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Cross-sectional investigation on sheep sarcosporidiosis in Sardinia, Italy

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ABSTRACT

An epidemiological survey on sarcosporidiosis was carried out with a cross sectional investigation on macroscopic and microscopic *Sarcocystis* spp. in Sarda breed sheep slaughtered in different abbatoirs of Sardinia, Italy. For the macroscopic survey, muscular samples (diaphragm, abdominal and intercostals muscles, cutaneous muscles and muscles of the thigh) from 769 slaughtered Sarda sheep, oesophagus (n = 365) and laryngeal and pterygoid muscles (n = 521) were macroscopically investigated and Polymerase Chain Reaction (PCR) on selected macroscopic cysts was performed for a molecular identification of macroscopic *Sarcocystis* species. For the microscopic investigation 112 heart samples from slaughtered Sarda sheep were collected and investigated with two different protocols: unstained (compression) examination and a molecular technique. The overall prevalence of infection for macroscopic forms of sarcocysts was of 23.3% (179/769) with prevalences higher in the oesophagus (31.6%; 125/395) compared with the other investigated tissue type; two different morphotypes, classified as large oval (LO) macroscopic cysts, identified as *Sarcocystis gigantea*, and slender fusiform (SF) sarcocysts, were identified. The examination of heart samples revealed an overall prevalence of 77.7% (87/112) for *Sarcocystis* spp.; the nested-PCR analysis of heart samples allowed to identify the microscopic species, *Sarcocystis tenella* and *Sarcocystis arieticanis*, with prevalences of 95.5% (107/112) and 17.8% (5/112) respectively. Reported results highlight the high prevalence of *Sarcocystis* infection in the island and suggests the need of an improvement of control and prevention strategies for this parasitosis.

1. Introduction

Members of the genus *Sarcocystis* (Apicomplexa, Eimerinae, Coccidea, Sarcocystidae) (Dubey, 1988) are among the most common protozoan parasites of striated muscles of livestock such as cattle, sheep and goats (Mirzaei Dehaghi et al., 2013). These parasites have an obligatory two-host life-cycle with carnivores as definitive hosts and herbivores and omnivores as intermediate hosts (Tenter, 1995).

Four species of *Sarcocystis* (*Sarcocystis gigantea*, *Sarcocystis medusiformis*, *Sarcocystis tenella* and *Sarcocystis arieticanis*) have been described in sheep (Dubey et al., 1989), although two other species, *Sarcocystis mihoensis* and *Sarcocystis gracilis*-like, originally isolated in roe deer, were described also in this domestic small ruminant (Saito et al., 1997; Giannetto et al., 2005).

S. gigantea and *S. medusiformis* transmitted by felids, are generally considered non-pathogenic and produce macroscopically visible cysts (Bahari et al., 2014); while *S. tenella* and *S. arieticanis*, producing microscopic cysts (Bahari et al., 2014) are transmitted by canids and considered pathogenic (Heckeroth and Tenter, 1999). Dogs are also recognized as the definitive hosts of *S. mihoensis* and together with foxes of *S. gracilis*-like (Giannetto et al., 2005).

S. tenella, *S. gigantea* and *S. arieticanis* are distributed worldwide, while infections with *S. medusiformis* have been reported only from Australia, New Zealand, Iran and Italy (Heckeroth and Tenter, 1999; Scala et al., 2008).

Sheep become infected with *Sarcocystis* spp. by ingesting sporocysts with contaminated food or water. The presence of macroscopic *Sarcocystis* spp. in sheep, causes great concern to the meat industry as part or even the whole infected carcasses may be rejected for human consumption, resulting in serious economic losses (Dubey et al., 1988; Oryan et al., 1996). The microscopic species, on the other hand, may cause serious pathological condition in the infected animals, especially during acute forms and also result in heavy production losses (Fayer, 1976; Munday, 1979, 1986). The severity of the disease in sheep caused by *S. tenella* and *S. arieticanis* seems to be related with the dose of ingested sporocysts and of the immune status of the host (Heckeroth and Tenter, 1999). During the early multiplication of the parasites by endopolygeny, a primary infection with one of the pathogenic *Sarcocystis* species may lead to acute sarcosporidiosis with encephalitis, encephalomyelitis, and haemorrhagic diathesis which can cause the death of the animal (Heckeroth and Tenter, 1999). In pregnant sheep,

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acute *Sarcocystis* infection sometimes results in foetal death, abortion, or premature birth of the lamb (Munday, 1981; Fayer and Dubey, 1988). Sheep may be infected with different pathogenic and non-pathogenic *Sarcocystis* species at the same time (Gjerde, 2013).

The most common taxonomic criteria for the identification of *Sarcocystis* species, is the ultrastructure of the sarcocysts wall (Giannetto et al., 2005). Several authors have reported that the sarcocysts wall varies from being relatively simple to highly complex (Dubey et al., 1988; Obendorf and Munday, 1987; O'Toole, 1987; Mehlhorn et al., 1975).

Nevertheless in recent years molecular methods have been used for the identification of *Sarcocystis* spp., particularly those infecting domestic animals (Gjerde, 2013; Tenter, 1995). Several molecular studies on *Sarcocystis* spp., have been carried using nuclear ribosomal DNA unit, particularly the small subunit (18S) rRNA gene (Gjerde, 2013).

Several reports on the prevalence of ovine *Sarcocystis* infection have revealed that this parasitosis is still common even in developed countries (Mirzaei and Rezaei, 2014; Mirzaei Dehaghi et al., 2013; Savini et al., 1992) particularly in regions where sheep breeding is still carried out with extensive methods, like Mediterranean countries. Within this area, Sardinia plays an important role as epidemiological observatory as >3,300,000 (Ministero della Salute, 2013) sheep are raised with traditional extensive methods in the island. Many parasitological diseases, including zoonosis, are still to date widespread in Sardinia due the isolation of animals and parasitic population due to insularity but also for political, cultural and also breeding methods (Varcasia et al., 2011).

In Sardinia, (Italy) Scala and Nieddu, 1990, reported a prevalence of 66% for *S. gigantea*. This specie is commonly found in the oesophagus and other localization (as skeletal muscles, diaphragm, heart, tongue and larynx) of slaughtered sheep (Bahari et al., 2014). Furthermore the same authors found microscopic cysts with prevalences ranging between 36% to 81% in the oesophagus and heart respectively (Scala and Nieddu, 1990).

Despite this, data on epidemiology and molecular characterization of sheep sarcosporidiosis are quite outdated and mainly present in grey scientific literature (regional papers in Italian).

Hence, the goal of this study was to fill this gap of knowledge on sheep sarcosporidiosis with a cross-sectional investigation on macroscopic and microscopic species.

2. Material and methods

2.1. Macroscopic sarcocysts

During 2013 a total of 769 Sarda breed sheep, females, aged between 3–7 years slaughtered in 4 abattoirs (Thiesi (SS) n = 282; Tula (SS) n = 157; Settimo S. Pietro (CA) n = 103; Nule (NU) n = 227) of Sardinia Island, Italy, were investigated for *Sarcocystis* infection. During the post-mortem inspection, diaphragm, abdominal and intercostal muscles, cutaneous muscles and muscles of the thigh were examined for the detection of macroscopic cysts of *Sarcocystis* spp. In addition, the oesophagus and laryngeal/pterygoid muscles were investigated in 365 and 521 of these 769 sheep, respectively. Macroscopic cysts were classified according to Dubey et al. (1989).

In order to confirm the taxonomy of macroscopic cysts, a molecular study was carried out on 30 individual cysts samples isolated during the macroscopic examination. Individually isolated sarcocysts were washed twice with distilled water, placed in 1.5 ml Eppendorf tubes with ethanol 70% and stored at −20 °C until DNA extraction with a commercial kit (PureLink® Genomic DNa Mini Kit – Invitrogen, USA), according to the manufacturer's instructions. Only the partial 28S and 18S rRNA genes were amplified using the primers pairs KL5a (5′- GAC CCT GTT GAG CTT GAC 30) and KL2 (5 ACT TAG AGG CGT TCA GTC - 3′) and 1L (5'CCATGCATGTCTAAGTATAAGC-3′) and 1H (5′-TATCCCCATCACGATG CATAC-3′) as described by Mugridge et al. (1999) and Yang et al. (2001). Polymerase chain reaction (PCR) was performed in a 25 μl total volume containing 2.5 μl 10 × PCR buffer, 2.5 μl 2 mM dNTP mix, 0.1 μM each primer, 0.5 μl *Thermus aquaticus* DNA Polymerase (Thermo Scientific), 2.5 μl MgCl 2, 0.2 μg genomic DNA and the remaining volume of water. Following the initial 3 min denaturation step at 94 °C, 30 amplification cycles were carried out at 94 °C for 45 s and at 65 °C (62 °C using primer pairs 1L/1H) for 45 s, extension at 72 °C for 1 min and a final extension of 5 min at 72 °C. PCR products were purified with a commercial kit (High Pure PCR Product Purification Kit, Roche) and sequenced through an external service (MWG Eurofins). Obtained sequences were then first compared with BLAST databases (http://blast.ncbi.nlm.nih.gov/Blast.).

2.2. Microscopic sarcocysts

During the same period, a further investigation was carried out on other 112 Sarda breed sheep coming from 4 abattoirs of Sardinia (Thiesi (SS) n = 40; Tula (SS) n = 20; Settimo S. Pietro (CA) n = 18; Nule (NU) n = 34) for the identification of microscopic *Sarcocystis* spp., with two different protocols, light microscopy of unstained samples and molecular techniques. Heart was chosen as target organ according to previous studies (Pérez-Creo et al., 2013; Wheater et al., 1987).

All samples were examined immediately after slaughtering at light microscopy for the presence of microscopic species of *Sarcocystis* as described by Fukuyo et al. (2002). For each heart samples two samples of muscle tissue were obtained; from the atrioventricular septum (AVS) and from the left ventricle (LV). About 0.5 g of muscles (2 mm × 8 mm) were cut and squashed between two glass slides and examined by light microscopy (×100) and other 10 g of each samples were stored for the further molecular investigations. In this step of the survey, 10 g of tissue were processed from all 112 heart samples. After homogenization, 0.05 g of each sample was processed for the DNA extraction with a commercial kit (PureLink® Genomic DNa Mini Kit – Invitrogen, USA). A nested PCR targeting the multicopy 18S rRNA, was performed on all samples as previously described by Heckeroth and Tenter (1999). The nested PCR (ST-nested-PCR) for *S. tenella* was performed using the external primer pair ST1(5′- GGA TCG CAT TAT GGT CAT-3′) AP2 (5′- CCC GGG ATC CAA GCT TGA TCC TTC TGC AGG TTC ACC TAC-3′) and the nested primer pair 8 (5′ -TTT GAC TCA ACA CGG G-3′) and ST3 (5′ CGT TGC CGC GCG TTA A-3′). For *S. arieticanis* the nested PCR was performed using the external primer pair STA (5′-TTT CGC AAG GAA GAG GA -3′) and SA2 (5′- TGA AAC GGC GCG TAG A-3′) with the internal primers 2 (5′- AGG GTT CGA TTC CGG AG -3′) and SA1(5′- GCG GGA AGA GGA GAA T-3′). The PCR was performed in a 100 μl reaction volumes containing 10 mM Tris-HCl, 50 mM potassium chloride, 0.1% Triton X-100, 1.75 mM magnesium chloride, 0.1 mM each of deoxynucleotide triphosphate (dNTP), 100 pmol of each primer and 1.5 U of *T. aquaticus* DNA Polymerase (Thermo Scientific). Amplifications were carried out in an GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystem, USA) using the following PCR protocol conditions: initial denaturation at 94 °C for 4 min, 26 cycles at 93 °C for 2 min, 57 °C for 2 min, 72 °C for 2 min, with a final extension step of 5 min to complete the process.

PCR products were separated on 1.5% agarose gel and visualized after staining with Gel Red to check for appropriately sized product. PCR products were then purified using the NucleoSpin Gel and PCR clean up (Machery-Nagel, Germany) and sent to an external sequencing service (Eurofins MWG Operon, Germany). Sequences analysis was performed as described above for macroscopic *Sarcocystis* spp.

2.3. Statistical analysis

Data were analysed using the statistical package Epi-Info (version 7.0, CDC/WHO, Atlanta, GA, USA). The Chi-squared test (χ^2) was used to determine significant differences on prevalences between the different examined tissues. Differences were considered with statistical significance when the P value was <0.05. Diagnostic tests agreement (compression method vs PCR) was evaluated using K statistic test.