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Short communication

Molecular confirmation of *Sarcocystis gigantea* in a naturally infected sheep in Argentina: A case report

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ABSTRACT

The work describes a case of *Sarcocystis gigantea* infection in a 3-years-old Corriedale ewe from Buenos Aires Province, Argentina. The ewe was found dead with a poor body condition. Pathological and molecular studies were carried out in order to try and confirm the causative agent of the infection. At necropsy, approx. 100 whitish elliptic (3–5 mm to 5–8 mm) macrocysts with a hard consistency were observed along the esophageal and pharyngeal muscular layers. Microscopically, the macrocysts consisted of an eosinophilic wall, internal septa originated from the eosinophilic wall and basophilic parasitic cells were located among the septa. The sarcocysts were identified molecularly through PCR amplification and sequencing of a short segment of the 18S rRNA gene. Sequence analysis of the amplified DNA demonstrated 100% identity to *S. gigantea* sequences previously published. To our knowledge this is the first molecular confirmation of *S. gigantea* infection in sheep in the Americas.

1. Introduction

The protozoon *Sarcocystis gigantea* (Railliet 1886) is a cyst-forming coccidia of the phylum Apicomplexa, with an obligatory 2-host life cycle and is distributed worldwide (Dubey et al., 2015). *S. gigantea* replicates asexually and sexually in sheep (*Ovis aries*) and domestic cats (*Felis catus*), respectively. Sheep become infected by the consumption of sporocysts shed in the faeces of infected cats. During the asexual stage, large sarcocysts can be detected macroscopically in muscles of the esophagus, larynx and tongue and, to a lesser extent, in the diaphragm and the rest of the carcass. Sarcocysts are usually up to 1 cm long, globular, dull white and, despite of their size, they are intracellular. *S. gigantea* is a mildly pathogenic parasite, therefore the infection is usually chronic and subclinical in sheep (Dubey et al., 2015). In contrast with *Sarcocystis hominis* in cattle and *Sarcocystis suihominis* in swine, *S. gigantea* does not infect humans (Fayer et al., 2015). Nevertheless, carcasses infected with macroscopic sarcocysts are commercially condemned (Martínez-Navalón et al., 2012; Dubey et al., 2015).

Although *Sarcocystis* spp. identification could be carried out by watching the structure and thickness of the cyst wall by light and electron microscopy (Dubey et al., 2015), it has been reported that some species have morphologically indistinguishable sarcocysts (Gjerde, 2016) and hence can only be unambiguously identified by molecular methods. Furthermore, molecular diagnostic methods are more time/cost effective than microscopic observation (Stojecki et al., 2012).

The aim of this study is to confirm by molecular methods, that an accidental finding of *S. gigantea*-like sarcocysts during a postmortem examination, indeed belonged to this species.

2. Materials and methods

2.1. Animals, sample collection and histopathology procedures

The case occurred in a 3-year-old Corriedale ewe, with a single term pregnancy, from a commercial flock of 380 sheep in Buenos Aires Province, Argentina. The production system was extensive but since it was a season with poor supply of grass, this and other ewes were kept in paddocks and fed with commercially balanced feed and hay. Before death, the ewe examined in this current study showed a poor body condition.

Post mortem examination was performed on the ewe and tissue samples were recovered for histopathology analysis as described by Campero et al. (2003).

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2.2. DNA extraction

DNA was extracted from 5 × 6 μm sections of formalin-fixed paraffin-embedded (FFPE) tissue which contained the sectioned sarcocyst. The QIAamp FFPE Tissue DNA Kit, QIAGEN, Hilden, Germany) was used, as per manufacturers' instructions, with the sample being finally eluted in 30 μl of DNase/RNase free water.

2.3. PCR amplification of Sarcocystsis DNA

The reaction mixture used in the PCR amplification of the *Sarcocystis* 18S rRNA gene has been previously described (Bartley et al., 2016) with the following alterations. The primers used were F1 5′-TGCATGTCTAAGTATAAGCTT-3′ and R1 5′-GGTCAGAAACTTGAATGATC-3′. The PCR reaction conditions were as follows: 94 °C for 5 min followed by 40 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min and a final extension period of 5 min at 72 °C. Following PCR amplification 6 μl of product was analysed by agarose gel electrophoresis (2% agarose in 1x Tris Acetate EDTA (TAE) buffer), stained with gel red (1:10,000) (Biotonium, Hayward, CA, USA) and visualised under UV light, using the AlphaImager system.

2.4. PCR purification and sequence analysis

Positive PCR amplicons for the *Sarcocystis* 18S rRNA gene were purified using a commercially available kit (Wizard® SV Gel and PCR Clean-up System Promega, Madison WI, USA), as per manufacturers' instructions. Samples were eluted in a final volume of 30 μl (DNase/RNase free water) and the nucleic acid concentration was determined by spectrophotometry (Nanodrop ND1000). For sequencing, 100 ng of the purified PCR amplicon was analysed (MWG Operon), with each primer (F1 and R1) to create an overlapping forward and reverse consensus. This consensus sequence was compared against previously published sequences using the NCBI-BLAST database.

3. Results

The only clinical sign observed in the ewe before its death was a progressive loss of body condition associated with weakness. At necropsy, approx. 100 whitish elliptic (3–5 mm to 5–8 mm) macrocysts with hard consistency were observed along the esophageal and pharyngeal muscular layers (Fig. 1A and B). The serous layer of the esophagus, at the level of the bronchi bifurcation, had translucent amber gelatinous appearance (edema) among macrocysts.

Microscopically, macrocysts consisted of an eosinophilic wall, internal septa originated from it and basophilic parasitic cells were located among the septa (Fig. 1C and D). A mild lymphohistiocytic infiltration was surrounding the macrocysts.

The PCR amplification for *Sarcocystis* DNA yielded positive results. Sequence analysis (BLAST) of the 227 bp consensus generated during this study demonstrated 100% identity to the *S. gigantea* (isolate S1.1) 18S rRNA gene sequence previously published in GenBank (KC209733) (Gjerde, 2013) The 227 bp consensus sequence was submitted to GenBank (accession number MG002175).

4. Discussion

Although sarcocystosis has been confirmed by molecular diagnosis in several ungulates in Argentina and Brazil (da Silva et al., 2009; Moré et al., 2013, 2016; Bittencourt et al., 2016; Reissig et al., 2016), and specifically *S. gigantea* is considered prevalent worldwide (Dubey et al., 2015; Kalantari et al., 2016; Pipia et al., 2016), *S. gigantea* infection in the Americas has been reported scarcely and based on morphological observations only (Dubey et al., 1988; Robles, 2007; Damboriarena et al., 2016). Whether the prevalence of *S. gigantea* is low or this species is underdiagnosed in this continent remains to be investigated. Nonetheless, different prevalences have been described from several areas (Britt and Baker, 1990). On the other hand, the lower prevalence of *S. gigantea*, in comparison with others *Sarcocystis* spp. could be explained because of the definitive host of this species. In this regard, *Sarcocystis* spp. transmitted via felids are less prevalent than those transmissible via canids, partially because cats are poor producers of sporocysts, and sarcocysts of feline transmitted species require several months in the host to become infective (Dubey et al., 2015).

Infected carcasses are commercially condemned (Martínez-Navalón et al., 2012; Dubey et al., 2015) even when *S. gigantea* is not a cause of zoonotic disease (Fayer et al., 2015). Our finding in an adult sheep is in agreement with previous studies (Bertero et al., 1980; Munday and Obendorf, 1984); nevertheless Bertero et al. (1980) did not find macrocysts in their epidemiological study in lambs because *S. gigantea* macrocysts are found mainly in adult sheep (Munday and Obendorf, 1984) like in this case. An earlier diagnosis in young sheep assessed by molecular techniques should be evaluated.

Microscopically, the sarcocysts found in this case are similar to

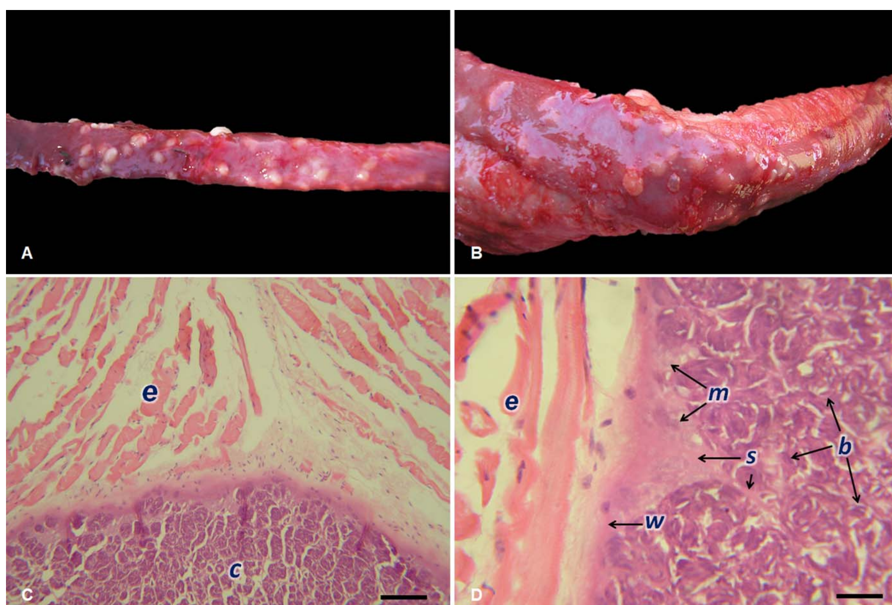

Fig. 1. A and B) Macroscopic sarcocysts in esophagus. C) Part of a cyst (c) in the esophagus muscle layer (e). H & E 100×. Barr = 100 μm. D) Esophagus muscle layer (e), cyst wall (w), internal septa (s), metrocytes (m), bradyzoites (b). H & E 400×. Barr = 20 μm.

those described by Dubey et al. (2015) for *S. gigantea*. Nevertheless, further structural description was not possible because neither periodic acid-Schiff (PAS) staining nor electron microscopy could be performed in this study.

Differential diagnosis may include *S. medusiformis* and *Sarcocystis moulei*. *S. medusiformis* also produces macrocysts in sheep and is transmitted by cats; however, its mature sarcocysts are smaller and more slender than those produced by *S. gigantea* and are primarily found in the diaphragm, abdominal muscles, and the carcass (Dubey et al., 2015). *Sarcocystis moulei* of goats is also morphologically similar to *S. gigantea* and could possibly infect sheep (Kalantari et al., 2016), however, in this case report the sequence obtained does not match with these species.

The large size of the tissue cyst containing millions of bradyzoites may favor the obtaining of an acceptable DNA quality and yield. Moreover a clean single band was amplified using the F1–R1 primers. Although a complete 18S gene sequence would be of a greater benefit, the formalin procedure limited the length of the PCR amplicons (309 bp). Fortunately there is a consensus sequence for 227 bp and sequencing for confirmation of *S. gigantea* infection was achieved. On the other hand, other engineered primers might have amplified a longer region but further procedures were not performed in this study, unfortunately.

To our knowledge, this is the first molecular confirmation of natural *S. gigantea* infection in sheep from the Americas. Studies examining the prevalence and economic impact of sarcocystosis in sheep and other ruminants in the Buenos Aires Province region should be made.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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