

## Molecular identification of *Sarcocystis* spp. in cattle: partial sequencing of Cytochrome C Oxidase subunit 1 (COI)

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### Abstract

*Sarcocystis* spp. are protozoan parasites with an obligatory two-host life cycle, with herbivores as intermediate hosts and carnivores as definitive hosts. Cattle are intermediate hosts for several species of *Sarcocystis*: indeed, in addition to *S. cruzi*, *S. hirsuta* and *S. hominis*, at least four new species were recently identified in bovine muscle: *S. bovifelis*, *S. rommeli*, *S. bovini* and *S. heydorni*. Since it is not possible to unambiguously discriminate between *S. hominis* and the new species either morphologically or by the analysis of the 18S ribosomal (rRNA) gene, the aim of the present study was to use molecular techniques to discriminate cattle *Sarcocystis* species, taking advantage of the higher discriminative power of the Cytochrome C Oxidase subunit I mitochondrial (mtDNA COI) gene. Therefore, 119 bovine muscle samples were tested to identify *S. hominis*-like sarcocysts using a multiplex PCR of the 18S rRNA gene; later, positive samples were tested using a newly designed primer set for the PCR amplification of COI gene. Species identification was achieved by sequencing the amplified products: 16 sequences were confirmed to belong to *S. bovifelis*, while 12 sequences didn't constitute the best BLAST match of any of the published sequences, allowing to speculate the possible presence of *S. hominis*. This study confirms the higher discriminatory power of COI mitochondrial gene; besides, our work provides the first report of *S. bovifelis* in Italy.

### Introduction

Sarcocystosis is a parasitic disease distributed worldwide, whose causative agents are protozoan parasites belonging to the phylum Apicomplexa. *Sarcocystis* spp. have an obligatory two-host life cycle: in the intermediate host occurs the asexual cycle with cyst formation, while the sexual cycle occurs in the definitive host (Dubey, 2015).

Definitive hosts, comprising carnivores

and omnivores as well as humans, usually become infected by ingesting the sarcocysts in muscles, while intermediate hosts become infected through the ingestion of oocysts and sporocysts in feed or water contaminated by infected feces (Dubey, 2015).

Cattle are intermediate hosts for different species of *Sarcocystis*, among which *S. hominis* and *S. heydorni* are considered zoonotic; besides, *Sarcocystis* species have been suggested to be associated with bovine eosinophilic myositis, an inflammatory myopathy characterized by multifocal grey-green lesions in striated muscle which result in considerable economic losses (Vangeel *et al.*, 2012).

Among cattle *Sarcocystis*, full life cycles are known only for *S. cruzi*, *S. hirsuta* and *S. hominis*. Indeed, during the last three years, five new *Sarcocystis* species have been named in cattle: *S. bovifelis*, *S. sinensis* (actually considered a water buffalo species), *S. rommeli*, *S. bovini* and *S. heydorni*, leading to considerable confusion about the identification of cattle *Sarcocystis* because of incomplete descriptions and lack of morphological and molecular information. The timeline in Figure 1 reports the first evidence in scientific literature of cattle species known to date.

As reported above, morphological diagnosis of cattle *Sarcocystis* is difficult as some of them share sarcocyst morphologies (Moré *et al.*, 2014; Gjerde, 2016); for example, sarcocysts of *S. bovifelis* are morphologically indistinguishable from *S. sinensis* in water buffaloes and from the other *S. sinensis*-like species reported in cattle in recent years (*S. bovini* and *S. rommeli*), and they can't either be discriminated by *S. hominis* sarcocysts using light microscopy, while just some differences can be identified by transmission electron microscopy (TEM) (Moré *et al.*, 2014; Gjerde, 2016).

Molecular methods, particularly sequencing of 18S ribosomal RNA (rRNA), have been increasingly used to complement morphological data in the identification of *Sarcocystis* species. However, different methods and DNA regions used to compare the same taxonomic groups can lead to conflicting results.

For example, 18S rRNA gene is well suited for discrimination between more distant species but less for closely related species, where very few molecular differences are observed; on the other hand, in the last decade Cytochrome C Oxidase subunit I mitochondrial gene (mtDNA COI) has been chosen as the standard barcoding marker due to its high interspecific variation, low intraspecific variation (Hebert *et al.*, 2005). The COI gene has already proved to be a useful genetic marker for the

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*Sarcocystidae* in ruminants (Gjerde, 2013), and it is better suited than the nuclear 18S rRNA gene to differentiate between closely related *Sarcocystis* spp., such as the mentioned *S. sinensis*-like species of cattle and *S. hominis* (Gjerde, 2016).

As they can't be unambiguously discriminated by the analysis of the 18S rRNA gene, referring to *S. hominis*, *S. bovifelis*, *S. bovini* and *S. rommeli* we will use the term "*Sarcocystis hominis*-like" from now on, not including *S. sinensis* as it is now no longer considered a cattle species (Dubey *et al.*, 2014).

The aim of the present study was to use molecular techniques to discriminate cattle *Sarcocystis* species, taking advantage of the higher discriminative power of the COI gene to discriminate *S. hominis*-like sarcocysts, considering the recent taxonomic revision of cattle *Sarcocystis*.

### Materials and Methods

#### Samples collection and DNA extraction

A convenience sampling of 119 bovine carcasses was carried out over a 7 years' time span (2011-2018); 25 gr sample of striated muscle were collected directly from different slaughterhouses in Piedmont region (North-West Italy) and stored at -20°C. DNA extraction was performed using

DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to manufacturer's tissue protocol; the lysis step was carried out at 56°C overnight with Proteinase K. DNA samples were kept frozen at -20°C.

### PCR amplification of 18S sequences

The DNA samples obtained were initially tested to discriminate *S. cruzi*, *S. hirsuta* and *S. hominis*-like using the multiplex PCR protocol described by Chiesa *et al.* (2013). The samples producing amplicons of 180 bp, corresponding to the *S. hominis*-like species, were subsequently tested using the primers developed for the amplification of the mitochondrial COI gene (see below).

### PCR amplification of COI sequences

#### Primer design

As previously reported, *S. hominis*, *S. bovis*, *S. rommeli* and *S. bovifelis* can only be distinguished using the mitochondrial COI gene; therefore, our initial aim was to design a set of primer that would amplify a COI gene fragment from cattle *Sarcocystis*. To achieve this, we aligned the barcode region of COI from *Sarcocystis* partial COI sequences available from Genbank to identify conserved regions suitable for primer designing (Table 1).

Sequences of *S. cruzi*, *S. hirsuta*, *S. rommeli*, *S. bovini*, *S. bovifelis*, and *S. heydorni* were aligned using MEGA Software. Besides, the sequences of another member of the phylum Apicomplexa (*Toxoplasma gondii*, GenBank accession no. KT363924.1) were included in the alignment to evaluate possible cross reactions.

Based on these sequences, we designed different sets of primers with Primer3Plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>); specifici-

ty of the primers for the *Sarcocystis* genus was examined in-silico using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). The sequences of the primers pair, selected for specificity and amplicon parameters, are as follows:

i) forward COI primer: 5'-TGTACAT-ACTTACGGCAGGT-3' corresponding to positions 11-30 of *S. bovini* sequence (GenBank accession no. KT901022.1); ii) reverse COI primer: 5'-CCGTAGGTATG-GCGATCAT-3', matching positions 895-913 of *S. bovini* sequence (GenBank accession no. KT901022.1).

The primers were synthesized by Sigma Aldrich (St. Louis, MO).

#### Optimization of the PCR amplification conditions

The new PCR protocol was therefore optimized in order to obtain the expected amplicon. The final PCR mixture contained 2.5 µl of template DNA (5-20 ng/µL), 0,5 mM of each primer, 2.5 mM MgCl<sub>2</sub>, 0,2 mM of each dNTP, 1 U recombinant Taq DNA polymerase, 10 × PCR Buffer and distilled water to a total volume of 25 µL.

The final thermal cycle conditions used

were: initial denaturation of 94° for 5 min; 35 cycles of 95° for 1 min, 60° for 45 s and 72° for 30 s, and final extension of 72° for 5 min.

#### PCR amplification

PCR amplification of COI sequences was performed on the samples previously resulted positive for *S. hominis*-like species after the amplification of the 18S rRNA gene (Chiesa *et al.* 2013).

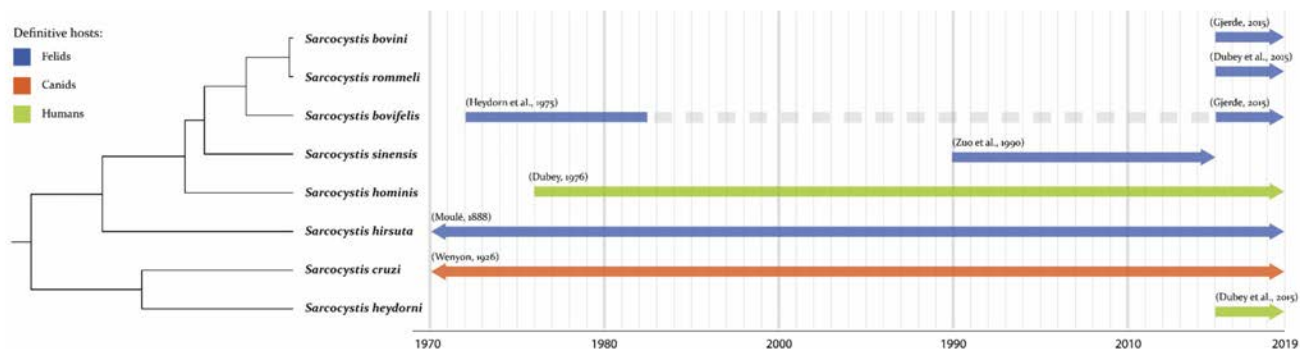
PCR products were observed in 2% agarose gel stained with SYBR safe stain (Invitrogen; Groningen, The Netherlands) and observed in a blue light transilluminator (Invitrogen; Groningen, The Netherlands).

#### Sanger sequencing

Species identification was achieved by sequencing the amplified products: they were purified with Exo-Sap (USB Europe, Staufen, Germany) treatment according to the manufacturer's recommendations. 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

**Table 1.** COI sequences accession numbers in Genbank used for primers design.

Species	Genbank accession number
<i>S. cruzi</i>	KT901095.1, KC209600.1, LC171861.1
<i>S. hirsuta</i>	KT901077.1, KC209634.1, KT901023.1
<i>S. rommeli</i>	KY120286.1, KY120291.1
<i>S. bovini</i>	KT901022.1, KT901288.1, KT900999.1
<i>S. bovifelis</i>	KT901287.1, KC209696.1, KT900997.1
<i>S. heydorni</i>	KX057995.1, KX057994.1
<i>Toxoplasma gondii</i>	JX473253.1, AF023246.1



**Figure 1.** First evidence in scientific literature of cattle species, definitive host of the species and evolutionary history inferred by unweighted pair group method with arithmetic mean (UPGMA), based on COI partial sequences (850 bp).

fragments were purified by DyeEX (Qiagen, Hilden, Germany) and resolved by capillary electrophoresis using an ABI 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).

### Average evolutionary divergence

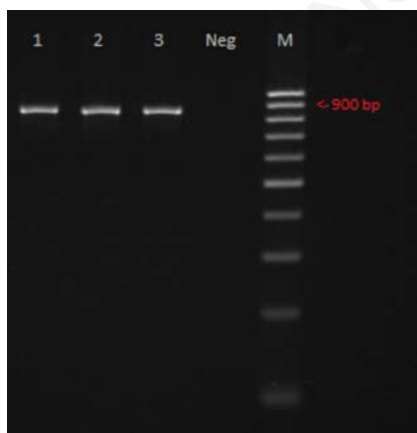
Finally, the Average Evolutionary Divergence over all Sequence Pairs of cattle *Sarcocystis*, based on the 18S rRNA gene and on the COI gene was estimated by MEGA 7.0 software (Kumar *et al.*, 2016).

## Results

The amplification of the 18S rRNA gene resulted in bands of ~180 bp in 30 samples, corresponding to *S. hominis*-like. After amplification with the new protocol, COI amplicons of the expected size (~850bp) were produced in all the samples (Figure 2).

The sequencing of the amplified products resulted in 28 readable sequences; among those, 16 showed 100% identity with published sequences from *S. bovis* (accession number KT900992.1) while 12 showed a homology of 89% to *S. bovis* (accession number KT900992.1) (Figure 3) and of 88% to *S. bovis* (accession number KT901021.1). Two samples did not result in readable sequences.

The Average Evolutionary Divergence over all Sequence Pairs of the 7 cattle species, based on the 18S rRNA gene, was 0.039 (Table 2), while the Average Evolutionary Divergence based on the COI gene was 0.226 (Table 3).



**Figure 2.** Amplification result of the *Sarcocystis* COI gene. M = 100 bp ladder; 1,2,3= positive samples; Neg = negative control.

## Discussion and Conclusions

DNA sequencing have been successfully applied in the classification of foodborne pathogens, improving diagnostic methods and phylogenetic analyses. *Sarcocystis* spp. is not a front-page parasite, but can lead to serious economic outcomes in the beef sector (Chiesa *et al.*, 2013). The recent rise of new species in cattle posed a new challenge for parasitologists and food safety scholars, as applying different methods and analyzing different genes to compare the same taxonomic groups can often lead to conflicting results (Gjerde, 2016).

Until recently, molecular species identification of various *Sarcocystis* spp. have been based almost exclusively upon sequences of the nuclear rDNA unit, particularly the 18S rRNA. However, the mitochondrial COI gene appears to have a better phylogenetic signal than the other more used genes: as previously described by some authors, the evolution of the COI gene looks like to be sufficiently rapid to discriminate between closely related species and investigate intraspecific diversity (Hebert *et al.*, 2003).

The use of COI gene for identifying protozoa is still in its initial stage, but has already proven to be a useful genetic marker for the Sarcocystidae family (Gjerde, 2013, 2016), leading to the discrimination of closely-related species previously impos-

sible to differentiate, such as *S. bovis*, *S. bovis*, *S. rommeli* and *S. sinensis*, and to the definition of new *Sarcocystis* species (Gjerde, 2016; Hu *et al.*, 2016, 2017).

Moreover, the discrimination of *Sarcocystis* species, and, of particular interest for this study, cattle *Sarcocystis* species, assumes a much more important role because humans can act as definitive host for two cattle sarcosporidian species, *S. hominis* and *S. heydorni*, with a resulting zoonotic risk for consumers of raw or undercooked meat (Bucca *et al.*, 2011).

The high presence of *S. hominis* in North-West Italian cattle, tested with molecular methods based on the analysis of 18S rRNA gene, has previously brought into question the zoonotic characteristics of this species (Chiesa *et al.*, 2013); besides, further doubts are due to the absence of reports, by the network of surveillance for foodborne diseases, of cases referable to sarcocystosis in Piedmont, a North-West Italian region characterized by a high consumption of raw beef. This has been explained by the low pathogenicity of *S. hominis* and the possibility that the infection goes unnoticed, as the symptoms reported in literature are common to most foodborne pathogens (Chiesa *et al.*, 2013). On the top of that it can now be numbered the overestimation of the prevalence of *S. hominis*, likely misidentified in consequence of the low discriminative power of

**Table 2.** The number of base substitutions per site from averaging over all sequence pairs are shown, based on the 18S rRNA gene.

Genbank ID	Species	1	2	3	4	5	6	7
KT901021.1	<i>S. bovis</i>	1						
KY120285.1	<i>S. rommeli</i>	2	0,01					
KT900992.1	<i>S. bovis</i>	3	0,007	0,014				
KF954731.1	<i>S. hominis</i>	4	0,025	0,028	0,025			
KT901158.1	<i>S. hirsuta</i>	5	0,039	0,043	0,041	0,041		
KX057997.1	<i>S. heydorni</i>	6	0,052	0,049	0,052	0,055	0,062	
KT901167.1	<i>S. cruzi</i>	7	0,048	0,051	0,046	0,05	0,059	0,024

**Table 3.** The number of base substitutions per site from averaging over all sequence pairs are shown, based on the COI gene.

Genbank ID	Species	1	2	3	4	5	6	7
KT901021.1	<i>S. bovis</i>	1						
KY120292.1	<i>S. rommeli</i>	2	0,004					
KT900992.1	<i>S. bovis</i>	3	0,059	0,057				
This study	Presuntive <i>S. hominis</i>	4	0,134	0,132	0,129			
KT901077.1	<i>S. hirsuta</i>	5	0,238	0,237	0,24	0,228		
KT901095.1	<i>S. cruzi</i>	6	0,313	0,315	0,337	0,333	0,35	
KX057995.1	<i>S. heydorni</i>	7	0,306	0,304	0,325	0,339	0,368	0,19



18S rRNA gene, which was, until recently, the standard target gene for molecular diagnosis of cattle sarcocystosis.

As clarified by the different estimates of Average Evolutionary Divergence based on the 18S rRNA gene and on the COI gene, this study confirm the higher discriminatory power of COI mitochondrial gene, because of the greater number of bases substitutions per site between sequences of different cattle *Sarcocystis* species: considering the

recent taxonomic confusion, the identification of a gene which can be used as the reference gene for the unambiguous identification of *Sarcocystis* species which are phylogenetically very close is of great importance.

Besides, this study demonstrates the presence of *S. bovifelis* in italian cattle: this is, to our knowledge, the first report of *S. bovifelis* in Italy and thus the role of domestic felids in the cattle sarcosporidiosis will

need to be reconsidered. We couldn't achieve an unambiguous identification of 12 sequences: these were all identical and 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. In this regard, an explanation could be found in the current absence of mtDNA COI gene sequences for *S. hominis* in Genbank : our hypothesis is that the 12 sequences correspond to the par-

### Sarcocystis bovifelis isolate B4.36 cytochrome c oxidase subunit I (cox1) gene, partial cds; mitochondrial

Sequence ID: [KT900992.1](#) Length: 1038 Number of Matches: 1

▶ [See 1 more title\(s\)](#)

Range 1: 11 to 866 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1038 bits(562)	0.0	759/857(89%)	2/857(0%)	Plus/Minus
Query 3	GTATCGGCCTCGAGGCCACGGTCATCATGTGGTGTGCCAAACTAGAGTACCCAGTATC	62		
Sbjct 866	GTATCGGCCTCGAGGCCACGGTCATCATGTGGTGTGCCATACCAGGGTACCTAGTACA	807		
Query 63	GATATGCATCCCATTGCCAGAATCATTGAGTGCCCGCCAAATACCTGCCCGCCCTTAACG	122		
Sbjct 806	GATATGCATCCCATTGCCAGAATCATTGAGTGCCCGCCAAATACCTGCCCGCCCTTAACG	747		
Query 123	GTAAGTGCATGCTGGATATAATACCGAAGCCAGGGAGAATCAGGATGTAACCTCCGGG	182		
Sbjct 746	GTGCTCAAACACTGGGATATTATGCCAAACCCGGTAGAATCAGGATATAAACCCTCCGGG	687		
Query 183	TGCCCCAAGAACCAGAACAAGTGTGGTACAGTACGCTATCACCAGGAGTACAGG-CATC	241		
Sbjct 686	TGCCCCAAGAACCAGAACAAGTGTGGTACAGTACACTGTCCCCGGCCAG-GCAGCCATC	628		
Query 242	GTAGAACTCGCAGTTGAAAGTGCAAATCGGCCGCAAGCATAAGCACCGCAGTAAATAT	301		
Sbjct 627	GTAGAACTCGCAGTTGAAATGCAGATCGGCAGCCAAACATAAGTACGGCAGCGTAAATAT	568		
Query 302	CGGTAAGGTAATTATCAACATTATGGAGGTAATATGATTGCCAGATGTACAGGATTCC	361		
Sbjct 567	CGGTAAGGTAATTATCAACATTATGGAGGTAATATGATTGCCAGATGTACAGGATTCC	508		
Query 362	ACACCGCAGCGACCCAAACGGCACCAGCAACGATGGTACCCAGGAGTTTATGGATCC	421		
Sbjct 507	ACATCGCCATGCCGTTACGGCCCAACGAATACCGCTGTACCTAGGAAAGTTGATGGAGCC	448		
Query 422	GAGGATGCTGCTCATACCCAGGAACAACAGGCCAAGAATTACCCAGTCAACGGCCTCCGT	481		
Sbjct 447	TAGAACAAGTCTCATACCCAGGAACCCAGGCCAAGAATTATCCAGTCAACGGCCTCCGT	388		
Query 482	ATTCATAGTCCCAGTATTGGTACTGAGTGGAGCATAGAGAGTCCAGCCAATGGCAGCACC	541		
Sbjct 387	ATTCATAGTCCCAGTATTGGTACTGAGTGGAGCATAGAGAGTCCAGCCAATGGCAGCACC	328		
Query 542	AAACTCCGCAATGAACGAGTGAACAGTAATACCGATCCAAGCGGCAGCAGGAAGTACGA	601		
Sbjct 327	AAACTCCGCAATGAATGAGTGAACAGTAATACCGATCCAAGCGGCAGCAGTAAGTACGA	268		
Query 602	AATGGCGTTAATTCTGGGCAAAACCACTTCGGCGGCTCCAATGTAGATGGGTACGAAGTA	661		
Sbjct 267	AATGGCGTTAATTCTGGGCAAAACCACTTCGGCGGCTCCAACGTAGATGGGTACGAAGTA	208		
Query 662	GTTACCAAAACAGAGAAACAATGCGGGCATCAAGAACAATGAAATATCATTGTAAGGCCATG	721		
Sbjct 207	GTTACCGAAGCCGGAGAAACAATGCGGGCATCAAGAACAATGAAATATCATTGTAAGGCCATG	148		
Query 722	CAGCGTAAACAGCACGTTGTAAGTAGCTACGCAGTACGAGGCAAAACATCCTTGCCCGGC	781		
Sbjct 147	CAGTGTGAACAGCACATTGTAAGTAGCTACGCAGTACGAGGCAAAACATCCTTGCCCGGC	88		
Query 782	AGTAGACAGTTTATACCCGACCCACATTTCGACATCAGAGTACCAACACGCTAAAGATACC	841		
Sbjct 87	AGTCGATAGTTTATACCCGACCCACGTTTCGACATCAGAGTACCAACACGCTAAAGATACC	28		
Query 842	TGCCGTAAGTATGTACA	858		
Sbjct 27	TGCCGTAAGTATGTACA	11		

Figure 3. The comparison of one of the twelve nucleotide sequences of the *Sarcocystis* COI gene with NCBI information (89% homology with sequences of *S. bovifelis*).

tial COI gene of *S. hominis*. The estimated phylogenetic distance between our sequences and the other cattle *Sarcocystis* sequences is consistent with this hypothesis; further studies are ongoing to confirm it. Finally, it wasn't possible to obtain readable sequences from two samples, probably because of the presence of coinfections in bovine muscles (Chiesa *et al.*, 2013), which could result in the overlapping between two or more sequences.

The molecular characterization of the COI gene of *S. hominis* should be considered of primary importance for the taxonomy clarification of cattle *Sarcocystis* species, allowing an appropriate risk assessment of public health issues arising from consumption of contaminated beef and providing new insights on the role of *Sarcocystis* species in the etiology of the eosinophilic myositis in cattle.

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