ∼3−5 mm long were separated from llama tissues with sterilized tweezers and washed with PBS. Bradyzoites were released after cutting cysts in half with a scalpel, diluted 1:200 with PBS, and quantified by microscopic observation ($100 \times$) using a Neubauer chamber. Each cyst contained between 1.5 to 2×10^7 parasites, consistent with observations of previous studies (Carletti et al., 2013).

2.2. Samples and DNA extraction

Blood samples (2−5 ml) were aseptically collected from the jugular vein of llamas into Vacutainer tubes containing 3.8% sodium citrate (Becton Dickinson). Llamas

belonged to small and medium producers from different localities of the provinces of Jujuy (n = 121), La Pampa (n = 54) and Catamarca (n = 37), in Argentina; and the locality of Turco-Oruro, in Bolivia (n = 13). Whenever possible, the sex and age of animals were documented. None of the sampled animals presented any clinical signs. In a group of 80 llamas of the province of Jujuy, blood samples were removed before slaughter, and the presence of cysts was determined by visual inspection of 10 cm^2 patches in the regions of the neck and intercostal muscles after slaughter. The examined regions were chosen because of their reliability to determine cyst infestation (Rooney et al., 2014; Moré et al., 2016). Animals were not euthanised explicitly for the purposes of this research, and sample collection followed accepted animal welfare guidelines.

Samples were kept at -20 °C until use. DNA was extracted from 200 µL of each blood sample using the DNA Puriprep-S kit (Inbio Highway, Argentina). DNA from *S. aucheniae* cysts and muscle samples from *S. aucheniae*-negative llamas was extracted using the DNA Puriprep-T kit (Inbio Highway, Argentina). In all cases, DNA was eluted using 200 µL elution buffer and kept at -20 °C until further use.

2.3. Design of the duplex semi-nested PCR primers

The 18S rRNA gene sequence of an *S. aucheniae* isolate (GenBank accession number AF017123) was aligned with orthologs of *Toxoplasma gondii* and *Neospora caninum* (GenBank Accession numbers, L24381 and L24380, respectively), two other coccidian protozoa that also infect llama (Moré et al., 2008), using MUSCLE (https://www.ebi. ac.uk/Tools/msa/muscle/). Two forward primers were designed to bind to DNA regions common to the three coccidians (cocc18S-F1: 5′-GAAAGTTAGGGGCTCGAAGA-3′, and cocc18S-F2: 5′- GACGGAAGGGCAC CACCAGG-3′). In contrast, the reverse primer, Sauch-R (5′-CCAATCCATACTTG GAAAAACGG-3′), was designed to bind to a *S. aucheniae*-specific region (Fig. 1). Primers cocc18S-F1 and Sauch-R, and cocc18S-F2 and Sauch-R resulted in generation of fragments of 730 and 583 bp, respectively. On the other hand, primers (Lg_16sRNA_F: AAGGAACTCGGCAAACACGA and Lg_16sRNA_R:
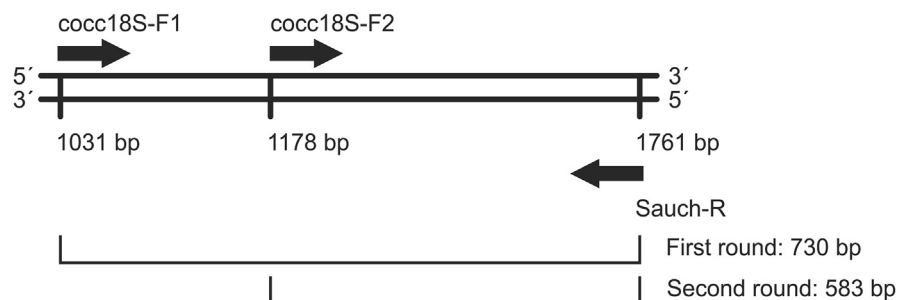

Fig. 1. General scheme of the semi-nested PCR for the amplification of the hypervariable region of *S. aucheniae* 18S rRNA gene.

ATTTGTTCATCCCCGCCTCT) target specifically the 16S mitochondrial gene of *Lama glama* (GenBank Accession number AY011176.1), amplifying a 257 bp DNA region. Primer specificity and lack of loop formation was verified using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and Oligoanalyzer software (www.idtdna.com/analyzer/applications/oligoanalyzer/), respectively.

2.4. Duplex semi-nested PCR

The amplification of a hypervariable region of the *S. aucheniae* 18S rRNA gene was performed using two consecutive PCR rounds. Both reactions were carried out in a final volume of 13 μL, containing 0.4 μM of each *S. aucheniae* primer and 0.1 μM of each *L. glama* primer, 0.2 mM of each dNTP (Thermo Fisher Scientific), 0.4 U Go-Taq polymerase and its corresponding buffer (Promega, Madison, USA), and as template, 1 μL genomic DNA sample described in section 2.2 in the first round, or the amplicon of the first round for secondary amplification. The primers used in the first round were: cocc18S-F1, Sauch-R, Lg_16sRNA_F, and Lg_16sRNA_R, and in the second round: cocc18S-F2, Sauch-R, Lg_16sRNA_F, and Lg_16sRNA_R. The cycling conditions used were as follows: denaturing at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 62 °C or 63 °C for 30 s, for the first and second rounds, respectively, 72 °C for 30 s, and a final extension period of 5 min at 72 °C. Genomic DNA extracted from a piece of llama muscle or from a *S. aucheniae* cyst was used as positive control for llama or parasite DNA, respectively. A negative control containing no template was run in parallel in both rounds to rule out amplicon contamination. PCR products were separated by horizontal electrophoresis (80 V, 30 min) in ethidium bromide-containing 1.5 % agarose gels, in TAE buffer (40 mM Tris-acetate,/1 mM EDTA, pH 8). A 1 Kb Plus DNA ladder (Life Technologies) was included. Products were observed in a UV transilluminator, and images were documented using an Uvitec gel documentation equipment.

For the sensitivity test, 100 μL aliquots of *S. aucheniae*-negative llama blood were spiked with known amounts of *S. aucheniae* bradyzoites (2×10^6 to 0), obtained as described in 2.1. DNA was extracted from each blood sample as described in 2.2, and subjected to molecular diagnosis as described above.

2.5. Sequence analysis

PCR products amplified from DNA of three field llama blood samples originating from Bolivia, Catamarca, and Jujuy were purified from agarose gels using GFX PCR DNA and gel band purification kit (GE Healthcare), quantified by Nanodrop spectrophotometric measurements, and sequenced using a 3730XL equipment (Applied Biosystems) at the Sequencing Service of Macrogen, South Korea (http://dna.macrogen.com/eng). After alignment, sequences were edited using the

BioEdit software and deposited in the GenBank (provisional accession numbers MG832003, MG832004 and MG832005). Sequences were used as a query to find significant hits using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and a global sequence alignment between the three obtained sequences, and the corresponding fragments of six other *S. aucheniae* 18S rRNA sequences deposited in the Genbank (KF383266, KF383267, KF383268, KT382799, KU527117 and AF017123) was carried out by MUSCLE. Percentages of sequence identity with respect to sequences obtained after semi-nested PCR amplification were calculated with NEEDLE (https://www.ebi.ac.uk/Tools/psa/emboss_needle/).

2.6. Statistical analysis

The significance of the differences in positive detection associated with age, sex, and origin was analyzed by Chi Square test. The correlation between positive and negative llamas as diagnosed by finding of cysts in carcasses and PCR was analyzed using the "Diagnostic test evaluation calculator" (https://www.medcalc.org/calc/diagnostic_test.php), considering cyst-positive and negative animals as true positives and true negatives, respectively.

3. Results

A semi-nested PCR based on the amplification of a fragment of the hypervariable region of the 18S rRNA gene was set up for the molecular diagnosis of *S. aucheniae* infections. A *S. aucheniae*-specific reverse primer (Sauch-R) was used in both amplification rounds (Fig. 1). The method was first applied to genomic DNA isolated from macrocysts and then, from blood of llamas. As shown in Fig. 2, a single amplicon of about 550 bp was obtained as a result of this semi-nested protocol,
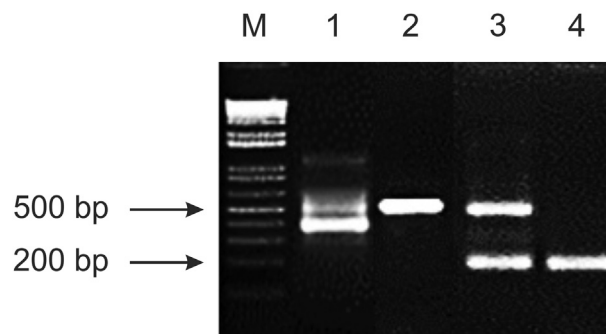

Fig. 2. Molecular detection of *S. aucheniae* DNA by semi-nested PCR. Fragments of *S. aucheniae* 18S rRNA gene were amplified by semi-nested PCR from DNA samples extracted from a *S. aucheniae* macrocyst using the protocol described in Martín et al. (2016) (lane 1, amplicon size ~400 bp) or in the present work (lane 2, amplicon size ~550 bp). A duplex format of this assay was set up, including primers to amplify a ~257 bp fragment of *Lama glama* DNA isolated from blood of llama. Lanes 3 and 4 show a positive and a negative result, respectively. M: 1 kb Plus DNA marker.

consistent with an expected size of 583 bp, when DNA from either *S. aucheniae*-macrocysts or infected llama blood was used as template (Fig. 2, lanes 2 and 3). This novel protocol constitutes a significant improvement with respect to a semi-nested PCR scheme previously reported by our group, in which several scattered bands were obtained upon amplification (Fig. 2, lane 1) (Martín et al., 2016). Primers that targeted a specific fragment of 257 bp of the llama mitochondrial 16S rRNA gene were included as quality control of the analyzed samples in both PCR rounds. Accordingly, two bands, of ∼550 and 250 bp or a single band of ∼250 bp were obtained when *S. aucheniae*-positive or negative llama blood samples were analyzed, respectively (Fig. 2, lanes 3 and 4).

The sensitivity of the semi-nested PCR was evaluated in DNA samples extracted from aliquots of *S. aucheniae*-negative blood spiked with different amounts of *S. aucheniae* bradyzoites. Detection was achieved until a parasitemia of 0.5 bradyzoites/ml blood. The same sensitivity limit was observed with or without inclusion of primers specific for llama DNA (results not shown). This method showed a 200-fold higher sensitivity than that described in our previous work (Martín et al., 2016).

After standardization of the method, field samples from four different geographic regions belonging to Argentina and Bolivia were analyzed. Out of 225 llama blood samples, 18.7 % were positive for *S. aucheniae* DNA detection. Amplification products of the second PCR round corresponding to three samples from Bolivia, Catamarca, and Jujuy were each sequenced (GenBank Accession numbers MG832003, MG832004, MG832005). Sequence alignments showed that these three sequences were identical. Maximal BLAST hits were obtained for the 18S rRNA gene of *S. aucheniae* (KU527117), followed by other *S. aucheniae* 18S rRNA sequences. A global alignment between MG832003 and other deposited *S. aucheniae* 18S rRNA sequences in the GenBank is shown in Fig. 3. Maximal percentages of identity (98.0 %) were obtained between sequences KU527117 and KT382799, corresponding to *S. aucheniae* cysts obtained from a llama of Jujuy, and a guanaco of Santa Cruz, Argentina, respectively (Regensburger et al., 2015; Moré et al., 2016), followed by AF017123 (96.7 %), corresponding to a cyst found in an alpaca of Australia (Holmdahl et al., 1998), and KF383267 (96.7%), KF383268 (96.3 %) and KF383266 (96 %), the latter three corresponding to cysts from llamas of Jujuy, Argentina (Carletti et al., 2013). These results suggest that the observed genetic variability in 18S rRNA sequences is not associated to host species or geographic origin of isolates. Further studies with other molecular markers are needed to gain further insight into the genetic diversity of this parasite.

The average detection of the parasite was similar (22−23 %) between the geographic locations of Catamarca, Jujuy and Turco (Table 1). In contrast, the percentage of positive samples of La Pampa was considerably lower (9.3 %) than the percentages

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MG832003      GGCTT-ATTTGACTC-ACACGGG-AAACTCACCAGGTCCAGACATAGGAAGGATTGACAG
KF383266.1    GGCTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATTGACAG
KT382799      GGCTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATTGACAG
KU527117.1    GGCTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATTGACAG
AF017123.1    GGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACATGGGAAGGATTGACAG
KF383267.1    GGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACATGGGAAGGATTGACAG
KF383268.1    GGCTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATAGACAG
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MG832003      ATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAG
KF383266.1    ATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAG
KT382799      ATTGATAGCTCTTTCTTGATTCTATGGGTAGTGGTGCATGGCCGTTCTTAGTTGGTGGAG
KU527117.1    ATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAG
AF017123.1    ATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAG
KF383267.1    ATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAG
KF383268.1    AGTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAG
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MG832003      TGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAAATAGGATTTCAA
KF383266.1    TGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAAATAGGA-TTCAA
KT382799      TGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAAATAGGATTTCAA
KU527117.1    TGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAAATAGGATTTCAA
AF017123.1    TGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAAATAGGA--TCAA
KF383267.1    TGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAAATAGGA--TCAA
KF383268.1    TGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAAATAGGA--TCAA
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MG832003      GAACATTACTACTTTCGGGTAGGTTTTTGTATCACTTCTTAGAGGGACTTTGCGTGTCTA
KF383266.1    GAACACTACTACTTTCGGGTAGGTTTTTGTATCACTTCTTAGAGGGACTTTGCGTGTCTA
KT382799      GAACATTACTACTTTCGGGTAGGTTTTTGTATCACTTCTTAGAGGGACTTTGCGTGTCTA
KU527117.1    GAACATTACTACTTTCGGGTAGGTTTTTGTATCACTTTTTAGAGGGACTTTGCGTGTCTA
AF017123.1    GAACATTACTACTTTCGGGTAGGTTTTTGTATCACTTCTTAGAGGGACTTTGCGTGTCTA
KF383267.1    GAACATTACTACTTTCGGGTAGGTTTTTGTATCACTTCTTAGAGGGACTTTGCGTGTCTA
KF383268.1    GAACATTACTACTTTCGGGTAGGTTTTTGTATCACTTCTTAGAGGGACTTTGCGTGTCTA
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MG832003      ACGCAAGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCTGCAC
KF383266.1    ACGCACGGAAGTTTGAGGCAATAACACGTCTGTGATGCCCTTAGATGTTCTGGGCTGCAC
KT382799      ACGCAAGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCTGCAC
KU527117.1    ACGCAAGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCTGCAC
AF017123.1    ACGCAAGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCTGCAC
KF383267.1    ACGCAAGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCTGCAC
KF383268.1    ACGCAAGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCTGCAC
              ***** *************************** ***********************

MG832003      GCGCGCTACACTGATGCATCCAACAAGTTGTTCAAAACCTTGGCCGAGAGGTTTAGGTAA
KF383266.1    GCGCGCTACACTGATGCATCCAACAAGTTGTTCAAAACCCTGGCCGAGAGGTTTAGGTAA
KT382799      GCGCGCTACACTGATGCATCCAACAAGTTGTTCAATACCTTGGCCGAGAGGTTTAGGTAA
KU527117.1    GCGCGCTACACTGATGCATCCAACAAGTTGTTCAATACCTTGGCCGAGAGGTTTAGGTAA
AF017123.1    GCGCGCTACACTGATGCATCCAACAAGTTGTTCAAAACCTTGGCCGAGAGGTTTAGGTAA
KF383267.1    GCGCGCTACACTGATGCATCCAACAAGTTGTTCAAAACCTTGGCCGAGAGGTTTAGGTAA
KF383268.1    GCGCGCTACACTGATGCATCCAACAAGTTGTTCAAAACCCTGGCCGAGAGGTTTAGGTAA
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MG832003      TCTTGTGAGTATGCGTCGTCAT-CGTGATGGGGATAGATTATTGCAATTATTAATCTTTA
KF383266.1    TCTTGTGAGTATG------CATACGTGATGGGGATAGATTATTGCAATTATTAATCTTTA
KT382799      TCTTGTGAGTATGCGTCGTCAT-CGTGATGGGGATAGATTATTGCAATTATTAATCTTTA
KU527117.1    TCTTGTGAGTATGCGTCGTCAT-CGTGATGGGGATAGATTATTGCAATTATTAATCTTTA
AF017123.1    TCTTGTGAGTATG------CAT-CGTGATGGGGATAGATTATTGCAATTATTAATCTTTA
KF383267.1    TCTTGTGAGTATG------CAT-CGTGATGGGGATAGATTATTGCAATTATTAATCTTTA
KF383268.1    TCTTGTGAGTATG------CAT-CGTGATGGGGATAGATTATTGCAATTATTAATCTTTA
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MG832003      ACGAGGAATGCCTACTAGGCGCAAGTCAGCAGCTTGTGCCGATTACGTCCCTGCCCTTTG
KF383266.1    ACGAGGAATGCCTAGTAGGCGCAAGTCAGCAGCTTGTGCCGATTACGTCCCTGCCCCTTG
KT382799      ACGAGGAATGCCTAGTAGGCGCAAGTCAGCAGCTTGTGCCGATTACGTCCCTGCCCTTTG
KU527117.1    ACGAGGAATGCCTAGTAGGCGCAAGTCAGCAGCTTGTGCCGATTACGTCCCTGCCCCTTG
AF017123.1    ACGAGGAATGCCTAGTAGGCGCAAGTCAGCAGCTTGTGCCGATTACGTCCCTGCCCCTTG
KF383267.1    ACGAGGAATGCCTAGTAGGCGCAAGTCAGCAGCTTGTGCCGATTACGTCCCTGCCCTTTG
KF383268.1    ACGAGGAATGCCTAGTAGGCGCAAGTCAGCAGCTTGTGCCGATTACGTCCCTGCCCTTTG
              ************** *************************************** ***

MG832003      TACACACCGCCCGTCGCTCCTACCGACTGAGTGTTCCAGTGAATTATTCGGACTGCTCCC
KF383266.1    TACACACCGCCCGTCGCTCCTACCGATTGAGTGTTCCGGTGAATTATTCGGACTGTTCCG
KT382799      TACACACCGCCCGTCGCTCCTACCGATTGAGTGTTCCGGTGAATTATTCGGACTGTTCCG
KU527117.1    TACACACCGCCCGTCGCTCCTACCGATTGAGTGTTCCGGTGAATTATTCGGACTGTTCCG
AF017123.1    TACACACCGCCCGTCGCTCCTACCGATTGAGTGTTCCGGTGAATTATTCGGACTGTTCCG
KF383267.1    TACACACCGCCCGTCGCTCCTACCGATTGAGTGTTCCGGTGAATTATTCGGACTGTTCCG
KF383268.1    TACACACCGCCCGTCGCTCCTACCGATTGAGTGTTCCGGTGAATTATTCGGACTGTTCCG
              ************************** ********** *************** ***

MG832003      CTTTT
KF383266.1    TTTTT (96.0%)
KT382799      TTTTT (98.0%)
KU527117.1    TTTTT (98.0%)
AF017123.1    TTTTT (96.7%)
KF383267.1    TTTTT (96.7%)
KF383268.1    TTTTT (96.3%)
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Table 1. Molecular detection of *S. aucheniae* infection in llamas (n = 225) of Argentina and Bolivia by duplex semi-nested PCR.

Factor		Animals	Positive	
		(n)	(n)	(%)
Age	<1 year	17	2	11.8
	1−3 years	90	16	17.8
	>3 years	118	24	20.3
Sex	Female	123	24	19.5
	Male	102	18	17.6
Origin	Jujuy	121	26	21.5
	Catamarca	37	8	21.6
	La Pampa	54	5	9.3
	Turco	13	3	23.1

found in the other studied regions, although this difference was not statistically significant.

Blood samples (n = 80) were collected from a group of llamas previous to their slaughter at an abattoir in Jujuy. After slaughter, the neck and intercostal muscles were analyzed for the presence of cysts. Table 2 shows the results of the molecular detection of the parasite in blood and detection of cysts in the carcasses. Considering llamas in which cysts have been visually observed or were not found as true positives or true negatives, respectively, the sensitivity of the semi-nested PCR was determined as 27% and its specificity 75%.

No significant difference was found between the sex or age of the animals and positive detection by PCR when the samples originating from the abattoir (n = 80), or when all samples (n = 225) were considered. However, finding of cysts in carcasses upon slaughter was significantly higher (p < 0.01) in female llamas and in animals of both genders older than 3 years of age (Table 3).

4. Discussion

In a previous work (Martín et al., 2016), we could demonstrate that *S. aucheniae* DNA can be detected in llama blood by semi-nested PCR. In the present work we significantly improved the limit of detection, resulting in a 200-fold higher

Fig. 3. Alignment of *S. aucheniae* 18S rRNA sequences obtained from llama blood and cysts from llama, guanaco and alpaca. Sequences were truncated to correspond in size to the amplicon as obtained after semi-nested PCR amplification of DNA extracted from llama blood (MG832003). The determined sequence MG832003 was aligned with KT382799 (nt 1233 to 1775) from a cyst found in a guanaco of Santa Cruz, Argentina, AF017123K (nt 1233 to 1768), from a cyst of an alpaca from Australia, and KF383266 (nt 1237 to 1774), KF383267 (nt 1238 to 1773), KF383268 (nt 1235 to 1771), and KU527117 (nt 1235 to 1779) obtained from cysts found in llamas of Jujuy, Argentina. Identity percentages with respect to MG832003 are shown at the end of the alignment.

Table 2. Comparison of the detection of *S. aucheniae* infection in llamas by molecular (PCR).

PCR	Cysts	
	Negative	**Positive**
Negative	21	38
Positive	7	14

Table 3. Visual and molecular detection of *S. aucheniae* infection in 80 llamas after slaughter.

Factor		Animals	Positive visual inspection		PCR-positive	
		(n)	**(n)**	**(%)**	**(n)**	**(%)**
Age	1–3 years	32	10	31.3[a]	7	21.9
	>3 years	48	42	87.5[a]	14	29.2
Sex	Female	42	36	85.7[b]	10	23.8
	Male	38	16	42.1[b]	10	26.3

[a, b] Significant at $p < 0.001$.

sensitivity (0.5 *vs.* 100 parasites/ml blood); and a significant improvement in the specificity of the assay was also achieved (one *vs.* several bands). In addition, inclusion of llama-specific primers that avoid false negative PCR results showed to be useful as a methodological control.

As shown in Table 2, a considerable number of cyst-infested llamas could not be detected by this PCR, demonstrating that this diagnostic tool does not reliably predict the presence of cysts. However, it can be suitable to identify active foci of SAC sarcocystosis transmission, to evaluate the effectiveness of measures of parasite control on the incidence of sarcocystosis, to study the time course of the infection, and to identify the range of definitive hosts that may contribute to disseminate the parasite into the environment.

Lack of PCR parasite detection in animals that contain cysts suggests that parasite circulation in the bloodstream is absent or below the detection threshold of this assay after encystment. On the other hand, molecular detection of parasites in the blood of llamas harboring cysts is consistent with the notion that a previous parasite exposure does not prevent recurrent infection.

Importantly, the PCR diagnostic test showed a relatively high specificity, i.e. most of the animals that had no cysts, also tested negative by PCR. Although it was not possible to collect information on the breeding type of sampled animals, it may be speculated that cyst-negative, PCR-negative animals were bred under sanitary conditions that do not favor *S. aucheniae* infection. Accordingly, in a previous study we

have observed that poor sanitary conditions, itinerant grazing and/or exposure to pastor dogs are risk factors that are associated with a higher seroprevalence of SAC sarcocystosis (Romero et al., 2017). It is also possible that genetic components determining a higher resistance of some animals to both, parasite invasion of the bloodstream, and cyst formation, could be responsible for the observed results.

Our results coincide with those of other studies carried out in Bolivia, where females and older animals were observed to have a higher risk of bearing macroscopic cysts than males and younger animals (Castro et al., 2004; Rooney et al., 2014). As possible explanations for these observations, it was suggested that females have lower immune defenses associated with pregnancy and parturition, while older animals have an increased time of exposure to infective foci and also, infections had longer time periods to develop into the final cyst stage (Rooney et al., 2014).

PCR is likely detecting the initial parasite stages present in the llama bloodstream after ingestion of infective parasite forms, but before invading muscles. Cysts, on the other hand, represent the last stage of the asexual cycle. Thus, the presence of the parasite in blood and the occurrence of cysts in muscles may likely depend on a range of different factors. For example, innate immune responses present in young animals could prevent cyst formation but not bloodstream invasion by the parasite.

In summary, we here describe a new sensitive and specific molecular tool for *S. aucheniae* detection that will help to reveal unknown aspects of this parasite and will facilitate the development of control strategies for SAC sarcocystosis.

Declarations

Author contribution statement

Cecilia Decker Franco: Performed the experiments; Wrote the paper.

Sandra Romero, Alejandro Ferrari: Contributed reagents, materials, analysis tools or data.

Leonhard Schnittger: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Monica Florin-Christensen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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