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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 is not possible to unambiguously discriminate between S. hominis and the new species either morphologically or by the analysis of the 18S ribosomial (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 sarcocystis 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 greygreen 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 buffaloes 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 indiscriminable 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 ribosomial 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 Correspondence: Selene Rubiola, Dipartimento di Scienze Veterinarie, Università degli studi di Torino, Largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy. Tel.: +39.011.6709334 - Fax: +39.011.6709224. E-mail: selene.rubiola@unito.it

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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. bovifelis, S. rommeli* and *S. bovini* 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. *hey-dorni* 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/cgibin/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 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 μ l of template DNA (5-20 ng/ μ L), 0,5 mM of each primer, 2.5 mM MgCl2, 0.2 mM of each dNTP, 1 U recombinant Taq DNA polymerase, 10 × PCR Buffer and distilled water to a total volume of 25 μ L.

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

The final thermal cycle conditions used

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

Species	Genbank accession number
S. cruzi	KT901095.1, KC209600.1, LC171861.1
S. hirsuta	KT901077.1, KC209634.1, KT901023.1
S. rommeli	KY120286.1, KY120291.1
S. bovini	KT901022.1, KT901288.1, KT900999.1
S. bovifelis	KT901287.1, KC209696.1, KT900997.1
S. heydorni	KX057995.1, KX057994.1
Toxoplasma gondii	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 \sim 180 bp in 30 samples, corresponding to *S. hominis*-like. After amplification with the new protocol, COI amplicons of the expected size (\sim 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. bovifelis* (accession number KT900992.1) while 12 showed a homology of 89% to *S. bovifelis* (accession number KT900992.1) (Figure 3) and of 88% to *S. bovini* (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. bovifelis*, *S. bovini*, *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	S. bovini	1							
KY120285.1	S. rommeli	2	0,01						
KT900992.1	S. bovifelis	3	0,007	0,014					
KF954731.1	S. hominis	4	0,025	0,028	0,025				
KT901158.1	S. hirsuta	5	0,039	0,043	0,041	0,041			
KX057997.1	S. heydorni	6	0,052	0,049	0,052	0,055	0,062		
KT901167.1	S. cruzi	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	56	7
KT901021.1	S. bovini	1						
KY120292.1	S. rommeli	2	0,004					
KT900992.1	S. bovifelis	3	0,059	0,057				
This study	Presuntive S. hominis	4	0,134	0,132	0,129			
KT901077.1	S. hirsuta	5	0,238	0,237	0,24	0,228		
KT901095.1	S. cruzi	6	0,313	0,315	0,337	0,333	0,35	
KX057995.1	S. heydorni	7	0,306	0,304	0,325	0,339	0,368 0,19	



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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: <u>KT900992.1</u> Length: 1038 Number of Matches: 1 <u>See 1 more title(s)</u>

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Query 242 GTAGAACTCGCAGTTGAAGTGCAAATCGGCCGCAAGCATAAGCACCGCACCAATAATAT 301 Sbjct 627 GTAGAACTCGCAGTTGAAATGCAGATCGGCAGCCAACATAAGTACGGCACCGGTAAATAT 568 Query 302 CGGTAAGGTAATTATCAACATTATGGAGGTAGGGCAGCCAACATAAGTACGGCACCGGTAAATAT 568 Query 302 CGGTAAGGTAATTATCAACATTATGGAGGGTAAATATGATTGCCCAAGATGTACAGGATTCC 361 Sbjct 567 CGGTATGGTAGTGATGAGCATTACGGAGGCACCAACGAACTAGGTAGTACGCCAGGATGTACAGGATTCC 508 Query 362 ACACCGCAGCGACCCAACGGACCAACGAACTAGGTAGTAGCCCAAGGAAGTTTATGGATCC 421 Sbjct 507 ACATCGCCATGCCGTTACGGCCCCCAACGGACATGGAGACTGCCAAGGAAGTTGATGGAGGCC 448 Query 422 GAGGATGCTGCTCATACCCAGGGAACACAGGCCAAGGAATTACCGATCCAAGGAAGTCCAAGGCCCCGT 481 Sbjct 447 TAGAACCTGGCCCATGCCGAGGAACCACGGGCACCAGGCCAAGGAAGTACCGAGGCCCCGCT 481 Sbjct 387 ATTCATGGTCATGATATTGGTACTGAGGGGGCACCAGGCACTAGGAGGACTCAAGGAGGAAGTACGA 601 Sbjct 387 ATTCATGGTCATGATAGAGGGCAACAGTGGAAGCACGTCCAAGGAGCATGAGGGCAGCAGCAGGAGCACGAGGAAGTACGA 601 Sbjct 327 AAACTCCGCGAATGGAACGAGTGCAACGAGTACCGAGCACTCCAAGGGCAGCAGCAGGAGCAATAGGGGTACGAAGGAGGAAGAACGGGCGCACCAACGGCGCAGCAGCA	Sbjct	686	tocccoAAG	AACCAGAACA	agtgctggtacagtacactgtc	ccccecceccec	ccatc 628
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Query302CGGTAAGGTAAGTTATCAACATTATGGAGGTAAATAGATTGCCCAGATGTACAGGATTCC361Sbjet567CGGTATGGTAGTGAGGAGTAGAGCATTACGGAGGTAAATACGATTGCCCCAGATGTACAGGATTCC508Query362ACACCGCCAGCGACCCAACGGCACCAACGAACATGGTAGTAGCCCAAGAAGTTTATGGAAGCC421Sbjet507ACATCGCCATGCCGTTACGGCCCCAACGAACAACGGCCAAGGAATTACCGCTGGTACCTAGGAAGGTTGATGGGAGCC448Query422GAGGATGCTGCTCATACCCAGGAACAACAGGCCAAGGAATTACCGATGCAACGGCCTCCGT481Sbjet447TAGAACACTGCTCATACCCAGGAACCACCGAGGCCAAGGAATTACCCAGTCGACGGCCTCCGT388Query482ATTCATAGTCCCGATATTGGTACTGAGTGGAGCATAGAGAGTCCAGCCAATGGCAGCACC541Sbjet387ATTCATGGTCATGATTGGTACTGAGTGGAGCATACAGAGGTCCAAGCCAATGGCAGCAGCACC328Query542AAACTCCCGCAATGAACGAGTGCAACAGTAATACCGATCCAAGCGGCAGCAGGAAGTACGA601Sbjet327AAACTCCCGCAATGAACGAGTGCAACAGTAATACCGATCCAAGCGGCAGCAGGAAGTAACGA268Query602AATGGCGTTAATTCTGGGCGAAAACCACTTCGGCGGCTCCAAAGGTGGCAGCAGTAAGTA	Sbjct	627	GTAGAACTC	GCAGTTGAAA	tőcágátcőgcagccáacátáá	dt Acgoc Accoc ta	ATAT 568
Sbjet 567 CGGTATGGTAGTGACGACACGACCAACGACGACCAACGAACATGGTAGTACCCAAGAAGTTTATGGATCC 421 Query 362 ACACCGCCAGCGACCCCAACGGCACCCAACGAACATGGTAGTACCCAAGAAGTTTATGGAACC 421 Sbjet 507 ACATCGCCATGCCGTTACGGCCCCAACGAACAACGGTGATGCTAGTACCTAGGAAGTTGATGGAGCC 448 Query 422 GAGGATGCTGCTCCTTACCCCAGGAACAACAGGCCAAGGAATTACCCAGTCAACGGCCTCCGT 481 Sbjet 447 TAGAACACTGCTCATACCCAGGAACACCAGGCCAAGGAATTACCCAGTCAACGGCGCTCCGT 388 Query 482 ATTCATAGTCCCGATATTGGTACTGAGTGGAGCATACAGGAGTCCAAGCCAATGGCAGGCA	Query	302	CGGTAAGGT	AATTATCAACA	ATTATGGAGGTAAATATGATTG	SCCCAGATGTACAGGA	ATTCC 361
Query 362 ACACCEGCAGCGACCCAACGGCACCAACGAACATGGTAGTAGCACCAAGAAGTTTATGGATCC 421 Sbjet 507 ACATCGCCATGCCGTTACGGCCCCAACGAATACCGCTGTACCTAGGAAGCT 448 Query 422 GAGGATGCTGCTCATACCCCAGGAACAACAGGCCAAGAATTATCCCAGTCAACGGCCTCCGT 481 Sbjet 447 TAGAACACTGCTCATACCCCAGGAACAACAGGCCAAGAATTATCCCAGTCGAACGGCCTCCGT 388 Query 482 ATTCATAGTCCCGATATTGGTACTGAGTGGAGCATAGAGAGTCCAGCCAATGGCAGCACC 541 Sbjet 387 ATTCATGGTCATGATATTGGTACTGAGTGGAGCATACAGAGTCCAGCCAATGGCAGCAGCACC 328 Query 542 AAACTCCGCAATGAACGAGTGCAACAGTAATACCGATCCAAGCGGCAGCAGGAAGTACGA 601 Sbjet 327 AAACTCCGCAATGAATGAGTGCAACAGTAATACCGATCCAAGCGGCAGCAGCAGCAGTAAGTA	Sbjct	567	ĊĠĠŦĂŦĠĠŦ	ÁGTGÁTGÁGĆ/	ATTACGGAGGTAAATACGATTG	SCCCAGATGTACAGGA	ttčč 508
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Query 422 GAGGATGCTGCTCATACCCAGGAACAACAGGCCAAGAATTACCCAGTCAACGGCCTCCGT 481 Sbjct 447 TAGAACACTGCTCATACCCAGGAACCACGGCCAAGAATTATCCAGTCGACGGCCTCCGT 388 Query 482 ATTCATAGTCCCGATATTGGTACTGAGTGGAGCATAGAGAGTCCAGCCAATGGCAGCACC 541 Sbjct 387 ATTCATAGTCCCGATATTGGTACTGAGTGGAGCATACAGAGTCCAGCCAATGGCAGCACC 328 Query 542 AAACTCCCGCAATGAACGAGTGCAACAGTAATACCGATCCAAGCGGCAGCAGGAAGTAAGT	Sbjct	507	ACATCOCCA	TGCCGTTACG	SCCCCAACGAATACCGCTGTAC	CTAGGAAGTTGATGO	SÁGČČ 448
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Query482ATTCATAGTCCCGATATTGGTACTGAGTGGAGCATAGAGAGTCCAGCCAATGGCAGCACC541Sbjct387ATTCATGGTCATGATATTGGTACTGAGTGGAGCATACAGAGTCCAGCCAATGGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA	Sbjct	447	TAGAACACT	GCTCATACCC	AGGAACCCCAGGCCAAGAATTA	ATCCAGTCGACGGCCT	TCCGT 388
Sbjet 387 ATTCATGGTCATGATATTGGTACTGAGTGCGAGCGATACAGAGTCCAGCCAG	Query	482			STACTGAGTGGAGCATAGAGAG		SCACC 541
Query 542 AAACTCCGCAATGAACGAGTGCAACAGTAATACCGATCCAAGCGGCAGCAGGAAGTACGA 601 Sbjct 327 AAACTCCGCAATGAATGAGTGCAACAGTAATACCGATCCAAGCGGCAGCAGCAGGAAGTACGA 268 Query 602 AATGGCGTTAATTCTGGGCAAAACCACTTCGGCGGCTCCAATGTAGATGGGTACGAAGTA 661 Sbjct 267 AATGGCGTTAATTCTGGGCAAAACCACCTCGGCGGCTCCAATGTAGATGGGTACGAAGTA 208 Query 662 GTTACCAAAACCAGAGAACAATGCGGGCATCAAGAAACATGAATATCATTGTAAGGCCATG 721 Sbjct 207 GTTACCGAAGCCGGGAGAACAATGCGGGGCATCAAGAACATGAATATCATTGTAAGGCCATG 148 Query 722 CAGCGTAAAACAGCACGTTGTAAGTAGCTACGCAGTCGACGGCAAACATCCTTGCGCCAGC 781 Sbjct 147 CAGCGTGAAACAGCACATTGTAAGTAGCTACGCAGTCGACGACGACGAACATCCTTGCCCCGGC 88 Query 782 AGTAGACAGTTCATACCGCACCACATTCGACATCAGAGTACCAACAACATCCTTGCCCCGGC 841 Sbjct 87 AGTCGATAGTTCATACCGCACCACGTTCGACATCAGAGTACCAACAACGCTAAAGATACC 28 Query 842 TGCCGTAAGTATGTACA 858 55 Sbjct 27 TGCCGTAAGTATGTACA 11	Sbjct	387	ATTCATGGT	CATGATATTG	STACTGAGTGGAGCATACAGAG	TCCAGCCAATGGCAG	SCACC 328
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Sbjet 287 AATGGEGTTAATTETGGGEAAAAGTACETGEGGEGETECAACGATGGATGGGTACGAAGTA 288 Query 662 GTTACCGAAAACCAGAGGAACAATGCGGGGCATCAAGAACATGAATATCATTGTAAGGCCATG 721 Sbjet 207 GTTACCGAAAGCCGGAGAACAATGCGGGGCATCAAGAACATGAATATCATTGTAAGGCCATG 148 Query 722 CAGCGTAAAACAGCACGTTGTAAGTAGCTACGCAGTCGACGGCAAACATCCTTGCGCCAGC 781 Sbjet 147 CAGTGTGAAACAGCACATTGTAAGTAGCTACGCAGTCGACGGCAAACATCCTTGCCCCGGC 88 Query 782 AGTAGACAGTTCATACCGCACCACATTCGACATCAGAGTACCAACAACGCTAAAGATACC 841 Sbjet 87 AGTCGATAGTTCATACCGCACCACGTTCGACATCAGAGTACCAACAACGCTAAAGATACC 28 Query 842 TGCCGTAAGTATGTACA 858 55 Sbjet 27 TGCCGTAAGTATGTACA 11	Query	002					
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Sbjct 87 AGTCGATAGTTCATACCGCACCACGTTCGACATCAGAGTACCAACAACGCTAAAGATACC 28 Query 842 TGCCGTAAGTATGTACA 858 Sbjct 27 TGCCGTAAGTATGTACA 11	Ouerv	782	AGTAGACAG	TTCATACCOC	ACCACATTCGACATCAGAGTAC	CAACAACGCTAAAGA	TACC 841
Query 842 TGCCGTAAGTATGTACA 858 Sbjct 27 TGCCGTAAGTATGTACA 11	Shict	87	AGTCGATAG	TTCATACCOC	ACCACGTTCGACATCAGAGTAC		TACC 28
Sbjct 27 TGCCGTAAGTATGTACA 11	Ouerv	842	TGCCGTAAG	TATGTACA	358		
	Sbjct	27	TGCCGTAAG	TATGTACA	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 myosistis in cattle.

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