Veterinary Parasitology 198 (2013) 396–400

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short Communication

Molecular identification of *Sarcocystis aucheniae* as the macrocyst-forming parasite of llamas

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ARTICLE INFO

Article history:
Received 13 July 2013
Received in revised form 6 September 2013
Accepted 13 September 2013

Keywords:
Sarcocystis aucheniae
Sarcocystiosis
Macroscopic cyst
Molecular identification
Phylogenetic analysis
South American camelids

ABSTRACT

The domestic South American camelids (SACs), llama (*Lama glama*) and alpaca (*Lama paco*), are frequently found to be infected with *Sarcocystis* parasites. Infections give rise in skeletal muscle to macroscopic cysts (1–5 mm long) that resemble rice seeds, each containing several million living bradyzoites. The finding of cysts prevents commercialization of SAC meat, an important source of income for rural families in the Andean flatlands. Thus, development of diagnostic methods to facilitate the control of these infections is highly desirable, and the first step to this end is the unequivocal species identification of the causative agent. Based on the cyst form and size, the infecting parasite has been described as *Sarcocystis aucheniae*; however, this traditional approach is not reliable as similar cysts may contain different species. To date, molecular identification has been done for a single isolate of *S. aucheniae* from an alpaca in Australia. In order to verify the identity of the species present in SACs of South America, the complete 18S rRNA gene was PCR-amplified and sequenced from macrocyst DNA obtained from three llamas of the Andean flatlands. A phylogenetic Bayesian analysis was carried out using the analyzed and available 18S rRNA sequences of *Sarcocystis* spp. In the constructed tree, all of the new 18S rRNA gene sequences segregated in a single clade together with the 18S rRNA gene sequence reported from an alpaca in Australia, demonstrating that the isolated parasite is *S. aucheniae*, and that this parasite indiscriminately infects both domestic SACs. This work represents the first molecular identification of the causative agent of SAC sarcocystiosis in South America, and can contribute to the development of control methods for this neglected parasitosis.

1. Introduction

The main livestock in the Andean regions of Northern Argentina and Chile, Bolivia, Peru and Ecuador consists of domestic South American camelids (SACs), which include llamas (*Lama glama*) and alpacas (*Lama paco*) (Wheeler, 1991). Domesticated in pre-Incan times from their wild relatives, guanaco and vicugna, these ruminants are well adapted to agro-ecological areas where non-indigenous productive animals cannot thrive. As opposed to other livestock animals, SACs do not alter the fragile ecology of Andean pastures when walking or grazing, and they have a reduced consumption of water (Wheeler, 1991).

In addition to the relevance of domestic SACs for fine wool supply, their meat constitutes an important source of

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http://dx.doi.org/10.1016/j.vetpar.2013.09.007

animal protein for rural Andean communities (Vilca, 1991). A severe limitation for the production and commercialization of SAC meat is the frequent presence in skeletal muscles of abundant macroscopic cysts resembling rice seeds (Leguía, 1991). Slaughtered animals are confiscated by sanitary authorities when cysts are found, resulting in substantial existence-threatening economic losses for small-scale producers (Leguía and Casas, 1999).

These cysts have been described as the life cycle stage in the intermediate host of the protozoan parasite *Sarcocystis aucheniae*, which has been proposed as the causative agent of sarcocystiosis (Torres et al., 1981; Levine, 1986). However, molecular identification of *S. aucheniae* by phylogenetic analysis of the 18S rRNA gene has been exclusively carried out for a single isolate found in an alpaca in Australia (Holmdahl et al., 1999), and never verified in other SACs from any other region of the world.

Acute sarcocystiosis in SACs can be associated with severe clinical signs such as anorexia, fever, anemia, weakness, weight loss, nervous signs, abortion, and even death (La Perle et al., 1999; Gabor et al., 2010). In canids, which are the definitive host, ingestion of high doses of parasites can give rise to weight loss, anorexia, and fever, lack of coordination, diarrhea, and even death (Leguía et al., 1989; Cornejo et al., 2007). In the case of human consumption, uncooked *Sarcocystis*-infected meat can produce gastroenteritis, nausea, diarrhea, colic, shivering, and respiratory problems (Leguía, 1991). The observed symptoms are due to sarcocystin, a neurotoxin protein present in the cysts. However, human clinical cases are rare because the toxin is normally inactivated during cooking (Hiepe et al., 1981; Briggs and Foreyt, 1985; Chileno et al., 2011).

Recently, a high prevalence of anti-*Sarcocystis* antibodies has been demonstrated by immunofluorescence in llamas, the main domestic SAC in the Andean NW of Argentina, using both *Sarcocystis* sp. bradyzoites isolated from llama cysts and *S. cruzi* bradyzoites from cattle (Moré et al., 2008). This shows a high degree of antigen cross-reactivity among *Sarcocystis* spp., which makes serology unsuitable for species identification.

In order to verify the identity of the parasite forming macroscopic cysts in llamas in the New World, a molecular phylogenetic analysis was carried out, as this is a prerequisite for the development of molecular diagnostics and other control measures against sarcocystiosis of SACs.

2. Materials and methods

2.1. Isolation and microscopical examination of parasite samples

Macroscopic cysts were detected by visual examination of intercostal and cervical muscles of slaughtered llamas ($n = 3$) originating from the Andean flatlands of the northwestern region of Argentina. Six cysts of approximately 5 mm length were separated from the muscle with a scalpel, washed with PBS, weighed and measured. Their semi-liquid contents were released, diluted with PBS (1:100), and the parasites were quantified by observation in an optical microscope (100× magnification) using a Neubauer chamber. The length and width of 64 bradyzoites were measured at 1000× magnification using QImaging software (QImaging, Surrey, Canada) to determine the average length, width and their standard deviations.

2.2. DNA extraction, PCR amplification, and sequencing

Pools of 2–6 sonicated cysts obtained from three different llamas (J2, J3 and J5) were used to extract genomic DNA using the DNAeasy blood and tissue kit (Qiagen, Hilden, Germany). The isolated DNA was quantified in a Nanodrop spectrophotometer, and stored at −20 °C until further use.

Two overlapping fragments of the complete 18S rRNA gene were amplified in PCR reaction mixtures composed of 0.2 mM of each dNTP (Fermentas, Burlington, Canada), 0.03 U/μl Go Taq DNA polymerase and its corresponding buffer containing 1.5 mM Mg^{2+} (Promega, Madison, WA), 10 μM of each primer (Medlin et al., 1988; Gubbels et al., 1999), and finally filled up with water to a volume of 25 μl. Subsequently, 100 ng genomic DNA was added and the cycle step program carried out consisting of 10 min at 95 °C, followed by 40 cycles of denaturing at 95 °C for 45 s, hybridization at 55 °C for 45 s, synthesis at 72 °C for 1 min, and a final extension period of 5 min at 72 °C. Negative controls containing no template were run in parallel, to rule out contamination.

Amplification products were run with a DNA marker (1 Kb Plus, Invitrogen, Carlsbad, CA) in ethidium bromide-containing 0.8% agarose gels by electrophoresis, purified and directly sequenced (Macrogene, Seoul, Korea). In addition, amplification products were cloned in pJet 1.2 blunt cloning vector following the recommendation of the manufacturer (Fermentas, Burlington, Canada), and competent *E. coli* TOP 10 cells (Invitrogen, Carlsbad, CA) were transformed. Three qualified clones were selected, grown in overnight cultures, purified using the GFX Micro Plasmid Prep Kit (GE Healthcare, Buckinghamshire, UK), and quantified by spectrophotometry. The plasmid inserts were sequenced using an ABI 3500xL equipment (Applied Biosystems, Carlsbad, CA). Edited 18S rRNA gene sequences were deposited in Genbank under accession numbers KF383266, J2; KF383267, J3; and KF383268, J5.

2.2.1. In silico analysis

Phylogenetic analysis followed the protocol of Whipps et al. (2012). The same multiple sequence alignment was used, but including only the 200 sequences from the Sarcocystidae. The three new sequences were manually aligned to this pre-existing alignment. Bayesian phylogenetic analysis was performed using the MrBayes v3.2.1 program (Ronquist and Huelsenbeck, 2003). Two data partitions were used, one for the unpaired rRNA positions with the GTR+G+I model, and one for the paired (stem) positions with the doublet+G+I model. All model parameters were unlinked across the partitions, except for the tree topology, and default priors were used. The analysis consisted of 10 runs of 2 chains each, with 1,000,000 generations per run and a burn in of 100,000 generations. This yielded 90,000 trees for the consensus tree.

Identities between nucleotide sequences were estimated using the MatGat program (Campanella et al., 2004),

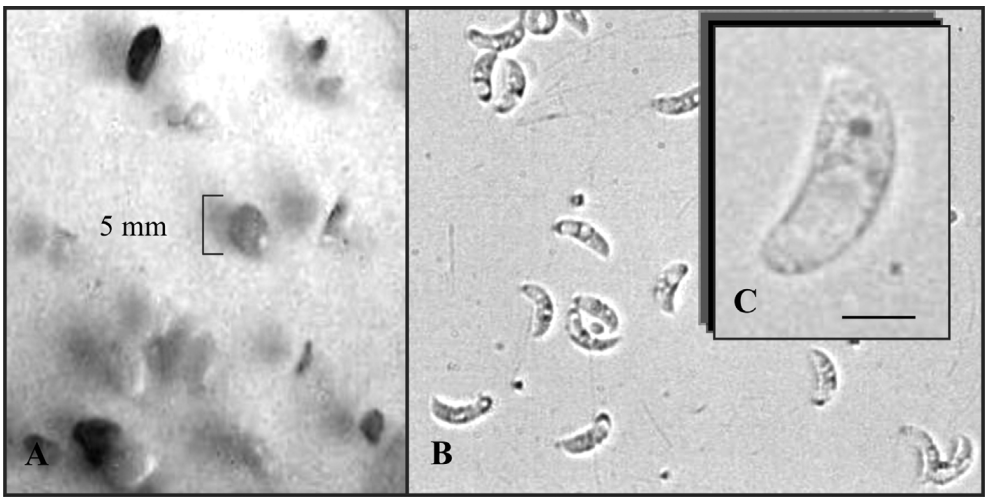

Fig. 1. Macroscopic cysts isolated from a *Sacrocystis*-infected llama and their content. Cysts isolated from llama muscles (A); microscopical image of parasites using a 400× (B) and a 1000× magnification (C); the bar corresponds to 3.5 μm (C).

based on an alignment of 1500 bp obtained by deleting positions including end gaps.

3. Results and discussion

Cysts are a frequent finding in the muscles of llamas (Fig. 1A). We carried out a careful microscopical examination of the semi-liquid contents of six such cysts, ranging in length from 3 to 5 mm and in wet weight from 17.3 to 33.1 mg. As expected for *Sarcocystis* parasites, an abundant number of half-moon shaped cells with oscillating motility were observed (Fig. 1B and C). By Neubauer chamber counting, the parasite quantity contained in the studied cysts was determined to range between 1.8 and 2.6×10^7 protozoa, while the average length and width of a bradyzoite was estimated as 17.64 μm ± 2.40 and 3.60 μm ± 0.80, respectively. These morphological metrics and characteristics are consistent with those described for the brayzoite parasite stage of *S. aucheniae* embodied in cysts (Gorman et al., 1984; Levine, 1986). In this context, it is important to note that Leguía et al. (1989) proposed the name *S. aucheniae* for those species that produce macroscopic slow-growing cysts in skeletal muscles, and distinguished it from the species *S. lamacanis* forming fast-growing microscopic cysts in the cardiac muscle of llamas.

In order to assess the identity of *Sarcocystis* isolates from South America and Australia, the full-length sequences of the 18S rRNA gene of macrocyst-parasite isolates J2, J3, and J5 originating from three individual llamas were aligned against the pre-existing database sequences of *Sarcocystis*. The sequences of isolates J2, J3, and J5 showed highly similar identities, ranging between 99.1 and 100% (Table 1). In contrast, the Australian isolate displayed a somewhat lower identity with respect to those originating from South America, ranging between 96 and 96.9%. The observed sequence differences could be due to different allelic forms of the 18S RNA gene, but it is also possible that they are due to 18S RNA gene paralogs (the methodology used does not allow differentiating between these two possibilities). Comparing 1500 aligned positions of 18S rRNA sequences, the highest identity of 92.2% for *S. aucheniae* Australia and 92.3% for South American isolates J2, J3, and J5 was exhibited with *Sarcocystis sinensis*, while the lowest identity of 86.7% for *S. aucheniae* Australia and 86.8% for South American isolates J2, J3, J5 was observed for *S. singaporensis* (data not shown). Notably, a considerably larger inter-species variation of the 18S rRNA gene is observed in Sarcocystidae (7.8–13.2%), as compared to some other apicomplexan pathogens as for example piroplasmids (0.3–8.2%) (Schnittger et al., 2003; Dahlgren and Gjerde, 2007; Gjerde, 2012; Whipps et al., 2012).

Table 1
Identity of 18S rRNA gene sequences between *S. aucheniae* isolates.

Isolate	J2 (%)	J3 (%)	J5 (%)
Sa Australia	96.9	96	96.9
J2	–	99.1	100
J3	–	–	99.1

The Bayesian tree shown in Fig. 2 demonstrates that the new 18S rRNA sequences originating from llama clustered in a single monophyletic group together with the corresponding sequence of *S. aucheniae* reported from an alpaca in Australia. The posterior probability value of 1.00 for this branch is very high, corroborating that *S. aucheniae* represents a well defined species as based on 18S rRNA comparison. Phylogenetic trees based on maximum likelihood and maximum parsimony yielded similar topologies likewise strongly supporting the monophyly of *S. aucheniae* with significant bootstraps (data not shown). Unfortunately, parasite material to sequence the 18S rRNA of *S. lamacanis* was not available, and the database 18S RNA sequences of *S. lamacanis* (AY840990, DQ100056) are extremely short (Medrano et al., 2006). With the exception of the *S. hardangeri* + *S. oviformis* + *S. ovalis* clade, *S. aucheniae* is the sister taxon to the crown group of *Sarcocystis* spp. that infects exclusively ruminants as intermediate host.

In summary, the segregation of the 18S rRNA sequences of *Sarcocystis* isolates from alpaca and llama into a single monophyletic group suggests that *S. aucheniae* infects these two SACs indiscriminately. This corroborates the hypothesis that the isolate from Australia is of South American

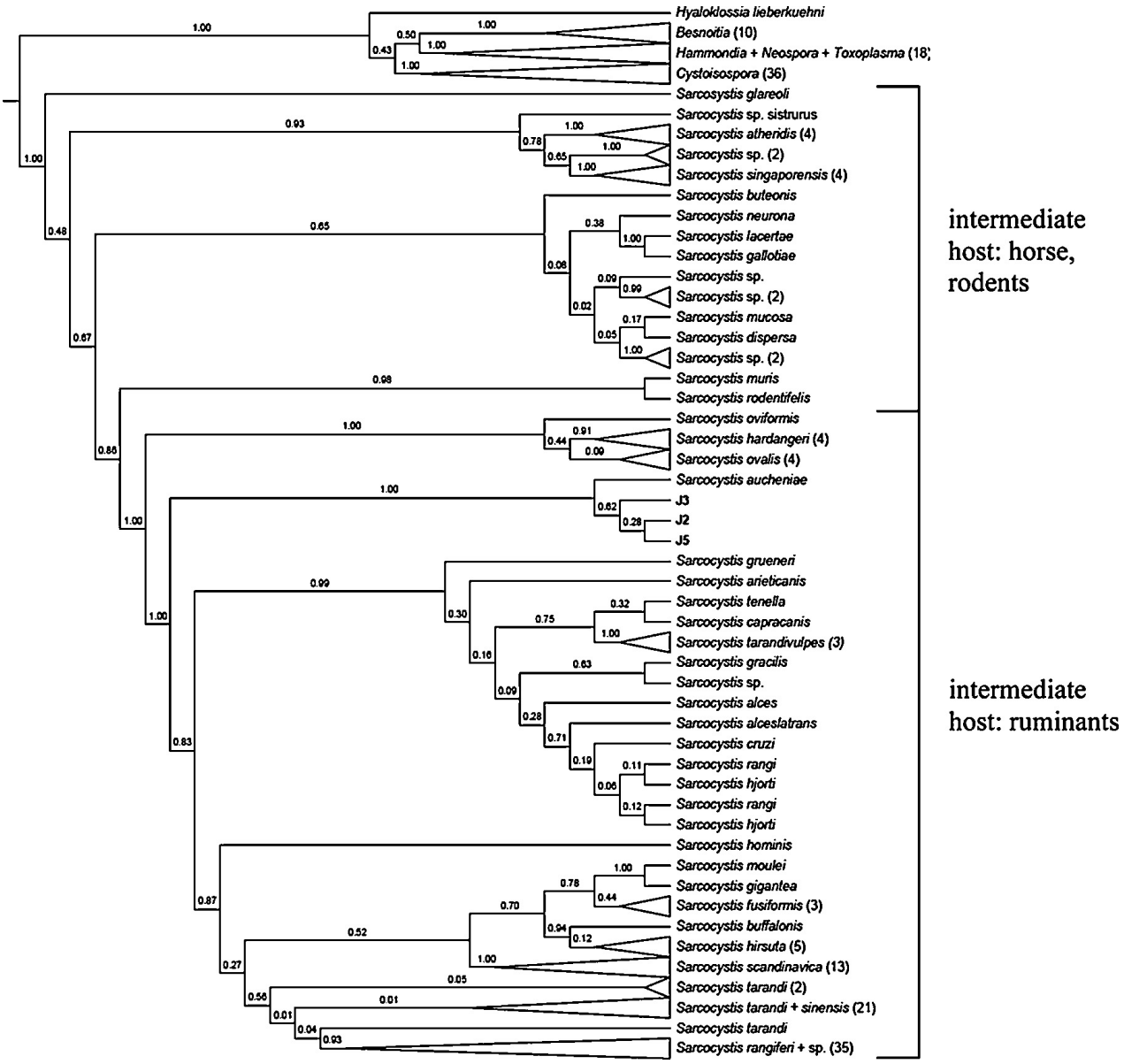

Fig. 2. Molecular phylogenetic analyis of *Sarcocystis* isolates of llama. Comparison of the full-length 18S rRNA gene sequences obtained from macrocysts of llamas (J2, J3 and J5) and of other *Sarcocystis* species by Bayesian analysis. The Toxoplasmatinae sequences were used as the outgroup. The segregation of intermediate hosts of *Sarcocystis* sp. is indicated.

origin and was imported with its alpaca host to Australia. The presented verification of the identity of *S. aucheniae* in macrocysts of llamas and alpacas will facilitate the development of diagnostics and other control measures against sarcocystiosis.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

We thank F. Delgado for technical assistance. Financial support from the National Research Council of Argentina (CONICET, PIP 114 20080100653) and the National Institute of Agricultural Technology (INTA, Argentina, Regional Project 320051) is gratefully acknowledged.

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