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 thedefinitive 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,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 N 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 postmortem 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′ -TATCCCCATCACGATGCATAC-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 TGCCGC 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 GGAGAA 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 (χ2) was used to determine significant differences on prevalences between the different examined tissues. Differences were considered with statistical significance when the P value was b 0.05. Diagnostic tests agreement (compression method vs PCR) was evaluated using K statistic test.

3 Results

3.1. Macroscopic sarcocysts

The overall prevalence of infection for macroscopic Sarcocystis spp., in examined sheep was of 23.3% (179/769). Prevalence of Sarcocystis spp., infection between the 4 abattoirs across the island are summarized in Table 1. In particular, it was possible to isolate two different morphotypes, identified as large oval (LO) macroscopic cysts (Fig. 1) and recognized as S. gigantea with a mean length of 7308.5 ± 1686.8 µm and a mean width of 5421.25 ± 1274.6 µm and slender fusiform (SF) sarcocysts with an average length of 7704.75 ± 549.9 µm and a mean width of 170.25 ± 30 µm (Fig. 2) localized especially in skeletal muscles and being similar to S. medusiformis.

Detailed prevalences of infection and Sarcocystis spp., found in the examined tissues are shown in Table 2 with the respectively Odds Ratio values. Differences were statistically significant (χ2 with 6 degrees of freedom = 336.11; P b 0.05). Oesophagus was the most infected tissue (31.6%; 125/395), followed by abdominal muscles (20.1%; 157/ 769). Macroscopic cysts classified as LO were mostly observed in the oesophagus (31.6%; 125/395) while SF cysts were mainly found in the abdominal muscles (12.3%; 95/769). Difference was statistically significant (χ2 = 63.36; P b 0.05).

The DNA extracted from the LO (N = 5) and SF (N = 4) macroscopic cysts was used as a template for genetic characterization of the two different macroscopic sarcocyst types: primers targeting the large ribosomal subunit (lsu) and the small ribosomal subunit of the rRNA gene yielded an 800 bp and 500 bp fragment, respectively, for all the samples. Type LO sarcocysts were identified as S. gigantea (showing a 99% nucleotide sequence identity for both genes with the sequences deposited in GenBank by Mugridge et al. (1999) and Gjerde (2013) (GenBank accession number: U85706.1 and KC209733.1) respectively. Type SF sarcocysts showed a 94% nucleotide sequence similarity with S. gigantea and S. moulei for both genes. The SF Sarcocystis could be considered consistent with S. medusiformis as described by Dubey et al. (1989) and Tenter (1995) though the sequences could not be compared with S. medusiformis, as sequences derived from this parasite are not present on GenBank. All sequences obtained from S. gigantea and S. medusiformis were submitted to GenBank and given the accession numbers (Genbank ID: KX223753; KX223754).

3.2. Microscopic sarcocysts

Sarcocysts were found in the 77.7% (87/112) of examined heart samples; no statistical difference in the frequency of sarcocysts between the two examined sites, AVS (63.4%; 71/112) and LV (51.8%; 58/112) was observed (χ2 = 3.09; P N 0.05). In Table 2 are detailed prevalences of infection found in the different abattoirs.

The nested-PCR analysis of heart samples allowed to identify the microscopic species S. tenella and S. arieticanis, with prevalences of 95.5% (107/112) and 17.8% (5/112) respectively; the difference between the prevalences of the two species was statistically significant (χ2 = 137.63; P b 0.05).
The alignment of our sequences with BLAST showed an homology of 100% and 99% within our samples sequences (Genbank ID KX223751; KX223752) and those of S. tenella (KP263759.1) with S. arieticanis (L24382.1) respectively, available in GenBank.
The 79.5% (83/112) of the examined heart samples tested positive both at the light microscopy method and PCR. The 21.4% (24/112) of heart samples were positive to PCR and negative to light microscopy. Conversely, the 3.6% (4/112) of heart samples were positive at light microscopy and at the same time PCR negative. No agreement between the two test was observed (K b 0.01).

4. Discussion

The results herein reported indicate the high distribution of macroscopic Sarcocystis spp., infection in slaughtered sheep of Sardinia, Italy. The morphological classification of the cysts allowed us to identify two macroscopic forms identified as LO and SF with morphological features comparable with those described by Dubey et al. (1989) and Tenter (1995) for S. gigantea and S. medusiformis, respectively. LO cysts were mainly localized in the oesophagus (57%) while SF cysts in the abdominal muscles (12.3%). This finding is in accordance with those reported by other authors (Oryan et al., 1996) and suggests that during the official inspection of the carcasses at slaughterhouses, abdominal muscles should be primarily inspected by the veterinary for Sarcocystis spp., infection. Molecular methods applied on the two morphotypes enabled to characterize for the first time in the island LO cysts as S. gigantea while it was not possible to confirm the morphological description of SF cysts as S. medusiformis due to the lack of sequences in the databases. Furthermore with the present survey it was possible to report and characterized by the molecular biology, for the first time in Sardinia the presence of the microscopic species, S. tenella and S. arieticanis.
On the evidence of the good performances obtained by the use of the light microscopy unstained (77.7% of prevalences vs 95.5% for the nestedPCR; χ2 Yates corrected = 13.89; P b 0.05) this technique simple, inexpensive and rapid could be considered as a useful tool suitable in the routine laboratory practice. On the other hand PCR assays may increase the detection sensitivity of Sarcocystis spp. and contribute to diagnostic precision (Mirzaei Dehaghi et al., 2013). The specificity of the 18S rRNA was previous confirmed by many authors that considered as a powerful tool for species-specific differentiation of the ovine Sarcocystis species (Heckeroth and Tenter, 1999). In our study S. tenella, one of the most pathogenic species in sheep (Dubey, 1988), was reported with a high prevalence (95.5%). Similar results had previously reported in other countries: 91.7% in Romania (Titilincu et al., 2008); 86.5% in Turkey (Beyazit et al., 2007), France 94.8% (Diéz-Baños, 1978), 87.6% in Slovaki (Mala and Baranova, 1995) 96.9% in Mongolia (Fukuyo et al., 2002); 97.0% in Iraq (Latif et al., 1999); and 76% in Italy (Giannetto et al., 2005).

5. Conclusion

The presence of macroscopic and microscopic sarcocysts in Sarda sheep is an important epidemiological data furthermore their presence seems to be evenly spread across the island. The lower frequency of macroscopic sarcocysts infection herein reported (23.3%) compared with microscopic sarcocysts (95.5%) may be due to the fact that Sardinian sheep for breeding reasons have more contact with dogs (shepherd dogs) than with cats (Varcasia et al., 2011). On the other hand Sarcocystis transmitted by felids play an important role for the monitoring of another important zoonosis like Toxoplasma gondii, that have the same lifecycle.
Although the macroscopic species seems to play a marginal pathogenic role, their presence in various organs causes important economic losses in the meat industry (Oryan et al., 1996) because during the official veterinary control at slaughterhouses, carcasses with a massive infection with macroscopic cysts must be condemned by veterinarians (Bahari et al., 2014; Reg CE 854/2004). On the other hand the high prevalences of the microscopic species could have an important impact on sheep production (Oryan et al., 1996) due to the negative effects on the growth and weight gain of the animals with heavy production losses and their role as a cause of abortion (Munday, 1979, 1986; Fayer and Dubey, 1988).
For these reasons, Sarcocystis spp., in sheep should be routinely investigated during clinical practice by practitioners, considering also that 47% of sheep abortions in Sardinia remains without a specific diagnosis (Firinu, 1989).
Molecular diagnosis has proved to be as a sensitive technique for specie-specific differentiation of Sarcocystis species and also other sheep protozoa like Toxoplasma and Neospora (Tamponi et al., 2015) confirming the importance of the molecular techniques for epidemiological and diagnostic surveys. Reported results highlight the high prevalence of Sarcocystis spp., infection in the island and suggests the need of an improvement of control and prevention strategies for these parasites.

Conflict of interest

The authors declare there is no conflict of interest which affects the outcome of this paper.

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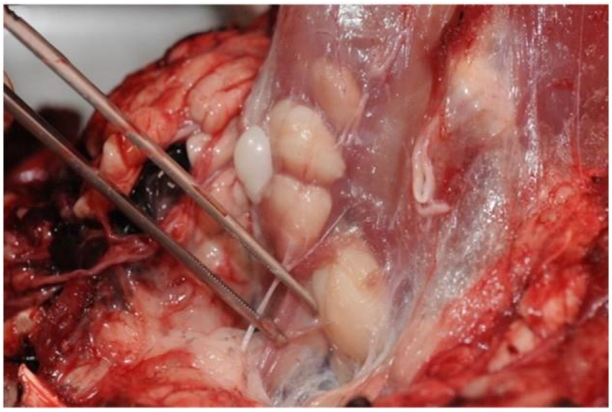

Fig. 1. Large oval (LO) macroscopic cysts.

Fig. 2. Slender fusiform (SF) sarcocysts localized in skeletal muscles.

Table 1

Prevalences of infection and Sarcocystis spp., found in the examined tissues.

Abattoirs	Macroscopic survey			Microscopic survey		
	Number of sheep	Number of positive sheep	P (%)	Number of sheep	Number of positive sheep	Prevalence of infection
Thiesi	282	55	19.5	40	29	72.5
Tula	157	34	21.6	20	16	80.0
Sinnai	103	26	25.2	18	14	77.8
Nule	227	64	28.2	34	28	82.3
Statistical analysis	χ^2 with 3 degrees of freedom = 5.78; P N 0.05			χ^2 with 3 degrees of freedom = 1.11; PN0.05		

Table 2

Prevalences of macroscopic and microscopic Sarcocystis spp. between abattoirs across the island.

Muscle tissue	Examined sheep (n)	Positive (n)	(P) %	Odds ratio	LO cysts (S. gigantea-like)		SF cyst (S. medusiformis-like)	P (%)
					n of positive sheep	P (%)	n of positive sheep	
Superficial muscles of the thigh	769	15	2.0%	0.33	6	0.8%	13	1.7%
Diaphragm	769	44	5.7%	1.00	17	2.2%	31	4%
Cutaneous muscles	769	63	8.2%	1.47	0		63	8.2%
Intercostal muscles	769	64	8.3%	1.50	28	3.6%	47	6.1%
Laryngeal[a] and pterygoid muscles	521	84	16.1%	3.17	84	16.1%	6	1.5%
Abdominal muscles	769	157	20.4%	4.23	90	11.7%	95	12.3%
Oesophagus[a]	395	125	31.6%	7.63	225	57%	5	1.3%

[a] Tissues not always available during the slaughterhouse inspection; LO cyst = large oval; SF cyst = slender fusiform (Dubey et al., 1989).