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Research paper

Unraveling *Sarcocystis miescheriana* and *Sarcocystis suihominis* infections in wild boar

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ABSTRACT

Sarcocystis species are worldwide spread cyst-forming protozoa that can infect wild boar but little is known about the prevalence of these parasites. In this study we assessed the prevalence of *Sarcocystis* spp. infections in wild boars from northeastern Portugal, for which novel PCR testing assays targeting *Sarcocystis* genus, *S. miescheriana* and *S. suihominis* were implemented, and risk factors potentially associated with these infections were evaluated. Samples from muscle tissue, namely diaphragm ($n = 102$), oesophagus ($n = 96$) and heart ($n = 101$), were collected from a total of 103 wild boar hunted between October 2011 and February 2012. Diaphragm muscle was used for the PCR detection of *Sarcocystis* nucleic acids since a higher proportion of samples showed the presence of cysts during histological examination. PCR assay targeting *Sarcocystis* genus yielded a 73.8% infection rate, which indicate a high level of exposure to these protozoan parasites among wild boars. These samples showed to be positive with the *S. miescheriana*-specific PCR assay and no sample was positive with the *S. suihominis*-specific assay, suggesting that a single species infecting wild boar is circulating in Portugal. These results were confirmed by the partial sequencing of the 18S rRNA gene amplified from selected samples from different geographic regions. Adults, young adults and female wild boars were found to be more likely infected. Hunters have an important role in the life cycle of *S. miescheriana* since potentially infected viscera and carcasses can be left behind promoting the protozoan dissemination to the scavenging final hosts. If hunting dogs bite and ingest infected meat they can perpetuate the life cycle of *Sarcocystis* spp. spreading oocysts or sporocysts in the environment.

1. Introduction

Protozoan parasites of the genus *Sarcocystis* are worldwide spread, cyst-forming coccidian known to infect farm animals like cattle, sheep and pigs (Dubey et al., 1989; Buxton, 1998). *Sarcocystis* spp. parasites have an obligatory two-host life cycle with mainly carnivores as definitive hosts and herbivores or omnivores as intermediate hosts (Dubey et al., 1989; Tenter, 1995). Different *Sarcocystis* species are assumed to be intermediate host specific, although, a single herbivore or omnivore may serve as the intermediate host of several species (Dubey et al., 1989; Heckeroth and Tenter, 2007). Swine can be intermediate hosts of *Sarcocystis miescheriana*, *Sarcocystis suihominis* and *Sarcocystis porcifelis*. However, the latter still requires confirmation since it has only been reported once (Dubey et al., 1989). Dogs, wolves, foxes and racoons are definitive hosts for *S. miescheriana*, man and non-human primates for *S. suihominis* and cats for *S. porcifelis* (Tenter, 1995). Wild boars and domestic pigs become infected after ingesting sporulated oocysts or free sporocysts shed by the definitive hosts. Both *S. miescheriana* and *S. suihominis* are considered pathogenic for wild and domestic pigs, naturally or experimentally infected, causing anorexia, fever, purpura, dyspnea, muscle tremors, constipation, alopecia and abortions, resulting in death in some occasions

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(Barrows et al., 1982; Daugschies et al., 1988; Li et al., 2007; Caspari et al., 2011). The pathogenic effects are associated with the second generation merozoites which are found in the endothelial cells of the heart, liver and kidney capillaries, causing bleeding of the affected organs (Avapal et al., 2004; Caspari et al., 2011; Chhabra and Samantaray, 2013). Mild infections are usually asymptomatic and associated with weight loss and poor body condition (Daugschies et al., 1988; Dubey, 1993).

The identification of *Sarcocystis* species is usually performed by the distinctive morphological features of the cyst: size, wall thickness and structure (Fayer, 2004). However, these features can vary with different stages of cyst development, location and also by the fixation methods used. Thus, the identification and description of *Sarcocystis* spp. based only in morphological features is not recommended (Tenter, 1995). Very few studies on *Sarcocystis* spp. occurrence have been performed in Europe in wild boar (Tropilo et al., 2001; Malakauskas and Grikienienė, 2002; Hvizdosová and Goldová, 2009; Coelho et al., 2014). Microscopic observation of histological sections (Dubey et al., 1989) or cyst isolation after digestion of a muscle sample (Heckeroth and Tenter, 2007; Goldová et al., 2008; Hvizdosová and Goldová, 2009) are methods often used for monitoring the occurrence of *Sarcocystis* spp. infection in wild boars. More recently, molecular studies based on PCR assays involving the sequencing of the 18S rDNA gene have been used to both differentiate *Sarcocystis* spp. and for epidemiological purposes (Fisher and Odening, 1998; Dahlgren et al., 2007; Dahlgren and Gjerde, 2007, 2008; Kia et al., 2011; Yan et al., 2013).

It is known from a recent preliminary report that *Sarcocystis* spp. infection occurs in wild boars from Portugal (Coelho et al., 2014). However, the species causing infection have not yet been identified. In the present study we assessed the prevalence of *Sarcocystis* spp. infections in wild boar from northeastern Portugal, for which novel nucleic acid testing assays targeting *S. miescheriana* and *S. suihominis* were implemented, and studied risk factors potentially associated with these infections.

2. Materials and methods

2.1. Sampling

Upon post-mortem analysis of wild boars hunted in the northeast of Portugal, between October 2011 to February 2012, samples from muscle tissue, namely diaphragm ($n = 102$), oesophagus ($n = 96$) and heart ($n = 101$), were collected from a total of 103 wild boar and kept refrigerated (4 °C) until further processing within 8 h. Information about geographic region of collection, gender and age of the animals were recorded. Wild boars age was determined based on the tooth eruption patterns and categorized in three age groups: juveniles (between 6 and 14 months old), young adults (between 15 and 25 months old) and mature adults (≥26 months old) (Saenz de Buruaga et al., 1991). Approximately 1 cm^3 of each muscle sample was fixed in 10% neutral-buffered formaldehyde for histological examination. Additionally, approximately 20 g of each sample was frozen at −20 °C for molecular analysis.

2.2. Histological examination

Diaphragm, oesophagus and heart samples were initially screened by histological analysis. Muscle tissues were processed using the conventional histological method. They were embedded in paraffin wax, sectioned at 3 μm and stained with Gill's hematoxylin and eosin, according to procedures previously described by Bancroft and Gamble (2002). Muscle sections were examined using a conventional light microscope at 200× and 400× magnification.

Table 1
Primers used to amplify and sequence parcial 18S rDNA gene.

Primer	Sequence (5′–3′)
Sar-F	AATAGAACCGAAATCCTATMTTG
Sar-R	CGCAAATTACCCAATCCTGAC
SmiesF	TGTCTCCCTATTGTTAATATTCTG
SsuihR	CTCCCACATTATCAATATAGACG

2.3. Digestion of muscle tissues and DNA extraction

The diaphragm muscle was used for molecular analysis since, when compared to oesophagus and cardiac muscle, a higher amount of wild boars were found to be positive for *Sarcocystis* spp. during histological examination. Due to the size limitation of the diaphragm sample from one animal, cardiac muscle was used instead.

A digestion step was performed. Briefly, 5 g of each sample were cut to smaller pieces with a sterile scalpel blade. The digestion of the muscle was performed at 37 °C for 20 min in a solution consisting of 150 ml of water acidified with 1.2 ml hydrochloric acid (25%) and 0.75 g of pepsin (1/10.000), with permanent stirring during 20 min. The digestion fluid was filtered and centrifuged at 2500 × *g* for 15 min. In order to concentrate the *Sarcocystis* spp. cysts the supernatant fluid was rejected and the sediment was washed with saline solution. This last procedure was repeated two times (Rommel et al., 1995). Genomic DNA was extracted from the sediment using a commercial kit (High Pure PCR Template Preparation Kit®, Roche, Germany), according to the manufacturer's instructions.

2.4. Primers design

Sequences of the 18S rRNA gene of *Sarcocystis* spp., including *S. miescheriana* and *S. suihominis*, were retrieved from GenBank-NCBI and aligned with the Clustal X software (Larkin et al., 2007). Primers were designed targeting conserved regions of the *Sarcocystis* gene, allowing the amplification of a fragment universal for the genus (Sar-F/Sar-R; Table 1). Additional species-specific primers were also designed in order to differentiate *S. miescheriana* (SmiesF, used with Sar-R) and *S. suihominis* (SsuihR, used with Sar-F). Validation of primers was achieved by testing DNA samples positive for *S. miescheriana* (kindly provided by Prof. Pozio, Istituto Superiore di Sanità, Italy) and a synthetic DNA identical to the *S. suihominis* 18S rRNA gene (containing the respective primer complementary regions). Samples positive for *Neospora caninum* and *Toxoplasma gondii* were also used to confirm the specificity.

2.5. Amplification assays

A PCR assay was implemented in order to amplify a 441 bp fragment of the 18S rRNA gene of *Sarcocystis* genus members. The reaction mixture contained 5 μl of DNA template extracted from muscle samples, 1 U of GoTaq® Flexi DNA Polymerase (PROMEGA, EUA), PCR buffer (1X) (PROMEGA; EUA), 2.5 mM $MgCl_2$ (PROMEGA; EUA), 10 mM dNTPs (PROMEGA; EUA) and 1 μM of each primer Sar-R and Sar-F, in a final volume of 25 μl. The PCR reaction was carried out in a thermocycler (MJ Mini Thermal Cycler®, BioRad, EUA) with the following program: 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 45 s at 72 °C and a final extension step of 10 min at 72 °C.

Muscle samples positive for *Sarcocystis* spp. were retested using the *S. miescheriana* and *S. suihominis*-specific primer sets. Primer pair SmiesF/Sar-R amplify a fragment of 352 bp only from *S. miescheriana*. The amplification assay was similar to the above mentioned but using 3.5 mM $MgCl_2$ in the reaction mixture and an annealing temperature of 57 °C. Primer pair Sar-F/SsuihR amplify