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Molecular differentiation of *Sarcocystis miescheriana* and *Sarcocystis suihominis* using a new multiplex PCR targeting the mtDNA cox1 gene in wild boars in southern Italy

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ABSTRACT

The increase of wild boar populations density and their meat consumption across Europe could expose humans to a plethora of foodborne diseases as sarcocystosis, caused by the zoonotic protozoan *Sarcocystis suihominis*. Humans become infected by eating raw or undercooked pig (*Sus scrofa domesticus*) containing *S. suihominis* sarcocysts. Despite this, to date very few data are available on the risk of infection by this parasite to wild boar (*Sus scrofa*) meat consumers. Thus, the present study aimed to assess the occurrence of *Sarcocystis* spp. in wild boars from southern Italy, applying both histology and a new multiplex PCR assay targeting the *cox1* gene. Between 2019 and 2020, 997 muscle tissues (i.e., $n = 269$ oesophagus, $n = 277$ diaphragms, $n = 298$ hearts, $n = 153$ tongues) from 311 wild boars were collected and screened by a combined histological and molecular approach. Overall, 251 (80.7%) animals tested were positive for *Sarcocystis* spp., and *S. miescheriana* whose definitive hosts are canids, was the only molecularly identified species. A statistically significant difference ($p < 0.05$) in the prevalence of *Sarcocystis* infection was found according to the wild boar age and muscle tissue. Findings outlined the low zoonotic potential of infection to humans via wild boar meat consumption in Italy and the importance of the application of new molecular methods in distinguishing different *Sarcocystis* species.

1. Introduction

During the last decades, a complex network of biological, environmental, and anthropic aspects provided a significant increase of the wild boar (*Sus scrofa*) populations density in most of Europe, with a high impact on human activities (Massei et al., 2015; Troiano et al., 2021). This overabundance led to an increasing boar consumption, to date popular not only among hunters and their families, but also in the international trade (Acevedo et al., 2014; Fredriksson-Ahomaa, 2019; Guardone et al., 2022) potentially enhancing the human exposure to foodborne pathogens, mainly through raw/undercooked meat and meat products (e.g., sausages) (Fredriksson-Ahomaa, 2019). Indeed, as known, wild boars can act as reservoirs of several zoonotic agents (Meng et al., 2009; Fredriksson-Ahomaa, 2019; Sgroi et al., 2020) including protozoan parasites of the genus *Sarcocystis* (Apicomplexa: Sarcocystidae), which infect mammals, birds, and reptiles (Shams et al., 2022). The life cycle of *Sarcocystis* spp. is based on a prey-predator relationship, usually characterized by a predator (mainly carnivores and omnivores) as definitive host and a prey as intermediate host (Rosenthal, 2021). While the definitive host harbours the sexual development of the parasite in the gut, producing oocysts that are excreted in the environment (Dubey, 2015), the intermediate host harbours the asexual parasitic

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stage, which develops in mature cysts containing bradyzoites in striated, cardiac, and smooth muscles (Poulsen and Stensvold, 2014; Rosenthal, 2021). The definitive host becomes infected ingesting mature sarcocysts containing bradyzoites, while the intermediate host becomes infected by the ingestion of sporocysts through food or water (Dubey, 2015). Domestic and wild swine are intermediate hosts of two *Sarcocystis* species, *Sarcocystis miescheriana* (*syn. Sarcocystis suicanis, Sarcocystis porcicanis*) of which the definitive hosts are wild and domestic canids (mainly dogs, foxes, wolves, jackals, raccoons) and *Sarcocystis suihominis* (*syn. Sarcocystis porcihominis*), of which the definitive hosts are humans and non-human primates (Dubey et al., 2015). A third species, *Sarcocystis porcifelis* (*syn. Sarcocystis suifelis*), was identified for the first time in the former Soviet Union in the '80s, although its description and taxonomy are still uncertain (Dubey, 2015; Guardone et al., 2022). Among the above-mentioned species, *S. suihominis* is a protozoan of zoonotic concern, which can infect humans by the ingestion of raw or undercooked pork meat (Rosenthal, 2021). Although infections are commonly asymptomatic, diarrhoea, nausea, anorexia, fever, and headache (probably due to a toxin released by bradyzoites) can occur in infected people, being the severity of clinical signs likely related to the amount of meat ingested (Dubey, 2015; Fayer et al., 2015; Rosenthal, 2021). Although the surveillance of zoonotic *Sarcocystis* species in meat is recommended by the European Commission, 2003 (section: "Other zoonoses and zoonotic agents - European Commission, 2003"; Taylor et al., 2010), to date, very few molecular reports of *S. suihomins* from Europe are available in wild boars (Calero-Bernal et al., 2016; Gazzonis et al., 2019).

In wild and domestic swine, *Sarcocystis* spp. can cause symptoms such as anorexia, fever, purpura, dyspnoea, muscle tremors, alopecia, weight loss and abortions, or can cause subclinical infections depending on the number of oocysts ingested (Daugschies et al., 1988; Reiner et al., 2002; Caspari et al., 2011). A case of fatal infection was reported in naturally infected domestic pigs (Caspari et al., 2011). Furthermore, a first report of macroscopic sarcocystosis due to *S. miescheriana* in a domestic pig leading to carcass condemnation has recently been documented in Italy (Rubiola et al., 2023).

Sarcocystis miescheriana and *S. suihominis* can be detected and differentiated in wild and domestic pigs through observation of the sarcocyst wall using both light and electron microscopy (Poulsen and Stensvold, 2014; Rosenthal, 2021). *Sarcocystis miescheriana* presents tightly packed, erect, finger-like cyst wall protrusions (cyst wall 3 to 6 μm) while *S. suihominis*, have longer and thinner hair-like protrusions along the cyst surface (cyst wall 4 to 9 μm) (Dubey, 2015). In recent years, molecular assays have been increasingly applied, allowing a more accurate and reliable identification of *Sarcocysts* spp. using different target genes. Although most studies have focused on the 18S ribosomal RNA (rRNA) gene (Coelho et al., 2015; Calero-Bernal et al., 2015, 2016; Moré et al., 2016; Imre et al., 2017), cytochrome C oxidase subunit I mitochondrial (mtDNA *cox1*) gene has proven to be one of the most promising tools to differentiate closely related *Sarcocystis* spp. having ungulates as intermediate hosts (Gjerde, 2013; Helman et al., 2022; Huang et al., 2019; Rubiola et al., 2020; Prakas et al., 2020a). In this regard, the molecular characterization of *S. miescheriana* and *S. suihominis cox1* gene has recently been provided, thereby paving the way to use this molecular marker as a novel diagnostic tool for the identification of *Sarcocystis* spp. in wild and domestic swine (Gazzonis et al., 2019).

As far as we know, the presence of *Sarcocystis* spp. in wild and domestic pigs in Italy has been poorly investigated and dated. Based on morphological examination, Piergili-Fioretti et al. (1985) reported a prevalence of 66.6% of *S. miescheriana* in wild boars in central Italy, while up to 74.4% of wild boars resulted positive in Italian islands (Sardinia, Leoni et al., 1995 and Sicily, Gaglio et al., 2012). So far, only one study investigating the occurrence and prevalence of *S. miescheriana* and *S. suihominis* in wild boars in northern Italy using molecular methods has been published (Gazzonis et al., 2019), reporting a high prevalence rate of *S. miescheriana* (97%) and a much lower prevalence of the zoonotic *S. suihominis* (1%), which was detected in a single wild boar (Gazzonis et al., 2019).

Considering the lack of data on *Sarcocystis* infection in wild boars, the aims of the present study were i) to perform a epidemiological survey on *Sarcocystis* spp. in wild boars in southern Italy, ii) to compare the prevalence and intensity of *Sarcocystis* spp. infection in analysed samples according to locality, sex and age of animals, and the type of muscles examined, and ii) to set up a multiplex-PCR (mPCR) to quickly discriminate the two currently known species, thus providing a useful molecular tool for the surveillance of the *Sarcocystis* spp. in domestic and wild swine.

2. Material and methods

2.1. Sample size

A sample size of 311 wild boars was calculated using the opensource software OpenEpi (Dean et al., 2003), inserting the following information: study population (84,000 wild boars; data supplied by *Piano Emergenza Cinghiale in Campania* - PECC 2016–2020), expected prevalence of *S. miescheriana* (97%) according to results from a recent molecular survey on wild boars from northern Italy (Gazzonis et al., 2019), confidence interval (99%) and desired absolute precision (1%).

Within a regional health plan PECC 2016–2020 sixteen veterinarians specialized in meat inspection were involved in the field to examine the boar carcasses.

The animals were collected in accordance with Italian and EU legislation, during the hunting seasons and with routine sanitary surveillance. Consequently, ethical approval was not deemed necessary.

For each wild boar, a specific form was filled, including hunting area, sex and, age. The age of animals was estimated by teeth examination (Massei and Toso, 1993), classifying wild boars into piglets (<1 year) (n° 31–10.0%); yearlings (1–2 years) (n° 129–41.5%) and adults (>2 years) (n° 151–48.6%).

Between 2019 and 2020, a total of 311 wild boar carcasses, 156 males (50.2%) and 155 females (49.8%) were collected from 4 provinces (i.e., n° 62, Avellino, n° 38, Benevento, n° 25, Caserta, n° 186, Salerno) of Campania region, southern Italy.

Based on the availability and condition of each carcass, a total of 997 muscle tissues (n° 269 oesophagus, n° 277 diaphragms, n° 298 hearts, and n° 153 tongue) were collected. Samples were delivered to the Department of Veterinary Medicine and Animal Productions (University of Naples, Italy) for the histological analyses.

2.2. Histological examination

All tissues were first sectioned, fixed with 10% formalin, paraffin-embedded for routine histological processing, and stained with hematoxylin and eosin for light microscopic examination. For each tissue, the presence and the number of cysts, and the associated histologic lesions were determined. In addition, individual cysts were identified and photographed using a Nikon Eclipse Ci- L plus at a high magnification of 400×. The longitudinal and transverse sections, wall thickness, and area occupied in the affected tissue were measured for each evaluable cyst using image analysis software (Imagej software). For each histologically positive sample, an aliquot of 25 mg was delivered to the laboratory of food inspection, Department of Veterinary Science (University of Turin, Italy) for the molecular analyses.

2.3. Molecular analysis

The DNA extraction was performed using the DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's tissue protocol. The lysis step was carried out at 56 °C overnight with Proteinase K. DNA samples were eluted in 50 μl of Elution Buffer and

kept frozen at −20 °C until further analysis. A new mPCR targeting the mtDNA *cox1* gene was set up to differentiate *S. suihominis* and *S. miescheriana* DNA. The partial sequences of *S. suihominis* and *S. miescheriana* mtDNA *cox1* gene available from GenBank (accession numbers: MH404228.1; MH404185-MH404227; MT070614-MT070635) were aligned together and examined for the presence of species-specific regions suitable for primer designing; to evaluate possible cross-reactions, the sequence of a phylogenetically related species (*Toxoplasma gondii*) was also aligned. The novel specific primer set was designed using Primer3Plus software (Untergasser et al., 2012). Based on the alignment results, three primers were designed to distinguish *S. suihominis* from *S. miescheriana*: a single common forward primer (*Cox1* SM) and two specific reverse primers (*Cox1* S - *S. suihominis* specific primer and *Cox1* M - *S. miescheriana* specific primer) (Table 1), resulting in ~400 bp and ~ 140 bp amplicons, respectively. Specificity of the primers was tested in silico using Primer-BLAST tools in NCBI (Ye et al., 2012); primers were synthesized by Sigma Aldrich (St. Louis, MO).

The primer set in vitro testing was performed on control DNA samples of *S. suihominis* and *S. miescheriana* previously isolated from the diaphragm of wild boars in north-western Italy (Gazzonis et al., 2019). The common forward and the different reverse primers were first assessed separately and then combined in a multiplex set-up to various compositions of PCR mixes and cycling conditions. The final PCR mixture contained 2.5 µl of template DNA (5–20 ng/µl), 0,5 µM of each primer, 2 mM MgCl2, 0.2 mM of each dNTP, 1 U Platinum Taq DNA polymerase, 10 x PCR Buffer and distilled water to a total volume of 25 µl. The PCR assay involved a denaturation step at 95 °C for 3 min, followed by 35 cycles at 95 °C for 60 s, 56 °C for 60 s and 72 °C for 30 s and final extension 72 °C for 3 min. A collection of *S. miescheriana* positive samples isolated from pigs and wild boars striated muscles in the Department of Veterinary Science of Turin University was used to further evaluate the sensitivity and specificity of the mPCR assay, together with a negative control (DNA from *Toxoplasma gondii*). PCR products were run on a 2% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA) and visualized using a Bio-Rad Universal Hood II Gel Imager (Bio-Rad Laboratories, Hercules, California, USA). Generated PCR products were sequenced to test the specificity of the mPCR assay: amplification products were purified through Exo-Sap treatment (USB Europe, Staufen, Germany) according to the manufacturer's instructions. Sequencing reactions were performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Sequenced fragments were purified using DyeEx Spin Kit (QIAGEN, Hilden, Germany) and run on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Following in silico and in vitro testing, the new mPCR assay was applied on each pool of tissue cysts-positive samples previously subjected to DNA extraction.

2.4. Statistical analysis

Confidence interval (95% CIs) and odds ratio (ORs) values were calculated for the proportions of infection herein found. The variability of cysts measurements was assessed by standard deviation values (± SDs). A chi-squared test was performed to compare the prevalence according to sex, hunting areas of wild boars, age and muscle tissues. Values of $p < 0.05$ were considered significant (Table 2). A *t-student* was carried out to compare the intensity of infection (mean of cysts number) in relation to muscle distribution. Values of $p < 0.05$ were considered significant (Table 3).

Table 1
Features of the mPCR used in this study.

Primer	Position (bp)	GenBank accession n.	Primer sequence (5′-3′)	Reference
Cox1 SM	363–382	MH404228.1	GAGCACCAATATCATGACGA	present study
Cox1 S	764–747	MH404228.1	ACGGATTTCGGGCTTAAC	present study
Cox1 M	504–487	MH404227.1	TCGAAGCGAACTGACTGC	present study

3. Results

Out of 311 wild boars, 251 (i.e., 80.7%, 95% CI: 76.3–85.1) were positive for *Sarcocystis* spp. by histological examination. The muscle sarcocysts shown an average circumference of 265.7 µm (± SD: 152.3, range 80.05–860.60 µm) with a mean area of 4671.00 µm^2 (SD: 4040.0, range 253.01–18,360.91 µm^2), and a highly variable range depending on their maturation stage (i.e., the content of bradyzoites and metrocytes). The major axis of the sarcocysts found had an average value of 96.7 µm (±SD: 71.8, range 27.2–394.7 µm), while the minor axis of the muscular cysts of 53.6 µm ± SD 19.3 (range 16.5–94.4 µm). The measured outer wall had an average value of 2.18 µm (± SD: 1.05, range 0.67–5.57 µm). In addition, microscopic observation allowed to differentiate immature cysts, characterized by a thick wall with metrocytes and generally smaller than mature cysts. The morphological description of sarcocysts with a thick of finger-like protrusions on the surface, filled with banana shaped bradyzoites, was consistent to *S. miescheriana*.

A statistically significant difference ($p < 0.05$) in the prevalence of infection according to age and muscle tissues was reported (Table 2). Based on age, piglets were less infected (61.3, 95% C.I. 44.1–78.4) compared to yearlings and adult wild boars (81.4% and 84.1%, respectively). Higher, but statistically insignificant ($p = 0.14$) prevalence was obtained in males (84.0%, 95% CI: 78.2–89.7) than in females (77.4%, 95% CI: 70.8–84.0). The positivity in the muscles showed a prevalence of 51.0% in heart (152/298, ± SD: 45.3–56.7), 44.2% in oesophagus (119/269, ± SD: 38.3–50.2), 41.9% in diaphragm (116/277, ± SD: 36.1–47.7) and 30.7% in tongue (47/153, ± SD: 23.4–38.0) with a statistical difference for heart (OR: 1.44) and oesophagus (OR: 1.10).

The mean number of sarcocysts for each wild boar was 3.4 (± SD: 5.8, variation = 1–55). In detail, for oesophagus the cysts mean was 2.2 (± SD: 3.2, variation = 1–34), 2.4 in diaphragm (± SD: 2.1, variation = 1–14), 2.3 in heart (± SD: 2.3, variation = 1–21) and 2.9 in tongue (SD: 4.2, variation = 1–29) (Table 3). There were no statistically significant differences regarding the intensity of infection in relation to sex, age, and muscle tissues.

No inflammatory reaction associated with parasitosis was observed in most tissues examined. In rare cases myositis due to muscle cell breakdown characterized by infiltration of eosinophils, lymphocytes, and macrophages was observed. The inflammatory infiltrate appeared uniformly distributed in the endomysium or, less commonly, organized

Table 2
Sarcocystis spp. infection according to variables investigated in this study.

Variable	Category	no. positive/ no. examined	Prevalence % (95% CI)	*p* value	OR
Hunting area	Avellino	46/62	74.2 (63.3–85.1)	0.37	–
	Benevento	29/38	76.3 (62.8–89.9)		
	Caserta	21/25	84.0 (69.6–98.4)		
	Salerno	155/186	83.3 (78.9–88.7)		
Sex	Male	131/156	84.0 (78.2–89.7)	0.14	–
	Female	120/155	77.4 (70.8–84.0)		
Age classes	Piglet	19/31	61.3 (44.1–78.4)	**0.01**	REF
	Sub-adult	105/129	81.4 (74.7–88.1)		2.76
	Adult	127/151	84.1 (78.3–89.9)		3.34
Muscle tissue	Heart	152/298	51.0 (45.3–56.7)	**<0.05**	1.44
	Oesophagus	119/269	44.2 (38.3–50.2)		1.10
	Diaphragm	116/277	41.9 (36.1–47.7)		REF
	Tongue	47/153	30.7 (23.4–38.0)		0.62

Table 3
Intensity of *Sarcocystis* spp. infections and muscles distribution.

	Intensity of infection	*t Student*	*p value*
Oesophagus	2.2 ± 3.2	0.6319	0.53
Diaphragm	2.4 ± 2.1		
Oesophagus	2.2 ± 3.2	0.3820	0.70
Heart	2.3 ± 2.3		
Oesophagus	2.2 ± 3.2	1.1884	0.24
Tongue	2.9 ± 4.2		
Diaphragm	2.4 ± 2.1	0.3384	0.74
Heart	2.3 ± 2.3		
Diaphragm	2.4 ± 2.1	1.0347	0.30
Tongue	2.9 ± 4.2		
Heart	2.3 ± 2.3	1.2350	0.22
Tongue	2.9 ± 4.2		

in multiple clusters at the endomysial or perimysial level (Fig. 1 a, b).

The mPCR assay demonstrated the high specificity of the newly designed primers, by identifying *S. miescheriana* DNA in 230 wild boars' carcasses out of 251 animals which resulted histologically positive (91,63%, 95% CI: 87.50–94.75). None of the tested samples revealed the presence of *S. suihominis* DNA.

4. Discussion

In the present study for the first time the presence of *Sarcocystis* spp. in wild boars from southern Italy was investigated. *Sarcocystis* spp. were confirmed combining a histological and a molecular tools. The obtained findings showed a high prevalence of *Sarcocystis* spp. in wild boars in the study area (80.7%); 230 out of 251 histologically positive samples were molecularly confirmed as *S. miescheriana.* Our prevalence results are slightly lower than the results reported by Gazzonis et al. (2019), which recorded an overall prevalence for *Sarcocystis* spp. of 97% (97% *S. miescheriana* and 1% of *S. suihominis* in coinfection with *S. miescheriana*) on 100 diaphragm samples from northern Italy. Similar results in terms of prevalence are reported from studies conducted in the last 20 years on *Sarcocystis* spp. in wild boars in Europe. In Portugal, a prevalence of 73.8% (on 103 wild boar) was detected (Coelho et al., 2015), while in Spain an overall rate of 72.2% was described on 910 wild boars (Calero-Bernal et al., 2016). Similarly, prevalence rates of 89.1% and 88.2% were reported in wild boars in two different studies in Lithuania (Malakauskas and Grikienienė, 2002; Prakas, 2011) and a prevalence of 87.1% was recorded recently in Latvia (Prakas et al., 2020b). Likewise, prevalence of 85% (on 20 wild boars) and 83.3% (on 30 wild boars) were reported in two studies on game meat in Slovakia (Goldová et al., 2008; Hvizdošová and Goldová, 2009). A lower prevalence was found in Poland (24.7%) (Tropilo et al., 2001) and Romania (60.4%) (Imre et al., 2017). Some studies are not easily comparable due to the different methodologies applied among investigations (compression, histology, PCR) (Guardone et al., 2022). In general, the application of molecular methods has in recent years broadened the knowledge on the prevalence of *Sarcocystis* spp. in various intermediate hosts in Europe (Prakas et al., 2023).

The mPCR assay applied to identify *Sarcocystis* spp. in wild boars that were histologically positive to the presence of sarcocysts did not detect the presence of *S. miecheriana* and/or *S. suihominis* DNA in 21 out of 251 samples. This result could be related to a low infection intensity, and the resulting absence of sarcocysts in the 25 mg of tissue submitted to DNA extraction and mPCR. In this context, the previous homogenization of a greater amount of tissue (e. g. 10–25 g), as described by Moré et al. (2011) and the subsequent extraction of a more representative aliquot of tissues might have resulted in a higher molecular detection of *Sarcocystis* spp. DNA. Future studies should be focused on harmonizing *Sarcocystis* spp. detection methods in order to get data effectively comparable among the different countries. In accordance with other studies (Coelho et al., 2015; Imre et al., 2017) the current research showed that adult and subadult wild boars are more exposed to *Sarcocystis* infection than piglets (Table 2). Calero-Bernal et al. (2015) reported an increased prevalence and intensity of infection with *Sarcocystis* spp. related to age, that could be due to a longer exposure to environmental oocysts (Imre et al., 2017; Gazzonis et al., 2019). Furthermore, Calero-Bernal et al. (2015) indicated a higher intensity of infection in older swine; nevertheless, the present study did not highlight any significant difference in mean intensity in relation with age. The high prevalence of *S. miescheriana* could be explained by a greater distribution of competent

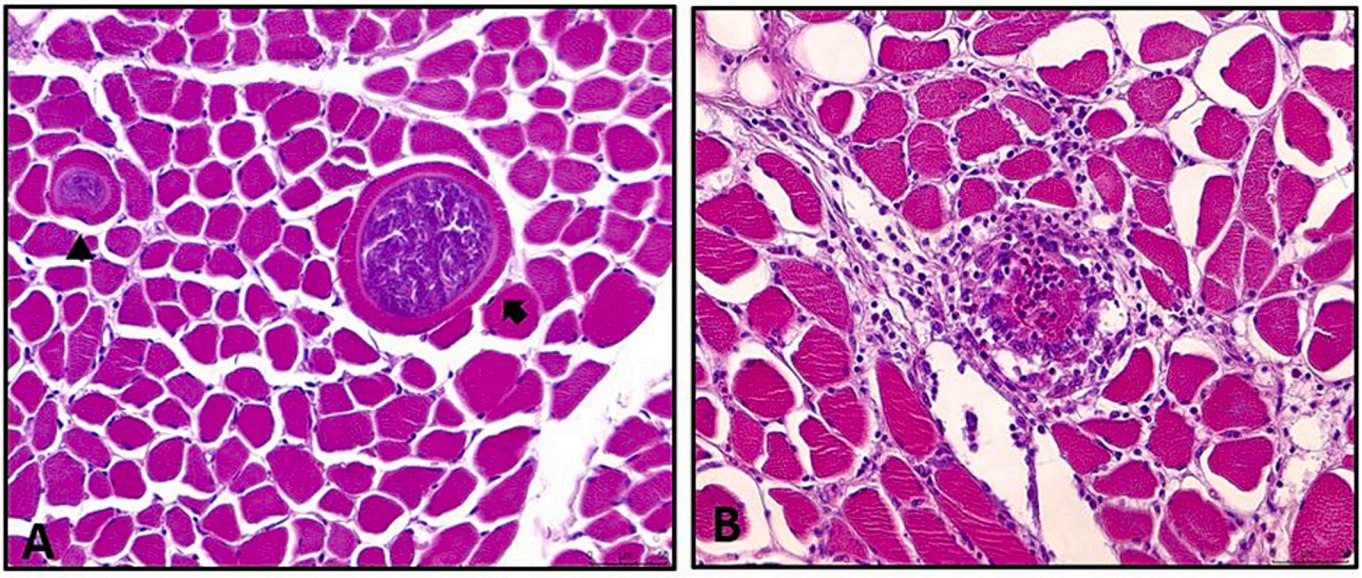

Fig. 1. a) *Sarcocystis* spp. infection, oesophagus, wild boar. A transverse section through the muscle fibers shows a large mature cyst with a thin wall completely filled with banana-shaped bradyzoites (arrow) and a young small cyst (arrowhead) with a thick wall filled with metrocytes at the periphery. Protrusions protrude from the cyst wall into the interior of the cyst, dividing the cysts into quarters. Note the central location of the cysts in the sarcoplasm and the complete lack of immune response. Hematoxylin and eosin (HE). High magnification x200. **b) *Sarcocystis* spp. infection, tongue, wild boar**. A transverse section of the tongue shows a substantial inflammatory infiltrate localized at the endomysial and intracellular levels, consisting mainly of lymphocytes and eosinophils. Concentrated eosinophils are found in foci in the center of the sarcoplasm. Hematoxylin and eosin (HE). High magnification x200.

definitive hosts (canids) in the same habitat of wild boars, which contribute to the contamination of the environment by sporocysts (Gazzonis et al., 2019). Indeed, as known, canids shed a large number of *Sarcocystis* spp. oocysts/sporocysts for several months without showing immunity to reinfection (Dubey et al., 2015; Moré et al., 2016); considering the great distances they are able to accomplish, wild canids can contaminate a large territory shared with the intermediate hosts (Dubey et al., 2015). In this context, for example, Mori et al. (2017) demonstrated that the territory of wolves (among the main definitive hosts of *S. miescheriana*) overlaps with the territory occupied by wild boars in Italy, which also represent the main prey of this canid (Mori et al., 2017; Buglione et al., 2020), supporting the prey-predator interface in which the life cycle of *S. miescheriana* is maintained. However, Lesniak et al. (2018) reported a not significant increasing prevalence of *S. miescheriana* in ungulates from wolf-inhabited areas of Germany. Likewise, red foxes (*Vulpes vulpes*) are recognized as the main scavenger wildlife species (Bassi et al., 2018), and a prevalence of 38.0% of *S. miescheriana* intestinal oocysts/sporocyts has been reported in these canids, confirming their role in in *Sarcocystis* spp. life-cycle maintenance (Bregoli et al., 2014; Moré et al., 2016). In addition, the rooting activity of wild boars (Massei and Toso, 1993; Fulgione et al., 2017), leads to an easier ingestion of several parasites from the ground, including oocysts/sporocysts (Pacifico et al., 2022). This feeding behaviour, together with the longevity and resistance of *Sarcocystis* spp. oocysts/sporocysts in the environment during different climatic conditions, including freezing (Dubey et al., 2015; Rosenthal, 2021), can raise *Sarcocystis* spp. infection rates. Besides, considering the well-known relationship between hunting dogs and wildlife pathogens (Pacifico et al., 2020), the role of these dogs in environmental diffusion of *Sarcocystis* spp. has also been described, mainly due to the habit of hunters of feeding them with wild game offal and raw meat (Basso et al., 2020). All these factors contribute to explain the high prevalence of the infection in wild swine and the lower exposure of domestic pigs (Imre et al., 2017). In our study, the analysis on *Sarcocystis* spp. distribution in muscles showed a higher infection rate in heart (51.0%, ± SD: 45.3–56.7, OR: 1.44) and oesophagus (44.2%, ± SD: 38.3–50.2, OR: 1.10) samples; this result is in accordance with Leoni et al. (1995), who described heart muscles as predilection site of infection. On the other hand, Coelho et al. (2015) reported a higher parasitic load in the diaphragm muscle, suggesting this tissue as the key sample for the molecular detection of *Sarcocystis* spp. cysts. Differences in prevalence and intensity of infection among muscles in wild boar are rarely reported, as many studies were performed testing only one muscle to investigate the presence of the parasite (Malakauskas and Grikienienė, 2002; Gazzonis et al., 2019). A higher intensity in tongue, sublingual tissue and diaphragm of wild boars was reported by Erber and Boch (1976), without difference in mean intensity among muscles analysed, according to Boch et al. (1978).

Based on the literature, the occurrence of *Sarcocystis* spp. infections in swine can be associated to pathological changes around mature cysts with inflammatory reactions characterized by the presence of lymphocytes and macrophages, although other studies did not find any inflammatory response around sarcocysts (Avapal et al., 2004; Dubey et al., 2015). In this study, no inflammatory reaction associated with the parasitosis was observed in most wild boar tissues, as previously outlined (Kia et al., 2011; Coelho et al., 2015). In certain cases, the eosinophilic myositis due to muscle cell breakdown herein observed was in accordance with Calero-Bernal et al. (2015) and Gazzonis et al. (2019). Eosinophilic myositis has already been reported in association with *Sarcocystis* spp. natural infections in wild and domestic ruminants such as sheep, red deer and cattle (Jensen et al., 1986; Basso et al., 2020; Rubiola et al., 2021), while only one report by Vangeel et al. (2012) pointed out the development of eosinophilic myositis in experimental infections in cattle. Nevertheless, more evidence is needed to investigate the putative causal relationship between eosinophilic myositis and *Sarcocystis* spp. (Dubey et al., 2015). Recently, the presence of macroscopic *S. miescheriana* sarcocysts was detected at slaughter in a domestic swine in Italy (Rubiola et al., 2023). Nevertheless, no macroscopic cysts were recorded in the wild boar tissues examined in the present study.

The occurrence of the zoonotic *S. suihominis* in wild boars has been rarely reported in Europe; for instance, Gazzonis et al. (2019) detected only one case of *S. suihominis* in co-infection with *S. miescheriana* (1/100) and Calero-Bernal et al. (2016) described a single positivity to *S. suihominis* in wild boars from Spain. Other molecular studies assessed the presence of only *S. miescheriana* (Coelho et al., 2015; Imre et al., 2017; Prakas et al., 2020b). The present study and previous findings (Gazzonis et al., 2019) indicate the rare circulation of *S. suihominis* in wild boars in Italy.

The presence of *S. suihominis* both in wild boars and domestic pigs is currently highly related to the sanitary condition, breeding management and slaughtering practices (Fayer et al., 2015; Kaur et al., 2016; Huang et al., 2019; Gazzonis et al., 2019). High prevalence of *S. suihominis* were described in India and China, where domestic pigs are usually free-range reared and pork products are traditionally consumed raw or undercooked (Kaur et al., 2016; Huang et al., 2019). Kaur et al. (2016) reported cases of human sarcocystosis related to a community with poor hygienic conditions in breeding management and where children had regular access to slaughterhouses, which often corresponded to their backyard. Likewise, high prevalence was described in countries where open air human defecation is still in practice, often in places accessible to domestic and feral swine (Chauhan et al., 2020). The human intestinal sarcocystosis is reported mostly in Asian countries (Fayer et al., 2015), with cases related to pork consumption in Germany via experimental infections (reviewed by Dubey, 2015). Nevertheless, due to the wild boars spread in urban and peri-urban areas (Fulgione and Buglione, 2022) and the increase in pigs outdoor farming systems in Europe, the risk of contact among wild and domestic pigs and humans' stool cannot be excluded.

5. Conclusion

Considering the significant public health concern, the traditional consumption of raw/undercooked meat products from boars and pigs may constitute a risk factor to humans. According to the European (EU) Regulation No 853/2004, trained hunters should be able to undertake an initial examination of wild game upon capture; furthermore, as stated by Commission Implementing Regulation (EU), 2023, carcasses with parasites infestations must be declared unfit for human consumption. Nevertheless, *Sarcocystis* lesions in wild and domestic swine go often unnoticed at meat inspection, being them not macroscopically visible. Moreover, the microscopical differentiation through the morphological examination of sarcocysts could be influenced by their age, other than the fixation methods employed. In this context, the use of molecular tools and in particular the amplification of the *cox1* mtDNA gene, could be a technically sound tool for the early detection of *Sarcocystis* spp. in meat. In the present study, the development and application of a mPCR protocol to differentiate *S. miescheriana* and *S. suihominis* confirmed the low prevalence of the latter species in wild boars from Italy, suggesting a low risk of infection to humans via consumption of wild boar meat.

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Ethics statament

The animals were collected in accordance with Italian and EU legislation, during the hunting seasons and with routine sanitary surveillance. Consequently, ethical approval was not deemed necessary.

CRediT authorship contribution statement

Laura Pacifico: Investigation, Writing – original draft. **Selene Rubiola:** Investigation, Methodology, Writing – original draft. **Francesco Buono:** Conceptualization, Methodology. **Mariafrancesca Sgadari:** Investigation. **Nicola D'Alessio:** Resources. **Stefano Scarcelli:** Investigation. **Giovanni Sgroi:** Formal analysis. **Maria Buglione:** Investigation. **Francesco Chiesa:** Conceptualization, Methodology. **Brunella Restucci:** Conceptualization. **Alessandro Fioretti:** Resources. **Petras Prakas:** Writing – review & editing. **Vincenzo Veneziano:** Conceptualization, Resources.

Declaration of Competing Interest

None.

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