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# Molecular diagnosis of *Sarcocystis aucheniae* by semi-nested polymerase chain reaction in blood from alpacas (*Vicugna pacos*) in Peru

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## ABSTRACT

**Background**: *Sarcocystis aucheniae* is the causative agent of sarcocystosis in South American camelids, such as alpacas. The disease is generally asymptomatic in its acute phase and is characterized by the presence of macroscopic cysts in skeletal muscles, which limits the commercialization of alpaca meat.

**Aim:** This study aimed to establish the analytical sensitivity and specificity of the semi-nested polymerase chain reaction (PCR) molecular technique for the diagnosis of *S. aucheniae* in the blood of alpacas from the province of Huancavelica, Peru.

**Methods**: The analytical sensitivity of semi-nested PCR was determined using very low concentrations of genomic DNA extracted from *S. aucheniae* macrocysts. The semi-nested PCR assay was compared with the results of the molecular test on blood and the necropsy method. For this purpose, genomic DNA was isolated from blood samples from Huancavelica ($n$ = 8) and Cajamarca ($n$ = 7) alpacas. Furthermore, a previous necropsy was conducted on the muscle tissue of these animals. Furthermore, 42 additional blood samples were collected from alpacas in Huancavelica to detect the 18S rRNA gene of *S. aucheniae* by semi-nested PCR. Macrocyst DNA was used as a positive control, whereas blood from a negative alpaca for the parasite was used as a negative control.

**Results:** The analytical sensitivity of semi-nested PCR had a detection limit of one picogram of DNA, and the specificity of the technique was 80%. Thirty-two percent ($n$ = 16) of Huancavelica alpacas tested positive for the parasite by semi-nested PCR.

**Conclusions:** Semi-nested PCR had both a satisfactory detection limit and a high degree of specificity for the diagnosis of *S. aucheniae* in alpaca blood.

**Keywords:** Alpaca, Blood, Diagnosis, Semi-nested PCR, *S. aucheniae.*

## Introduction

Alpaca farming represents an economic and social activity of significant importance to Peru's high Andean population. The local fauna has adapted to the conditions of the region, thus becoming an essential source of livelihood for local families. These families obtain an economic income from selling meat, fiber, and skins (Martin *et al.*, 2016). Moreover, this practice constitutes an integral component of Peru's cultural heritage, underscoring the imperative for its conservation (Aguirre, 2004; Mendoza *et al.*, 2020). However, alpacas are susceptible to a multitude of diseases that result in clinical and subclinical ailments, thereby constraining fiber and meat yield and quality (Windsor *et al.*, 1992; Ballweber, 2009). In the field of parasitic diseases, sarcocystosis is regarded as an emerging pathogen in South American camelids (Velásquez *et al.*, 2019). Sarcocystosis has been

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demonstrated to result in considerable economic losses for producers, primarily due to its high prevalence, morbidity, and meat confiscation in cases of macrocyst presence. The protozoan *Sarcocystis aucheniae* causes this parasitic disease in South American camelids, including alpacas and llamas (Saeed *et al.*, 2018). Several cases of this parasitic disease have been reported due to the presence of macrocysts in the animal's body at necropsy in countries such as Peru, Bolivia, Argentina, Chile, and Colombia, with Peru being the most affected, as it is home to 95% of the alpaca population in South America (Romero *et al.*, 2017; Saeed *et al.*, 2018). Concurrently, the prevalence of sarcocystosis in alpacas diagnosed by necropsy has been documented to exceed 70% in the elevated Andean regions of southern Peru (Medrano *et al.*, 2006; Gomez and Mallqui, 2018). Infection by the parasite under consideration occurs when camelids consume water and pastures contaminated with mature sporocysts or cysts of *Sarcocystis* spp. These are released into the intestine, pass through the intestinal wall, and enter the bloodstream, where schizogony, or the asexual phase, occurs. The size of cysts in tissues can vary, ranging from microscopic to visible macrocysts, depending on the species. When definitive hosts consume tissues such as meat or carcasses containing cysts, they develop into infectious forms within the intestines, which are subsequently excreted in feces into the environment. Infection of intermediate hosts is initiated through the ingestion of these sporocysts, thereby completing the parasite's life cycle (Wieser *et al.*, 2024). Modern techniques are available for the diagnosis of *Sarcocystis* spp. such as the analysis of ribosomal RNA gene expression, including 28S rRNA, 18S rRNA, as well as internal spacer region 1, and cytochrome c oxidase subunit I (cox1) (Bentancourt Rossoli *et al.*, 2024). This phenomenon exhibits variation according to the taxonomic group, given that these parasites co-evolved with their respective hosts (Prakas *et al.*, 2023). Currently, the diagnosis of sarcocystosis infection in South American camelids is primarily based on necropsy, which evaluates the presence of macrocysts in the animal's body (Baranauskaitė *et al.*, 2023). Immunological methods are subject to limitations due to the production of specific antibodies, a consequence of species-specific cross-reactivity (Moré *et al.*, 2010; Decker *et al.*, 2018). Conversely, contemporary methodologies entail the analysis of nucleic acids (DNA) extracted from biological samples (blood) obtained from subjects, which is regarded as the optimal approach. The first molecular detection of sarcocystosis using genomic DNA (gDNA) isolated from llama blood *in vivo* was performed in Argentina (Martin *et al.*, 2016). The same technique has also been successfully applied in the diagnosis of sarcocystosis in rodents in Japan (Shimozuru *et al.*, 2017), Nigeria (Kamani *et al.*, 2018), and Turkey (Usluca *et al.*, 2019). However, the low concentration of *Sarcocystis* spp. DNA in the bloodstream limits the use of conventional PCR. Conversely, the semi-nested PCR technique has been shown to offer enhanced sensitivity through the generation of sufficient concentrations of amplicons for visualization (Prakas *et al.*, 2024). This approach is particularly advantageous because sarcocystosis does not manifest specific symptoms during the acute phase, and it is improbable that macrocysts will be detected in muscle tissues during this stage (Saeed *et al.*, 2018), as the parasite would only be present in the blood. Therefore, the present study sought to optimize a semi-nested PCR technique to establish the analytical sensitivity and specificity of the technique for detecting very low parasite concentrations in alpaca blood.

## Materials and Methods

### *Blood sample collection*

Blood samples for molecular analysis were collected in 3 ml tubes containing EDTA 2K anticoagulant (Zhejiang Gongdong Medical Technology Co. Ltd., China) in a sterile and aseptic manner. Fifty blood samples were collected from adult alpacas via jugular vein puncture before slaughter at the municipal slaughterhouse in Huancavelica province (S 12°47'24"; W 75°02'14"). Subsequently, a visual inspection of the skeletal musculature of the neck and thorax was performed, where eight alpacas were found to be negative for the presence of *Sarcocystis* spp. macrocysts. Furthermore, seven blood samples were collected from macrocyst-free alpacas at the Granja Porcón research center, located in Cajamarca province (S 7°02'09"; W 78°37'56"). These samples, along with those obtained from Huancavelica's eight macrocyst-free alpacas, were analyzed using semi-nested PCR and compared with the necropsy method to determine specificity.

Blood samples were obtained from the negative control for sarcocystosis from 2 to 7-day-old offspring in a herd of alpacas at the Porcón farm. The two alpaca calves were designated as negative samples because of their exclusive reliance on their mother's milk, a factor that rendered them unacquainted with grass or water contaminated with sarcocystis oocysts (Raggi *et al.*, 1995; Huarachi, 2002). Blood samples from two young animals and 57 adult alpacas were identified and stored in a cooler (model 8885M01, BASA-Peru) with cooling gel at 4°C. The samples were then transferred to the animal health biotechnology laboratory at the Baños del Inca–Cajamarca agricultural experimental station, where they were stored in a refrigerator at 4°C (Samsung, RT35K5930S8/PE, Samsung, Mexico) until use.

### *Collection of sarcocystis macrocysts*

Portion of the cervical skeletal muscle (5 × 8 cm) infested with *Sarcocystis* spp. macrocysts was collected from two alpacas slaughtered at the Huancavelica abattoir. The extraction was performed using a combination of surgical scissors and toothless

anatomical dissection forceps (stainless steel, Neodent-Peru). Three macrocysts were obtained from each animal ($n$ = 6). The samples were placed in a cooler (model 8885M01, BASA-Peru) and transferred to the animal health biotechnology laboratory at the Baños del Inca Agricultural Experimental Station in Cajamarca. The storage temperature of the samples was −20°C (Samsung RT35K5930S8/PE, Samsung, Mexico) until processing.

***Extraction of genomic DNA from the blood of alpacas***

gDNA was extracted from 300 µl of blood from each sample using the Wizard® purification kit extraction protocol (Promega, USA). The quality and concentration of the samples were evaluated using the PCR MAX Lambda 64272 instrument (Bibby Scientific Ltd, UK), and the sample were subsequently stored at −20°C until further use.

***Genomic DNA extraction from Sarcocystis spp. macrocysts***

The macrocysts were prewashed with sterile buffered saline solution (PBS) at pH 7.4.

The samples were then subjected to a crushing process in a porcelain mortar with liquid nitrogen at a temperature of −196°C. The extraction of gDNA from the crushed material was conducted using the Wizard® purification kit (Promega, USA) in accordance with the manufacturer's instructions. Following the extraction process, the concentration and quality of the samples were analyzed at 260/280 nm using a spectrophotometer (PCR MAX Lambda 64272, Bibby Scientific Ltd, UK). The samples were then stored at −20°C until further use.

***Optimization and analytical sensitivity of semi-nested PCR***

Semi-nested PCR was optimized using gDNA from *S. aucheniae* macrocysts. To this end, the technique previously implemented for the diagnosis of *S. aucheniae* in llamas (*Lama glama*) by Decker *et al.* (2018) was applied, with slight modifications as follows: The reaction mixture contained 1 µl (5 mM) of each primer for the *S. aucheniae* gene, 6.5 µl of molecular water, and 12.5 µl of G2 green master mix (Promega, Madison, USA). As a template, 2 µl of gDNA sample was used at concentrations of 10, 20, 30, 50, and 100 ng/µl, with a final volume of 25 µl. The semi-nested PCR reaction was performed in two cycles. The initial amplification (Alpha Thermal Cycler–PCRmax AC196) used the forward 1 cocc18S-F1 and reverse Sauch-R-18S primers to yield a 730-base pair amplicon of the 18S rRNA gene. The second round of amplification was based on the largest sequence obtained in the first round, using the forward 2: scocc18S-F2 and reverse Sauch-R-18S primers to obtain a 583-bp amplicon of the same gene. The primers Lg-16sRNA-F and Lg-16sRNA-R were used to amplify a 257 base pair sequence corresponding to the mitochondrial 16S gene of alpaca. The primers used in this study have been previously described by Decker *et al.* (2018) (Table 1).

The thermal profiles for the first and second amplifications are presented in Table 2.

DNA extracted from *S. aucheniae* macrocysts was utilized at very low concentrations (1 ng, 0.1 ng, 0.01 ng, 1 pg, and 0.01 pg) to ascertain the analytical sensitivity of the molecular technique. This study aimed to ascertain the lowest detectable concentration of the target substance using polymerase chain reaction (PCR) targeting the 18S rRNA gene under optimized conditions. The same optimized semi-nested PCR technique was applied for the diagnosis of the parasite in blood, with an adjustment in the second round of amplification. In this experiment, 1 µl (5 mM) of primers for the amplification of the alpaca 16S mitochondrial gene was added, resulting in a final volume of 27 µl.

***Electrophoresis***

The PCR products were separated by horizontal electrophoresis (100 V/50 minutes) on a 1.5% agarose gel in 1% TBE buffer (40 mM Tris-borate/1 mM EDTA¾SIGMA, USA, pH 8), to which SYBR safe (Invitrogen, USA) was added. A 1 kb molecular weight marker (Promega, USA) was also used. The PCR products were visualized using a UV transilluminator (Visi-Blue™ Transilluminator, Jena Analytic).

***Purification and sequencing of PCR products***

For sequencing analysis, PCR amplicons obtained from genomic DNA (gDNA) extracted from blood samples ($n$ = 2, code D, and 27 C) and macrocysts ($n$ = 1, code 31 C) were randomly selected and purified before sequencing.

***Data processing and analysis***

The results obtained from the molecular test were entered into an Excel database (Microsoft Office LTSC Professional Plus 2021) and sorted accordingly.

**Table 1.** Primers for amplifying the 18S rRNA gene of *S. aucheniae* and the 16S mitochondrial gene of alpaca.

| Gene | Primer sequence | PCR amplicon (bp) | References |
|---|---|---|---|
| Forward 1: cocc18S-F1 | F: GAAAGTTAGGGGCTCGAAGA | 730 bp | Decker *et al.* (2018) |
| Reverse Sauch-R-18S | R:CCAATCCATACTTGGAAAAACGG | | |
| Forward 2: scocc18S-F2 | F: GACGGAAGGGCACCACCAGG | 583 bp | Decker *et al.* (2018) |
| Reverse Sauch-R-18S | R:CCAATCCATACTTGGAAAAACGG | | |
| Lg-16sRNA-F | F: AAGGAACTCGGCAAACACGA | 257 bp | Decker *et al.* (2018) |
| Lg-16sRNA-R | R: ATTTGTTCATCCCCGCCTCT | | |

**Table 2.** Semi-nested PCR cycling conditions.

| Amplification | PCR stage | Temperature | Time | Cycles | References |
|---|---|---|---|---|---|
| First | Initial denaturation | 95°C | 2 minutes | 30 | Decker *et al.* (2018) |
| | Denaturation | 95°C | 30 seconds | | |
| | Alignment | 62°C | 30 seconds | | |
| | Extension | 72°C | 30 seconds | | |
| | Final extension | 72°C | 5 minutes | | |
| Second | Initial denaturation | 95°C | 3 minutes | 30 | Decker *et al.* (2018) |
| | Denaturation | 95°C | 30 seconds | | |
| | Alignment | 63°C | 30 seconds | | |
| | Extension | 72°C | 30 seconds | | |
| | Final extension | 72°C | 5 minutes | | |

The percentage of positives and negatives for *S. aucheniae* was then calculated using GraphPad Prism 9.3.1 (GraphPad Software, Inc., San Diego, CA). The sequences were edited in Chromas v. 2.6.6 for bioinformatic analysis and then compared with the 18S rRNA sequences of *S. aucheniae* deposited in the GenBank database of the National Center for Biotechnology Information (NCBI, USA, http://www.ncbi.nlm.nih.gov/) using Standard Nucleotide BLAST (BLASTN).

***Ethical approval***

Blood samples and macrocysts were obtained from alpacas slaughtered at the Huancavelica Municipal Slaughterhouse. The negative control blood samples were obtained by certified veterinarians from the Faculty of Veterinary Sciences of the National University of Cajamarca, Peru (Veterinary Clinic, Ministry of Agriculture) in accordance with specific protocols and ensuring animal welfare, as provided for in Law No. 30407, the Animal Protection and Welfare Law.

## Results

Molecular testing using the semi-nested PCR technique, utilizing gDNA isolated from alpaca blood (20 ng/µl) and macrocysts (50 ng/µl), revealed the presence of *Sarcocystis* spp., for which a 580-bp amplicon of the 18S rRNA gene of *S. aucheniae* was obtained. Furthermore, the presence of four 250-base pair bands was observed, representing the 16S rRNA gene of alpaca, which was used as a quality control for the PCR process (Fig. 1).

With regard to the analytical sensitivity of the semi-nested PCR technique, only four of the five concentrations of gDNA used from alpaca blood samples (1 ng, 0.1 ng, 0.001 ng, 1 pg, and 0.1 pg) were detectable. The minimum detectable concentration of parasite DNA corresponding to the 18S rRNA gene was determined to be 1 pg (Fig. 2).

The semi-nested PCR technique was found to be repeatable, with the presence of the parasite being observed in alpaca blood samples using gDNA for 3 consecutive days at a working concentration of 20 ng/µl, with two replicates per sample. No discrepancies were observed in the amplification pattern following the initial reaction on days 2 and 3, thereby validating the reliability and reproducibility of the amplification outcomes (Fig. 3).

The specificity of the semi-nested PCR technique was evaluated using blood samples from 15 alpacas, 8 from the province of Huancavelica and 7 from the Porcón–Cajamarca farm. All samples were found to be negative for *S. aucheniae* macrocysts, as determined by necropsy. The application of the semi-nested PCR technique to the samples from Huancavelica, with a specificity of 80%, yielded positive results in three cases.

The semi-nested PCR technique was used to amplify the gDNA of 50 alpaca blood samples, encompassing the 42 alpacas that did not undergo necropsy and the 8 alpacas that did. Of the samples examined, 16 (32%) were positive, and 34 (68%) were negative for *S. aucheniae.*

The sequences of the parasites obtained from the amplification of the 18S rRNA gene of *S. aucheniae* from blood samples ($n$ = 2) and macrocysts ($n$ = 1) were analyzed using BLASTn with the sequences contained in the US National Center for Biotechnology Information database. Multiple matches were obtained for each sample; however, Table 3 includes the reference sequences showing the highest percentage of identity. A similarity percentage ranging from 98.17% to 100% was identified in 15 GenBank sequences (Table 3).

## Discussion

At present, there are no standardized technical criteria or diagnostic tests for detecting *S. aucheniae* in live alpacas. As stated by Saeed *et al.* (2018) the most widely accepted method for determining sarcocystosis is necropsy to identify macrocysts in the striated muscle of alpacas and llamas. This study is significance as it represents the first report of molecular

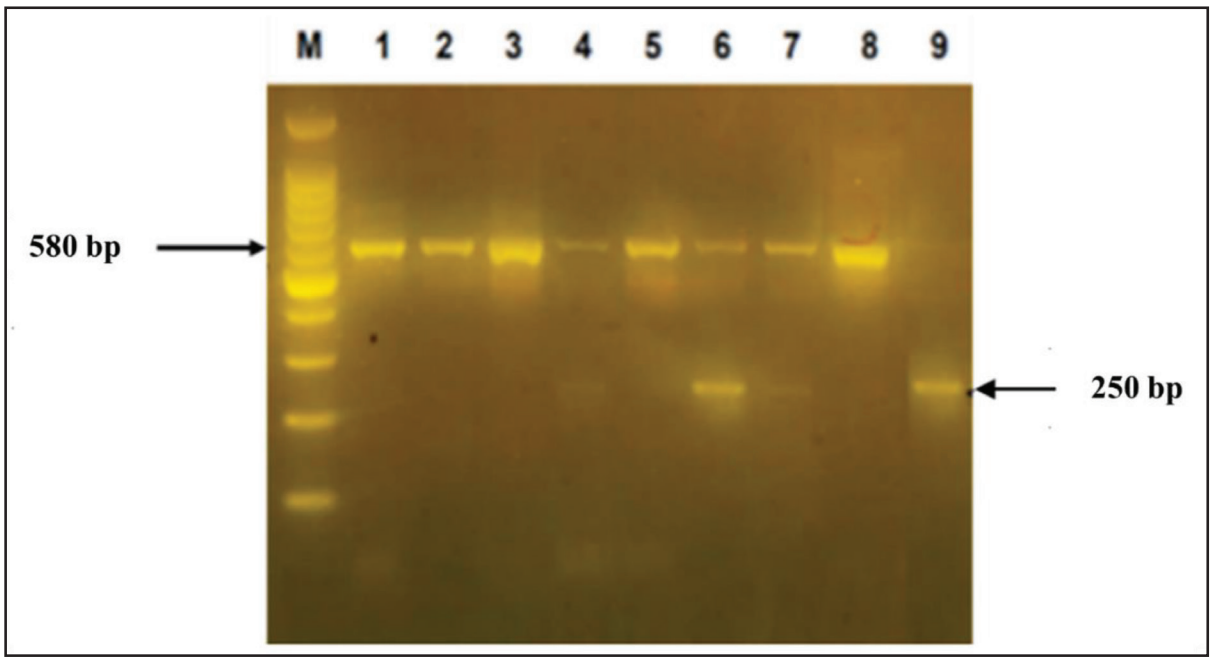


**Fig. 1.** Detection of *S. aucheniae* by the semi-nested PCR molecular technique. M: 100 bp marker. Lane 1: Positive control (macrocyst). Lane 9: Negative control. Lanes 2–8: Positive results for S. *aucheniae*. The 580 bp band corresponds to the 18S rRNA gene of the parasite and the 250 bp band to the 16S mitochondrial gene of alpaca. Lanes 4, 6, and 7 show both bands (580 bp and 250 bp), while lanes 2, 3, 5, and 8 show only the 580 bp band. Lanes 2, 3, 5 and 8 are positive results for *S. aucheniae* in alpaca blood, without using primers for the 16S rRNA gene.

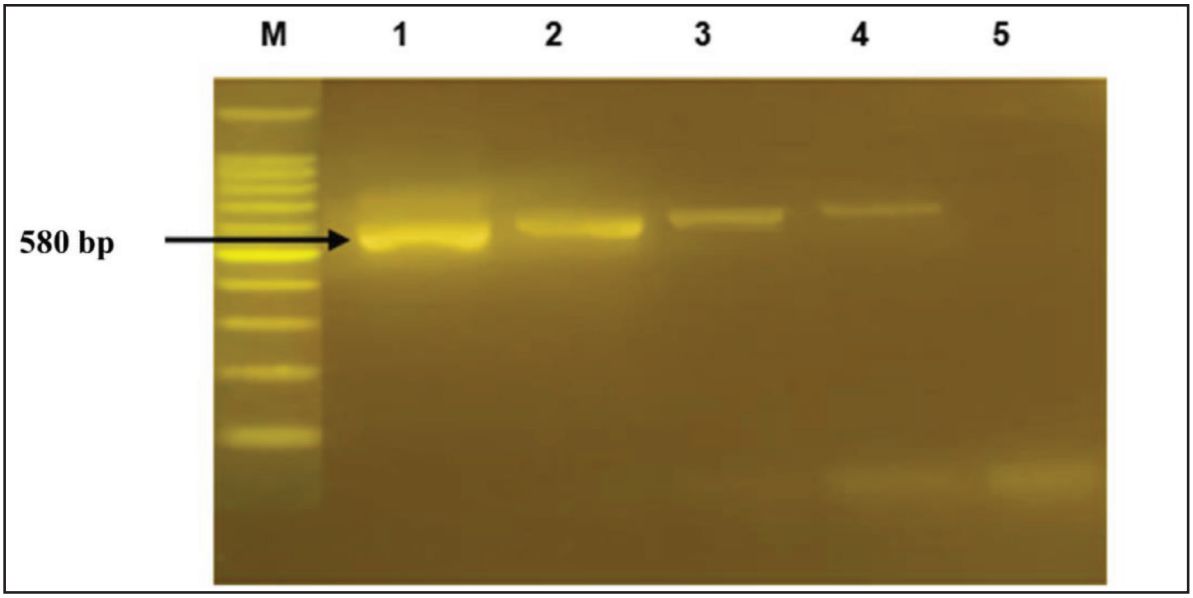


**Fig. 2.** Analytical sensitivity of semi-nested 1.5% agarose gel PCR of parasite gDNA at different concentrations. M: 100 bp marker. Lane 1: 1 ng; Lane 2: 0.1 ng; Lane 3: 0.001 ng; Lane 4: 1 pg; Lane 5: 0.1 pg of the 18S rRNA gene of *S. aucheniae*.

diagnosis of sarcocystosis in Peruvian alpacas. The 18S rRNA gene of *S. aucheniae* was detected through the amplification of a gene fragment of approximately 580 base pairs using semi-nested PCR in alpaca blood samples (Fig. 1). Consistent findings were reported by Decker *et al.* (2018) who identified *S. aucheniae* in blood samples from llamas in Argentina and Bolivia using the same molecular technique. In this particular

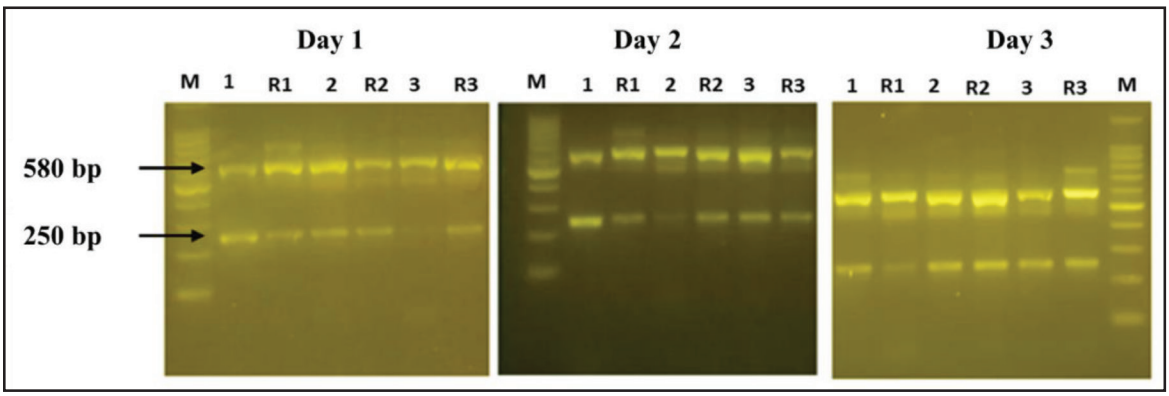


**Fig. 3.** Repeatability of the semi-nested PCR molecular test in the detection of *S. aucheniae*, 1.5% agarose gel; M: 100 bp marker; days 1, 2 and 3: Lanes 1, 3, 5: Sample; Lanes 2, 4, 6: Sample repeat; 18S rRNA gene (580 bp); 16S rRNA gene (250 bp).

**Table 3.** Percentage identity of the 18S rRNA gene sequences of *S. aucheniae* isolated from blood samples ($n$ = 2) and macrocysts ($n$ = 1) from alpacas, compared with reference sequences in the GenBank database.

| Sample codes for 18S rRNA gene sequences of *S. aucheniae* | Percentage of identity (%) | Code (GenBank) | References |
|---|---|---|---|
| D (blood sample) | 100 | MG832004.1 | Decker *et al.* (2018) |
| | 99.64 | KU527117.1 | Regensburger *et al.* (2015) |
| | 99.64 | KT382799.1 | More *et al.* (2016) |
| | 98.81 | MG832003.1 | Decker *et al.* (2018) |
| | 99.44 | KU527117.1 | Holmdahl *et al.* (1999) |
| 27 C (blood sample) | 99.8 | MG832004.1 | Decker *et al.* (2018) |
| | 99.61 | MG832003.1 | Decker *et al.* (2018) |
| | 99.44 | KT382799.1 | More *et al.* (2016) |
| | 99.44 | KU527117.1 | More *et al.* (2016) |
| | 97.96 | AF017123.1 | Holmdahl *et al.* (1999) |
| 31 C (macrocyst) | 100 | MG832004.1 | Decker *et al.* (2018) |
| | 99.81 | MG832003.1 | Decker *et al.* (2018) |
| | 99.63 | KT382799.1 | Regensburger *et al.* (2015) |
| | 99.63 | KU527117.1 | More *et al.* (2016) |
| | 98.17 | AF017123.1 | Holmdahl *et al.* (1999) |

instance, a fragment of approximately 550 base pairs was reported, thereby demonstrating the efficacy of the nested PCR molecular technique in the diagnosis of *S. aucheniae* in live South American camelids. These results are consistent with those of Martin *et al.* (2016) who designed the first semi-nested PCR for the detection of this gene in *S. aucheniae* in llama blood, obtaining a 450-bp amplicon.

About analytical sensitivity, the technique implemented has been able to amplify the 18S rRNA gene at very low concentrations of parasitic DNA, with a detection limit of 1 pg (Fig. 2). These results are analogous to those reported by Decker *et al.* (2018) who demonstrated that semi-nested PCR has a detection limit of up to one parasite per milliliter of blood, suggesting that the technique has acceptable accuracy. Notably, analytical performance at low concentration limits is often of great interest in molecular testing for infectious diseases because it defines the assay's diagnostic capability (Burd, 2010). Concurrent studies have determined the analytical sensitivity of nested PCR in the diagnosis of *Sarcocystis tenella* and *Sarcocystis arieticanis*, which infect sheep, where the 18S rRNA gene was also detected at concentration of up to 10 pg of DNA from each parasite (Heckeroth and Tenter, 1999).

The technique demonstrated high specificity (80%), with the majority of the alpacas analyzed not harboring macrocysts in their skeletal muscles, thus yielding negative results for the presence of *S. aucheniae* using semi-nested PCR. Concurrent findings have been documented by Moré *et al.* (2013) who attained a specificity of 95.5% for the multiplex PCR technique in the diagnosis of various *Sarcocystis* spp. infecting cattle. Similarly, a separate study documented a high specificity of 75% for the semi-nested PCR technique in the detection of *S. aucheniae* in llamas from Argentina (Decker *et al.*, 2018). The findings demonstrate that semi-nested PCR is capable of accurately detecting parasites in animals that do not harbor the parasite. Conversely, in this study, three Huancavelica alpacas exhibited positive results in blood by semi-nested PCR but negative results in necropsy. This phenomenon may be attributed to several factors, including, but not limited to, inadequate sanitary conditions, the presence of definitive hosts (canines), or other variables that elevate the risk of infection (Alva *et al.*, 1981; Saeed *et al.*, 2018). Consequently, the positivity of the test could be attributed to the acute phase of infection in the animals. Moreover, according to certain studies, necropsy is not an appropriate method for confirming the acute phase of *S. aucheniae* infection. This assertion is corroborated by the findings of Decker *et al.* (2018) who observed the parasite's presence in the blood of llamas that had initially tested negative by necropsy. In this regard, semi-nested PCR would be a useful technique for determining the presence of the parasite in the blood during the early stages of infection.

Regarding the blood samples from alpacas in Cajamarca ($n = 7$), the results of both PCR and necropsy tests were negative, which can be attributed to the implementation of effective health management practices. Despite the absence of recent data, a report was published approximately 28 years ago documenting the presence of *S. aucheniae* macrocysts in alpaca meat (Cabrera, 1996), indicating the potential existence of the parasite in Cajamarca. Similarly, a recent study confirmed the presence of *Sarcocystis* spp. oocysts in the feces of canines inhabiting the same alpaca-rearing areas (Ydrogo, 2017). Consequently, confirming the presence of this parasite in the definitive host using a molecular method would be highly relevant. As illustrated in Figure 1, 16 (32%) of the Huancavelica animals were positive for *S. aucheniae* using semi-nested PCR. This phenomenon could be attributed to breeding conditions that are conducive to transmission, such as transhumance and exposure to dogs, which have been identified as risk factors in Argentina (Romero *et al.*, 2017). Inappropriate practices, such as the provision of infected raw meat to canines, have been demonstrated to facilitate the parasite cycle by contaminating pastures and water with oocysts or sporocysts present in their feces (Guerrero, 1987).

In contrast, 34 alpacas (68%) exhibited a negative result for *S. aucheniae*, which could be indicative of a breeding method that does not permit parasitic infection due to stringent sanitary conditions. Although all the study samples originated from Huancavelica, they do not all stem from the same breeding area. Another possibility is that there are genetic components in some alpacas that determine greater resistance to parasite invasion. This hypothesis is supported by the findings of previous studies that have identified significant genomic regions in the sheep genome that are associated with resistance to parasites (Al Kalaldeh *et al.*, 2019). It is also conceivable that the parasite is not circulating in the animal's blood but may be present in the animal's skeletal muscle in the form of macrocysts.

Sequence analysis confirmed that the 18S rRNA gene amplified from gDNA from blood samples corresponded to *S. aucheniae*. The sequences exhibited identity percentages >97.96% with the 18S rRNA sequences deposited in GenBank, which were derived from blood samples of llamas originating in Argentina and Bolivia. Furthermore, a high percentage of identity was observed between the sequence of the gene isolated from macrocysts in this study and the sequences of the same gene from macrocysts (98.17%) from llamas, alpacas, and guanacos. These results indicate that the genetic variation observed in the 18S rRNA sequence is not related to the host species or the geographical origin of the isolates (Decker *et al.*, 2018). However, further studies are required to confirm this hypothesis.

The PCR exhibited repeatable results, indicating that the replicates of the test application in each sample over a period of 3 days remained constant (Fig. 3). This finding shows that the technique is robust; that is, its performance remains unaffected by minor alterations in testing procedures that may occur over time within a single laboratory (Waugh and Clark, 2021).

## Conclusion

This study demonstrates significant and substantial progress in the diagnosis of *S. aucheniae* in alpacas in Peru. The analytical sensitivity of the semi-nested PCR technique was as low as 1 pg of parasite gDNA for the 18S rRNA gene, indicating that the technique has an optimal detection limit. Conversely, the semi-nested PCR technique exhibited a high degree of specificity (80%).

The application of this technique revealed that 16 of the alpacas analyzed (32%) were found to be infected with the parasite, indicating that they would be in the acute phase of infection, that is, when it is circulating in the blood. Implementing this technique in early control programs for alpaca offspring could facilitate the zoning of areas free of *S. aucheniae.* Furthermore, it can facilitate the evaluation of pharmaceuticals intended for the management of both the intermediate and final hosts. This approach is designed to circumvent

the occurrence of false positives and negatives in the treatment.

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***Conflict of interest***

The authors declare no conflicts of interest.

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***Authors' contributions***

Conceptualization: MCG and MRJ. Methodology: DRV and ATS. Internal research: NBM and OECM. Resources: MCG and WAG. Formal analysis: MCG, DRV. Visualization: MCR and MCG.

*Data* ***availability***

Data supporting the results and conclusions of this research are available from the corresponding authors, DRV and MCG, upon reasonable request.

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