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Molecular differentiation of cattle *Sarcocystis* spp. by multiplex PCR targeting 18S and COI genes following identification of *Sarcocystis hominis* in human stool samples

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ABSTRACT

Sarcocystis spp. are protozoan parasites which can infect a wide range of vertebrates, including humans; the latter can act as definitive hosts for two cattle *Sarcocystis* spp.: *Sarcocystis hominis* and *Sarcocystis heydorni*. Reports of intestinal sarcocystosis are well documented in the literature, but PCR-based methods have been scarcely used to identify *Sarcocystis* species in human stools, and have been limited to the molecular analysis of 18S ribosomal RNA (18S rRNA) gene sequences. Since the mitochondrial cytochrome *c* oxidase subunit I (COI) gene is one of the most promising tools for distinguishing between closely related *Sarcocystis* spp., and taking into account the lack of publicly available *S. hominis* COI sequences, in the present study we obtained the first partial COI sequence of *S. hominis* from human stool samples of patient with gastrointestinal symptoms. We designed specific COI primers to develop a multiplex PCR method for the identification of *Sarcocystis* spp. in cattle. The submission of the COI sequence described herein and the unambiguous identification of *S. hominis* through the application of the new multiplex PCR is important for determining the prevalence of this zoonotic *Sarcocystis* spp. in meat and the risk for consumers.

1. Introduction

Sarcocystosis is a protozoan parasitic disease, distributed worldwide, caused by members of the genus *Sarcocystis*, belonging to the phylum Apicomplexa. *Sarcocystis* spp. have a strict two-host life cycle with herbivores or omnivores as intermediate hosts and carnivores or omnivores as definitive hosts (Dubey, 2015). These latter, which include humans, become infected via the ingestion of cysts in muscular tissues, while the intermediate hosts acquire infection by ingesting oocysts and sporocysts in feed or water contaminated by infected feces (Dubey, 2015). More than 200 *Sarcocystis* spp. are currently recognized and can be found in tissues of mammals, birds, reptiles and fishes (Fayer et al., 2015). Among them, only pigs and cattle are confirmed intermediate hosts of zoonotic *Sarcocystis* spp. (Dubey, 2015), while snakes serve as definitive hosts of *Sarcocystis nesbitti*, the only *Sarcocystis*

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sp. that has humans as intermediate hosts (Lau et al., 2014). Humans can therefore develop two different clinical forms of sarcocystosis: an intestinal form, caused by *S. hominis*, *S. heydorni* and *S. suihominis*, and a muscular form, caused by *S. nesbitti*.

While reports of human muscular sarcocystosis are rare and mostly confined to South East Asia (Esposito et al., 2014; Kwok and Ting, 2019), the intestinal form is well documented in the literature. Reports describe gastrointestinal symptoms, such as nausea, diarrhea and abdominal pain as the most common outcome of the infection, with a wide range of intensity, depending on the immune response of the host and on the number of ingested cysts, with most infections going unnoticed (Dubey et al., 2015a,b).

Reports of *Sarcocystis* in human stool samples from infected patients are mostly referred from European and Asian countries (Fayer et al., 2015; Poulsen and Stensvold, 2014). The detection of oocysts or sporocysts in stools by light microscopy is usually performed to confirm the diagnosis of intestinal Sarcocystosis in patients with gastrointestinal symptoms (Fayer et al., 2015); however, light microscopy cannot discriminate among different *Sarcocystis* spp. due to their morphological similarities. Diagnostic limitations restrict the possibility to understand the epidemiology and the public health implications of human sarcocystosis, and PCR-based methods would represent a significant step forward (Poulsen and Stensvold, 2014). These methods, however, have been scarcely used and the few reports concerning the use of molecular methods to identify *Sarcocystis* spp. in human stools are based on the molecular analysis of 18S ribosomal RNA (rRNA) gene sequences (Agholi et al., 2016). However, the suitability of this locus to differentiate between *Sarcocystis* spp. is questionable (Robertson et al., 2019).

The Internal Transcribed Spacer 1 (ITS1) locus and parts of 28S rRNA gene have been shown to be valuable targets for further characterization (Gjerde, 2013), but sequencing of the partial Cytochrome C Oxidase subunit I mitochondrial gene (mtDNA COI gene) is seen as the most promising tool for distinguishing between closely related *Sarcocystis* spp. (Gjerde, 2013, 2016a). This is even more needed for cattle *Sarcocystis* spp., where a number of new, closely related species were added to the well-known *S. hominis*, *S. hirsuta* and *S. cruzi* in the last three years (Dubey et al., 2015a,b, 2016; Gjerde, 2016a,b).

In particular, *S. bovifelis* and *S. bovini*, both, apparently, associated with cats (definitive hosts) (Gjerde, 2016a), are not easily distinguishable from *S. hominis* based on the 18S rRNA gene sequence. Indeed, as highlighted by Moré et al. (2013), sequence differences between *S. hominis*, *S. bovifelis* (referred to by Moré et al., as *S. sinensis*, but later proved to be *S. bovifelis*) and *S. bovini* are approximately 3% of the 18S rDNA, and using only size differences of the amplified 18S rDNA fragments may result in the misidentifications of *S. hominis*, *S. bovifelis* and *S. bovini* (Moré et al., 2013; Vangeel et al., 2007). For this reason, in this paper, we will use the term "*Sarcocystis hominis*-like" when referring to sequences derived from bovine muscular tissue cysts previously identified as *S. hominis* by 18S rRNA PCR (Chiesa et al., 2013; Vangeel et al., 2007).

The lack of publicly available *S. hominis* COI sequences constitutes the basic problem that hindered the development of a COI-based method for the identification of *Sarcocystis* spp. in cattle. In our previous study (Rubiola et al., 2019) 12 COI sequences obtained from *Sarcocystis hominis*-like positive samples, did not constitute the best BLAST match of any of the published sequences, with the best hit being the sequences of *S. bovifelis* with 89% of homology. Considering the importance of the molecular characterization of the COI gene of *S. hominis* to allow an appropriate risk assessment of public health issues arising from consumption of contaminated beef, the aims of the present study were: i) to prove the identity of the *S. hominis* sequences obtained in the previous study by matching them with *Sarcocystis* COI sequences derived from human stool samples; ii) to design specific COI primers for *S. hominis* and *S. bovifelis* and integrate the previous multiplex PCR method for the identification of bovine *Sarcocystis* spp. (Chiesa et al., 2013).

2. Materials and methods

2.1. Source material

Stool samples from patients living in Piedmont, North-West Italy, and hospitalized with gastrointestinal (GI) symptoms were collected between January 2014 and April 2016. The patients presented at the Infectious Diseases Unit of the Molinette Hospital in Turin - Italy with common GI complaints such as diarrhea, abdominal pain, cramps, nausea and vomiting. At the hospital, all samples were screened for common foodborne pathogens. Patients with confirmed infections, among which *Campylobacter jejuni* and *Salmonella* resulted the most common, were excluded from the study; only undiagnosed cases ($n = 60$) were enrolled.

The stool samples were collected in sterile plastic containers and transferred to the Laboratory of Food Inspection at the Department of Veterinary Science – University of Turin. On arrival, unpreserved fecal samples were stored at 4 °C; a fecal suspension was prepared within 1 week for DNA isolation: for each sample, 200 μl of saline solution was added to 1 g of feces. The suspension obtained were submitted to three cycles of freezing/thawing: all samples were frozen by immersion in LN2 (1 min) and thawed in a water bath at 100 °C (30 s) to break the oocyst walls the fecal suspensions for DNA isolation were then stored at −20 °C.

2.2. DNA extraction

DNA was extracted from human feces using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

2.3. Semi-nested PCR amplification from stool samples

DNA from the stool samples was used as template for the amplification of *S. hominis* specific fragments. For this purpose, considering the small amount of *Sarcocystis* DNA expected, two semi-nested PCR assays were designed, targeting, respectively, the 18S rRNA gene and the COI gene.

The first assay included a first amplification with two *Sarcocystis* genus specific primers, sarF (Vangeel et al., 2007) and sarcoREV (Chiesa et al., 2013), amplifying an expected region of ~240 bp, and a second amplification achieved with the *S. hominis*-like specific forward primer Hom1 (Chiesa et al., 2013) coupled with the reverse primer used in the first amplification, amplifying an expected region of ~180 bp. In both amplification rounds the PCR reaction contained 2.5 μl of DNA (5–20 ng/μl), 0.2 mM dNTPs (Invitrogen, Thermo Fisher Scientific, Vilnius, Lithuania), 2.5 mM $MgCl_2$, 0.5 μm of each primer, 1 U of Platinum Taq (Invitrogen, Thermo Fisher Scientific, Vilnius, Lithuania), 10 x PCR Buffer and distilled water to a total volume of 25 μl. *S. bovifelis* DNA obtained from a previous study was used as a positive control, while extracted DNA from negative cattle muscles as well as reagent blanks were included as a negative control. The amplification was performed in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, CA, USA) with the following cycling profile: one step of 3 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 56 °C, 30 s at 72 °C and a final extension step of 3 min at 72 °C.

The second assay, targeting COI gene, was performed on the samples that tested positive for *Sarcocystis hominis*-like spp. by the 18S rRNA PCR. The semi-nested PCR involved a first amplification round using the protocol described by Rubiola et al. (2019) and a second round of amplification using the same forward primer and a newly designed internal reverse primer. To achieve this, we aligned the barcode region of COI from *Sarcocystis* partial COI sequences available from GenBank to identify conserved regions, in the sequence of the fragment previously amplified, suitable for primers designing. Sequences of *S. cruzi*, *S. hirsuta*, *S. bovifelis*, *S. bovini* and *S. heydorni* were aligned using MEGA Software. Furthermore, to evaluate possible cross-reactions, the sequence of *Toxoplasma gondii*, another member of the phylum Apicomplexa, was also aligned.

Based on these sequences, we designed different primers with Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi); specificity of the primers for the Sarcocystis genus was examined in-silico using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The selected primer was named R2 COI and is shown in Table 1; it was synthesized by Sigma Aldrich (St. Louis, MO).

Nested PCR was performed using 1 μl first-round PCR product as template DNA, 1 mM of each primer, 3 mM MgCl2, 0.2 mM of each dNTP, 1 U recombinant Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific, Vilnius, Lithuania), 10 x PCR Buffer and distilled water to a total volume of 25 μl. The cycling conditions were 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 60 °C for 30 s, 72 °C for 30 s and a final extension of 72 °C for 5 min. PCR products were observed in 2% agarose gel stained with SYBR safe stain (Invitrogen, Carlsbad, CA) and observed in a blue light transilluminator (Invitrogen, Groningen, The Netherlands).

2.4. Sanger sequencing and phylogenetic analysis

PCR products from the second amplification of the 18S rRNA and mtDNA COI genes were sequenced to achieve species identification: the fragments were purified with Exo-Sap (USB Europe, Staufen, Germany) treatment according to the manufacturer's instructions. Forward and reverse sequencing reactions were performed using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems, Foster City, CA). Sequenced fragments were purified by DyeEX (Qiagen, Hilden, Germany) and sequence analysis was performed on an Applied Biosystems 310 Genetic Analyser (Applied Biosystems, Foster City, CA). The nucleotide sequences were analyzed using the BLASTN sequence similarity search at the NCBI database (Altschul et al., 1990). Phylogenetic analyses of the 18S rRNA and mtDNA COI genes were performed using the Neighbor-Joining method within MEGA 7. *Sarcocystis* spp. reference sequences included for 18S rRNA and COI mtDNA are shown in Table 2.

2.5. Set up of multiplex PCR assay

The partial sequences of the *S. hominis* mtDNA COI gene were used to set up a new multiplex PCR to discriminate closely-related species previously impossible to differentiate from *S. hominis* using 18S rRNA gene. To achieve this, partial sequences of the COI gene of *S. bovini* and *S. bovifelis* available from GenBank (accession numbers: LC171858.1, KT901022.1; KT901286.1, KC209696.1) were aligned together with the newly identified partial sequence of *S. hominis* COI gene (deposited in GenBank with accession number MH021119.1) 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.

Examination of the alignments of the mtDNA COI genes revealed no regions useful for the development of species-specific primers to discriminate *S. bovini* from *S. bovifelis*. In contrast, based on the alignment results, three primers were designed to

Table 1
Forward and reverse primers used for the semi-nested PCR amplification of COI sequences.

Primers	Position	Sequence	GenBank accession no.	References
F1 COI primer	11–30	TGTACATACTTACGGCAGGT	KT901022.1	Rubiola et al., 2019
R1 COI primer	895–913	CCGTAGGTATGGCGATCAT	KT901022.1	Rubiola et al., 2019
R2 COI primer	400–419	AGGCCAAGAATTATCCAGTC	KT901022.1	This study

Table 2
Reference sequences downloaded from GenBank and used in this study.

Species	Strain	COI mtDNA	18S rRNA
S. bovifelis	B4.8	KC209693.1	KT901136.1
	B1.15	KC209691.1	N
	B3.1	KC209692.1	KC209743.1
	B1.13	N	KT901123.1
S. bovini	B9.7	KT901022.1	N
	B7.2	KT901005.1	KT901150.1
	B4.15	N	KT901144.1
	B8.9	KT901021.1	N
S. hominis	Clone 1B HRF93A	N	JX679470.1
	2730ho	N	AF176944.1
	28h7ho	N	AF176945.1
S. hirsuta	B12.22	KT901075.1	KT901166.1
	B10.14	KT901055.1	N
	B9.1	KT901031.1	KT901163.1
	B10.5	N	KT901165.1
	B12.1	N	N
S. heydorni	Isolate 1	KX057994.1	KX057996.1
	Isolate 2	KX057995.1	KX057997.1
S. cruzi	B11.1	KT901095.1	KT901173.1
	B3.5	KT901093.1	N
	B4.13	KT901094.1	N
	B1.6	N	KT901167.1
	B2.1	N	KT901169.1

N: sequence not present in GenBank.

distinguish *S. hominis* from *S. bovifelis*: a single common forward primer (COI HB) and two specific reverse primers (COI H and COI B) (Table 3). Specificity of the primers was examined in-silico using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast); primers were synthesized by Sigma Aldrich (St. Louis, MO).

In order to design a multiplex PCR assay to simultaneously identify all the species of the genus *Sarcocystis* actually reported in cattle in Italy, and to allow the possible detection of other species, we modified the previous multiplex PCR approach described by Chiesa et al. (2013), which allowed the identification of *S. cruzi*, *S. hirsuta* and *S. hominis*-like, with the combination of a genus specific primer set (Vangeel et al., 2007) and the newly designed primer set for *S. hominis* and *S. bovifelis*, involving the use of 7 primers capable of amplify and separate gene fragments of different lengths.

The primer set designed to distinguish *S. hominis* from *S. bovifelis* was 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 mM of each primer, Sarco Rev, Sar F, Hirsuta, Cruzi, COI HB, COI H and COI B, 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; sequence and reference of primers sets are shown in Table 3.

The PCR assay involved a denaturation step at 95 °C for 3 min, followed by 35 cycles at 95 °C for 60 s, 58 °C for 60 s and 72 °C for 30 s and final extension 72 °C for 3 min. A collection of *Sarcocystis* positive samples isolated from cattle striated muscle in the Department of Veterinary Science of Turin University during several studies (Chiesa et al., 2013; Rubiola et al., 2019) was used to further evaluate the sensitivity and specificity of the multiplex PCR assay, together with a negative control (DNA from *Toxoplasma gondii*).

Table 3
Sequence and origin of the sets of primers.

Primers	Gene	Primer sequences	Reference
Sarco_Rev	18S	AACCCTAATTCCCCGTTA	Chiesa et al., 2013
SarF	18S	TGGCTAATACATGCGCAAATA	Vangeel et al., 2007
Hirsuta	18S	CATTTCGGTGATTATTGG	Chiesa et al., 2013
Cruzi	18S	ATCAGATGAAAATCTACTACATGG	Chiesa et al., 2013
COI_HB	COI	AATGTGGTGCGGTATGAACT	This study
COI_H	COI	GGCACCAACGAACATGGTA	This study
COI_B	COI	TCAAAAACCTGCTTTGCTG	This study

Table 4.1
Estimates of Evolutionary Divergence between the 18S rRNA sequence isolated in this study and other *Sarcocystis* spp. sequences deposited in GenBank. The number of base substitutions per site between sequences are shown. Analyses were conducted using the Tamura-Nei model (Tamura and Nei, 1993); evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). The bold sequence indicates our isolate.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	KT901144.1_S._bovini_isolate_B4.15																	
2	KT901150.1_S. _bovini_isolate_B7.2	0,000																
3	KT901136.1_S. _bovifelis_isolate_B4.8	0,008	0,008															
4	KC209743.1_S. _bovifelis_isolate_B3.1	0,008	0,008	0,000														
5	KT901123.1_S. _bovifelis_isolate_B1.13	0,008	0,008	0,000	0,000													
6	KT901165.1_S. _hirsuta_isolate_B10.5	0,063	0,063	0,070	0,070	0,070												
7	KT901163.1_S._hirsuta_isolate_B9.1	0,063	0,063	0,070	0,070	0,070	0,000											
8	KT901166.1_S. _hirsuta_isolate_B12.1	0,071	0,071	0,079	0,079	0,079	0,007	0,007										
9	KX057996.1_S. _heydorni_isolate_1	0,038	0,038	0,030	0,030	0,030	0,085	0,085	0,093									
10	KX057997.1_S. _heydorni_isolate_2	0,038	0,038	0,030	0,030	0,030	0,085	0,085	0,093	0,000								
11	KT901169.1_S. _cruzi_isolate_B2.1	0,039	0,039	0,031	0,031	0,031	0,079	0,079	0,088	0,000	0,000							
12	KT901173.1_S._cruzi_isolate_B11.1	0,039	0,039	0,031	0,031	0,031	0,079	0,079	0,088	0,000	0,000	0,000						
13	KT901167.1_S._cruzi_isolate_B1.6	0,039	0,039	0,031	0,031	0,031	0,079	0,079	0,088	0,000	0,000	0,000	0,000					
14	AF176944.1_S._hominis_strain_2730ho	0,000	0,000	0,008	0,008	0,008	0,063	0,063	0,071	0,038	0,038	0,039	0,039	0,039				
15	JX679470.1_S._hominis_clone_1B_HRF93A	0,000	0,000	0,008	0,008	0,008	0,063	0,063	0,071	0,038	0,038	0,039	0,039	0,039	0,000			
16	AF176945.1_S._hominis_strain_28h7ho	0,000	0,000	0,008	0,008	0,008	0,063	0,063	0,071	0,038	0,038	0,039	0,039	0,039	0,000	0,000		
17	**Sarcocystis_hominis_human_stool_sample**	**0,000**	**0,000**	**0,008**	**0,008**	**0,008**	**0,063**	**0,063**	**0,071**	**0,038**	**0,038**	**0,039**	**0,039**	**0,039**	**0,000**	**0,000**	**0,000**	

Table 4.2
Estimates of Evolutionary Divergence between the COI mtDNA sequence isolated in this study and other *Sarcocystis* spp. sequences deposited in GenBank. The number of base substitutions per site between sequences are shown. Analyses were conducted using the Tamura-Nei model (Tamura and Nei, 1993); evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). The bold sequence indicates our isolate.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	KT901021.1_Sarcocystis_bovini_isolate_B8.9															
2	KT901005.1_Sarcocystis_bovini_isolate_B7.2	0,002														
3	KT901022.1_Sarcocystis_bovini_isolate_B9.7	0,002	0,005													
4	KC209691.1_Sarcocystis_bovifelis_isolate_B1.15	0,055	0,055	0,057												
5	KC209692.1_Sarcocystis_bovifelis_isolate_B3.1	0,059	0,059	0,061	0,006											
6	KC209693.1_Sarcocystis_bovifelis_isolate_B4.8	0,056	0,056	0,059	0,001	0,007										
7	KT901075.1_Sarcocystis_hirsuta_isolate_B12.22	0,242	0,240	0,245	0,245	0,246	0,246									
8	KT901055.1_Sarcocystis_hirsuta_isolate_B10.14	0,235	0,233	0,238	0,239	0,241	0,241	0,007								
9	KT901031.1_Sarcocystis_hirsuta_isolate_B9.1	0,232	0,230	0,235	0,240	0,241	0,241	0,008	0,006							
10	KX057995.1_Sarcocystis_heydorni_isolate_2	0,302	0,302	0,306	0,323	0,328	0,325	0,374	0,369	0,370						
11	KX057994.1_Sarcocystis_heydorni_isolate_1	0,300	0,300	0,304	0,321	0,327	0,323	0,374	0,369	0,370	0,004					
12	KT901095.1_Sarcocystis_cruzi_isolate_B11.1	0,313	0,317	0,313	0,336	0,341	0,338	0,360	0,356	0,352	0,188	0,185				
13	KT901093.1_Sarcocystis_cruzi_isolate_B3.5	0,315	0,318	0,315	0,337	0,343	0,339	0,356	0,352	0,352	0,194	0,191	0,009			
14	KT901094.1_Sarcocystis_cruzi_isolate_B4.13	0,317	0,317	0,317	0,335	0,341	0,337	0,356	0,352	0,352	0,190	0,187	0,008	0,004		
15	**MH021119.1_Sarcocystis_hominis_human_stool_sample**	**0,131**	**0,133**	**0,134**	**0,128**	**0,128**	**0,129**	**0,234**	**0,231**	**0,228**	**0,339**	**0,337**	**0,334**	**0,335**	**0,337**	

3. Results

3.1. Semi-nested PCR amplification from stool samples

Out of 60 samples, the application of the first PCR assay, targeting the 18S rRNA gene, resulted in the expected amplicons in 6 samples: 2 samples demonstrated the presence of *Sarcocystis* spp. DNA after the first round of amplification, with a 240 bp amplicon, while 4 more samples resulted positive after the second round of the semi-nested PCR with bands of 180 bp.

The application of the second assay, targeting the COI gene, on the 6 samples previously resulted positives for *S. hominis*-like spp., resulted in the expected amplicons in all the samples: 1 sample demonstrated the presence of *Sarcocystis* spp. DNA after the first round of amplification, with a 850 bp amplicon, while the second round of amplification showed products of the appropriate size (~409 bp) in all the 6 samples.

3.2. Sanger sequencing and phylogenetic analysis

The sequencing of the amplified products obtained by the second amplification of the 18S rRNA gene resulted in 6 sequences, which showed 100% identity with published sequences from *S. hominis* (accession number AF176944). The estimation of Evolutionary Divergence between our sequences and other *Sarcocystis* spp. sequences deposited in GenBank is shown in Table 4.1; analyses were conducted using the Tamura-Nei model (Tamura and Nei, 1993). As shown in the table, intraspecific distance within our sequence and other *S. hominis* sequences is 0.000, interspecific distance between *S. hominis*, *S. bovini* and *S. bovifelis* ranges from 0.000 to 0.008, and interspecific distance between *S. hominis* and *S. cruzi*, *S. heydorni* and *S. hirsuta* ranges from 0.038 to 0.071.

The sequencing of the amplified product obtained by the first amplification of the mtDNA COI gene (~850 bp) resulted in a readable sequence which showed the highest similarity in GenBank with a *S. bovifelis* mtDNA COI sequence (accession number KT900992.1, 89% identity), followed by *S. entzerothi* (accession number MF596201.1, 89%) and *S. bovini* (accession number KT901021.1, 88% identity). Besides, the sequence showed 100% identity with the 12 COI sequences derived from bovine muscular tissue and identified as *S. hominis-like* by 18S rRNA PCR in our previous work (Rubiola et al., 2019). The sequencing of the 6 products amplified by the semi-nested PCR assay (~409 bp) resulted in 6 readable sequences, all of which showed the same grade of homology described before.

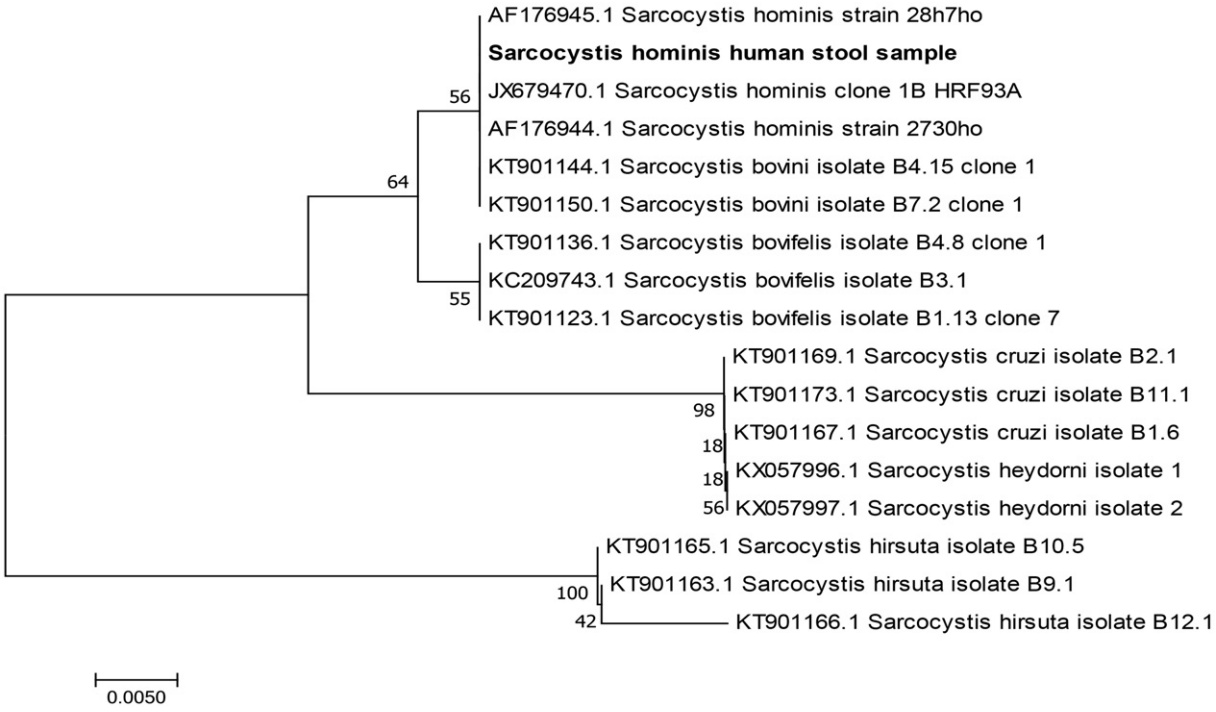

Fig. 1. (A) and (B) Neighbor-Joining trees inferred from *Sarcocystis* 18S rRNA sequences (A) and *Sarcocystis* COI mtDNA sequences (B) isolated in this study and sequences from GenBank. The evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (2500 replicates) are shown next to the branches. The bold sequence indicates our isolate.

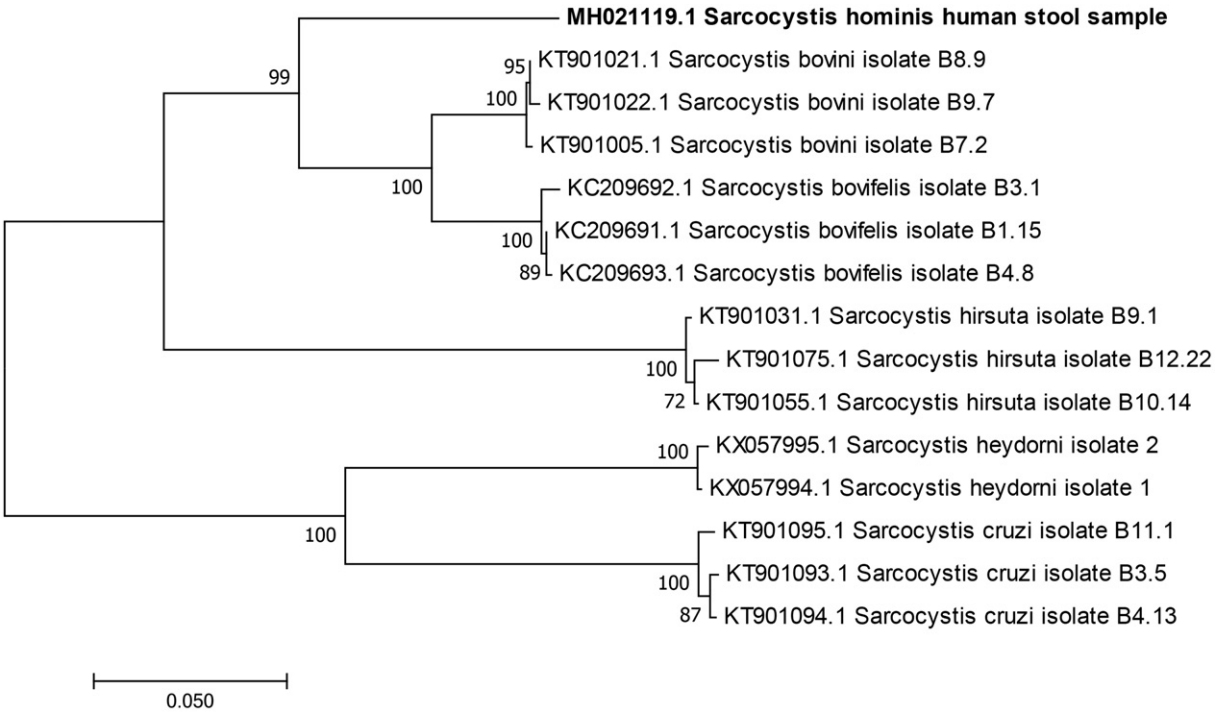

Fig. 1 (*continued*).

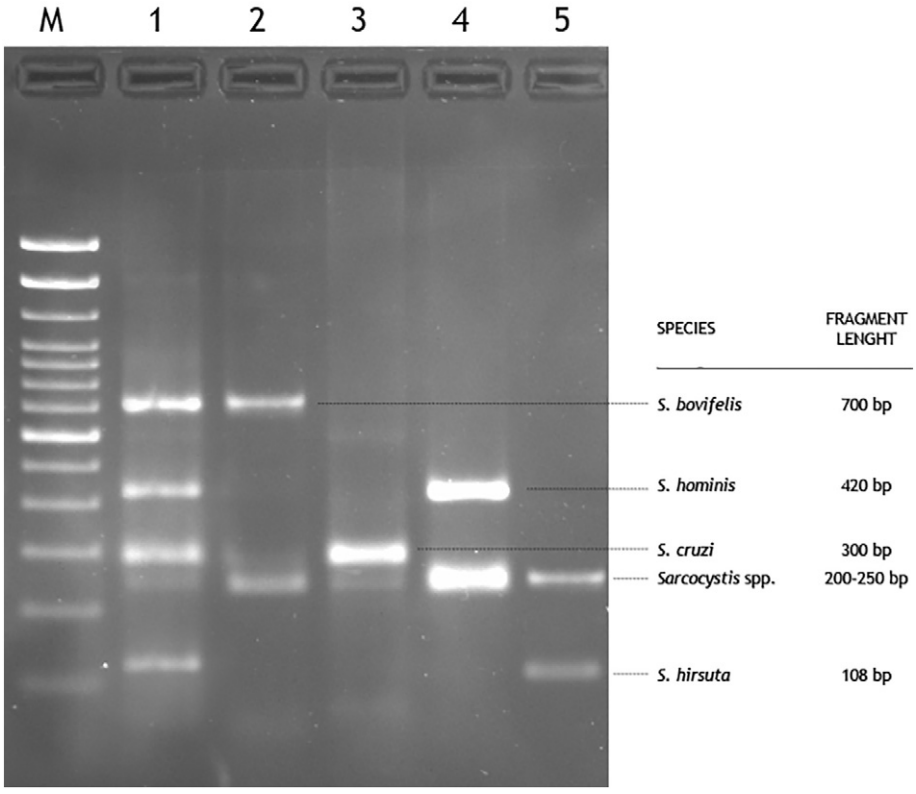

Fig. 2. Agarose gel electrophoresis of DNA fragments generated by multiplex PCR with the *Sarcocystis* spp. positive samples isolated from cattle striated muscle in the Department of Veterinary Science of Turin University. Lanes 1 to 5 correspond respectively to: the simultaneous presence of *S. bovifelis*, *S. hominis*, *S. cruzi* and *S. hirsuta* (lane 1); *S. bovifelis* (lane 2); *S. cruzi* (lane 3); *S. hominis* (lane 4); *S. hirsuta* (lane 5). In each sample, the *Sarcocystis* spp. fragment is generated. Lane "M" correspond to the 100 bp DNA molecular-weight size marker (Invitrogen, Thermo Fisher Scientific, Vilnius, Lithuania).

The estimation of Evolutionary Divergence between our sequences and other *Sarcocystis* spp. sequences present in GenBank is shown in Table 4.2; analyses were conducted using the Tamura-Nei model (Tamura and Nei, 1993). As shown in the table, considering the COI fragment amplified, interspecific distance between *S. hominis*, *S. bovini* and *S. bovifelis* ranges from 0.128 to 0.134, and interspecific distance between *S. hominis* and *S. cruzi*, *S. heydorni* and *S. hirsuta* ranges from 0.228 to 0.339.

A phylogenetic analysis of 18S rRNA gene and COI mtDNA gene on *S. hominis* isolates characterized herein and on representative sequences deposited in GenBank was inferred using the Neighbor-Joining method (Saitou and Nei, 1987); the Neighbor-Joining phylogenetic trees are shown in Fig. 1A and B. As shown in Fig. 1A and B, *S. hominis* is clearly distinguishable from *S. bovini* and *S. bovifelis* using the COI fragment amplified, with high bootstrap values supporting each major node, while the 18S fragment amplified doesn't allow a clear distinction among the species.

3.3. Multiplex PCR assay

Combining the primers reported in Table 3, all samples were correctly identified: the amplification resulted in bands of ~100, ~200–250, ~300, ~420 and ~700 bp for *S. hirsuta*, *Sarcocystis* spp., *S. cruzi*, *S. hominis* and *S. bovifelis*, respectively (Fig. 2); the difference in length of the *Sarcocystis* spp. fragments is due to the presence of gaps and insertions characterizing the 18S rRNA gene of each *Sarcocystis* sp. The specific primer pairs demonstrated to be species specific, amplifying only the target species.

4. Discussion

The discrimination of *Sarcocystis* spp. in cattle should be considered of primary importance because humans are definitive hosts for two species, *S. hominis* and *S. heydorni*, with a zoonotic risk for consumers of raw or undercooked meat (Bucca et al., 2011; Dubey et al., 2015a,b). On the other hand, cattle sarcocystosis can lead to serious economic outcomes in the beef sector, as the parasite is considered one of the causes of bovine eosinophilic myositis (BEM), a specific inflammatory myopathy with multifocal grey-green lesions in striated muscle, leading to carcass condemnation (Chiesa et al., 2013; Jensen et al., 2016; Vangeel et al., 2009). Thus, species identification of intra-lesional *Sarcocystis* is important for understanding the contribution of specific species to BEM pathogenesis, and the difference between the very high prevalence of sarcocysts in cattle and the low prevalence of BEM lesions (Gajadhar and Marquardt, 1992; Vangeel et al., 2013).

In fact, the few studies carried out in Italy at slaughterhouses reveal a prevalence of *Sarcocystis* infection between 80 and 96% (Bucca et al., 2011; Chiesa et al., 2013; Domenis et al., 2011; Meistro et al., 2015), which is in line with data reported from other countries (Cama, 2006; Moré et al., 2011; Vangeel et al., 2007; Wouda et al., 2006). These studies report the prevalence of *S. hominis* ranging from 42,7% (Domenis et al., 2011) to 68% (Meistro et al., 2015). However, in the recent taxonomic revision of cattle *Sarcocystis* mtDNA COI sequences have proved to perform better than 18S rRNA to differentiate closely related species (Gjerde, 2013). Despite the fact that the phylogenetic trees based on the shorter and less discriminative 18S rRNA gene sequences (Fig. 1A) and on mtDNA COI sequences (Fig. 1B) show a similar pattern of branching, the node support values and the evolutionary divergence reported in Tables 4.1 and 4.2 highlight the superior genotyping resolution provided by the mtDNA COI marker. As a consequence, the previous *S. hominis* prevalence have been overestimated by the detection techniques based on the lower discriminative 18S rRNA gene, which was, until recently, the standard target gene for molecular diagnosis of cattle sarcocystosis (Gjerde, 2016a; Rubiola et al., 2019).

Given the absence, until August 2019, of mtDNA COI gene sequences for *S. hominis*, and the resulting identification problems shown in our previous study (Rubiola et al., 2019), the first aim of our work was to use molecular techniques to obtain sequences of the COI gene of *S. hominis* from human feces. The study area (Piedmont Region, North-West Italy) is well known for raw beef consumption. In order to maximize the probability of *Sarcocystis* detection from human stool samples, we have chosen to enroll in the study samples from patients with undiagnosed GI symptoms, based on the reported pathology in the literature (Dubey et al., 2015a,b; Fayer et al., 2015).

In the current study, the results obtained by matching 12 undetermined *Sarcocystis* COI sequences, derived from our work on cattle muscular tissue (Rubiola et al., 2019), with *Sarcocystis* COI sequences obtained from human stool samples, supported, for the first time, the unambiguous identification of the *S. hominis* COI sequence. The estimated phylogenetic distance between our sequences and the other cattle *Sarcocystis* sequences confirmed the identification. If the positivity of human stool samples demonstrates the circulation of the parasite and the presence of an established transmission cycle between cattle and humans in the area of study, the data, however, cannot be used to speculate on the correlation between the infection and the gastrointestinal symptoms. Further focused studies should be conducted to explore the zoonotic potential of this parasite, which, in the literature, is reported to cause symptoms with a wide range of intensity and likely to go unnoticed (Dubey et al., 2015a,b).

Notably, our *S. hominis* COI sequence (GenBank accession number MH021119.1) has recently been used in the context of a prevalence study on *Sarcocystis* spp. in cattle in the Netherlands (Hoeve-Bakker et al., 2019), whose conclusions highlighted the limited number of variation among *Sarcocystis* spp. 18S genes and provided four additional *S. hominis* COI sequences (GenBank accession numbers MK497840.1, MK497841.1, MK497842.1, MK497843.1).

The second aim of this study, the development of a novel species-specific multiplex PCR assay for the simultaneous identification of all the species of the genus *Sarcocystis* actually reported in cattle in Italy, was therefore achieved taking advantage of the higher discriminatory power of the mtDNA COI gene sequences. This new protocol, which modifies and integrates the multiplex PCR described by Chiesa et al. (2013), is the first one which allow the unambiguous discrimination of *S. hominis* from the other

cattle *Sarcocystis*, being of the utmost importance for further studies on the prevalence of *S. hominis* in meat. The possibility to detect the presence of *Sarcocystis* spp. different from the reported *S. hominis*, *S. bovifelis*, *S. cruzi* and *S. hirsuta*, through the use of a genus specific primer set (Vangeel et al., 2007), makes this novel multiplex PCR assay a useful tool for the identification of unrecognized species, considering the number of new *Sarcocystis* spp. identified during the last years (Dubey et al., 2015a,b; Dubey et al., 2016; Gjerde, 2016a,b; Moré et al., 2014) and the lack of morphological and molecular information.

In conclusion, to our knowledge, this work provides the first published partial sequence of *S. hominis* mtDNA COI gene, aiding the development of a novel species-specific PCR for the molecular detection and species identification of *Sarcocystis* in cattle. The publicly available COI sequence described in this study (GenBank accession number MH021119.1) and the unambiguous identification of *S. hominis* through the application of the new multiplex PCR assay will permit the determination of the real prevalence of *S. hominis* in meat, allowing to evaluate its correlation with BEM and, from a public health perspective, to estimate the risk for the consumer.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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