A new molecular approach to assess the occurrence of *Sarcocystis* spp. in cattle and products thereof: preliminary data

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Abstract

The genus *Sarcocystis* consists of more than 200 species. Those protozoa are characterised by a biological cycle composed by two obligatory hosts, definitive and intermediate. Apart from being possibly pathogenic for the intermediate host, a number of authors consider the intestinal sarcocystosis a minor zoonotic disease. Humans, in fact, can act as definitive host for two sarcosporidian species, *S. suihominis* e *S. hominis*, being infected through the consumption of raw or undercooked pig and bovine meat, respectively. Other two species could parasitise cattle: *S. cruzi* and *S. hirsuta*, having canids and felids as definitive hosts, respectively. The three species differentiate from each other in dimensions and cystic wall morphology, this latter being the basis for taxonomical studies. In 2010, the European Food Safety Authority (EFSA) highlighted the absence of reliable methods for epidemiological studies on the presence of *Sarcocystis* spp. in animals and products thereof. On this basis, the present study has been developed a new molecular method for the identification of *Sarcocystis* in bovine meat. For the development of the polymerase chain reaction (PCR) protocol, a set of samples of bovine meat from cattle (N=15), slaughtered at the didactic abattoir at the Veterinary Faculty of Turin University, has been collected, sequenced and used as reference samples during the study. A second set of samples (N=29), gathered from the same abattoir (N=12) and from abattoirs of Piedmont region (N=17), has been used for applicability tests. The overall positive rate for *Sarcocystis* spp. in our samples has been 91% (40/44), with *S. cruzi* representing the species with higher rates (68%), followed by *S. hominis* (43%) and *S. hirsuta* (2%). Based on the results of specificity and applicability tests performed in this study, the newly developed protocol proved to be reliable and suitable for epidemiologic purposes.

Introduction

The genus *Sarcocystis* (from the greek *sarkos*=muscle; *kystis*=cysts), belong to the phylum Apicomplexa protozoa or sporozoa, includes a considerable number of species of coccidia, heterogeneous in terms of life cycle, host-specificity, morphology and pathogenicity (Dubey *et al*., 1989; Fayer, 2004). The latest revision of the Sarcosporidia taxonomy, dating from 1998, identified and classified 189 species; in the following years these have been joined by others, formerly unknown (Heckeroth and Tenter, 2007).

This kind of protozoa is characterised by a life cycle with two vertebrate hosts, intermediate and definitive, with a predator-prey relationship. In the definitive host, after the ingestion of tissue cysts present in the muscles of preyed animals, starts the sexual intestinal phase, followed by liberation of oocysts into the intestinal lumen, which are then shed in the feces. These are, in turn, ingested by intermediate hosts, where the extraintestinal phase (asexual multiplication) takes place, with the formation of intramuscular cysts, containing bradyzoites (Dubey *et al*., 1989; Fayer, 2004). Beyond the potential pathogenicity in the intermediate host (Heckeroth and Tenter 2007), many authors consider the intestinal sarcocystosis as a minor zoonosis. Humans can host the intestinal phase of two species of Sarcosporidia, *S. suihominis* and *S. hominis*, acquiring the infection through consumption of raw or undercooked infested pork and beef (Tenter, 1995), with nausea, diarrhea and vomiting as the main symptoms of the infection. In addition to those already mentioned, other species known in cattle count *S. cruzi* and *S. hirsuta*, which recognise, as definitive hosts, respectively, canines and felines. In cattle, the three species show unique characteristics in terms of sise and morphology of the cyst wall; until recently, the taxonomy of the genus was based on this latter feature (Fayer, 2004; Dubey and Lindsay, 2006). The epidemiological situation of bovine sarcocystosis recognises a prevalence as high as 90%, with data referring to different geographical areas: New Zealand, Belgium, France, Iraq, the Netherlands and Ethiopia (Tenter, 1995; Dubey and Lindsay, 2006; Heckeroth and Tenter, 2007; Vangeel *et al.*, 2007). The spread of this parasite in Italy is documented by studies conducted both in continental and island areas; among those, one study conducted in Sicily found a prevalence of 96% (Bucca *et al*., 2010). In another recent study bovine muscle samples were screened by histological techniques and subsequently analysed with molecular methods, showed a prevalence for *Sarcocysis* spp. above 80% (Domenis *et al*., 2011). In the *post-mortem* examination, the diagnosis can only regard the rare cases of animals suffering from eosinophilic myositis, which is an impressive inflammatory form, traditionally connected, in terms of etiology, to the presence of *Sarcocystis* spp. (Lindsay *et al*., 1995). Except for the above mentioned cattle, the infection is unapparent and the presence of the parasite can be appreciated only through histological techniques; for species identification, on the other hand, the observation of the cyst wall ultrastructure by electron microscope is needed (Odening, 1998). In recent decades, several groups of researchers have tested molecular methods for the identification of *Sarcocystis* spp. The immunoassay protocols in fact, are, in general, characterised by lack of specificity (Tenter, 1995) while electron microscopy lacks of sensitivity and is known to be far too expensive for routine practice (Dubey *et al.*, 1988). The study of the parasite genome and in particular of the nucleotide sequences coding for the 18S rRNA, provided valuable alternatives to electron microscopy (Vangeel *et al.*, 2007). Sequencing and random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR) have been studied by different authors for the identification of *Sarcocystis* spp. (Yang *et al.*, 2002; Guclu *et al.*, 2004; Vangeel *et al.*, 2007). The frequency of simultaneous infection by different species, however, significantly reduces the field of application of these methods.

In this perspective, we developed a multiplex PCR for the identification of species of the genus *Sarcocystis* in cattle.

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Materials and Methods

For the development of the PCR protocol a set of muscle samples, taken from 15 cattle at the slaughterhouse of the Faculty of Veterinary Medicine of Turin University, were analysed. DNA was extracted from 25 (±5) mg of muscle (diaphragm, heart or masseter), using a commercial kit (DNeasy Blood and Tissue Kit; Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted DNA was amplified with specific primers for the genus *Sarcocystis* according to Yang *et al.* (2001) and then sequenced with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were purified using the DyeEx 96 Kit (Qiagen) and sequenced by capillary electrophoresis using the ABI 310 Genetic Analyser (Applied Biosystems). Sequences were analysed with BLASTN software (Altschul *et al.*, 1990).

These sequences were subsequently used as reference samples for setup the multiplex PCR protocol. A second set of samples (N=29), used for applicability tests, were in part from the slaughter facility of the Faculty of Veterinary Medicine of Turin (N=12) and in part from different slaughterhouses in Piedmont region (N=17); the latter represented by cattle muscle samples positive for eosinophilic myositis.

The 18S rRNA gene sequences, of the three species of interest (*S. cruzi, S. hominis and S. hirsuta*) together with phylogenetically related species (*Toxoplasma gondii* and *Neospora caninum*), retrieved from GenBank were aligned with ClustalX software (Thompson *et al.*, 1999), for design the species-specific primers. The sequences used are listed in Table 1. Based on the alignment results four primers were designed: a reverse primer, common to all the three species and three forward primers specific for *S. cruzi*, *S. hominis* and *S. hirsuta* (Table 2).

The PCR reaction was set up for a final volume of 25 µL per reaction, containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1 U of Platinum Taq (Invitrogen, Paisley, UK), 0.2 mM dNTPs (Invitrogen), 2 mM $MgCl_2$, 12.5 pmol/µL of each primer and 2.5 µL of DNA, at a concentration of 100 ng/µL. The amplification conditions consisted of a denaturation step at 95°C for 3 min, followed by 40 cycles at 95°C for 60 sec, 56°C for 60 sec and 72°C for 30 sec and final extension at 72°C for 5 min.

In order to increase the sensitivity of the assay, in terms of representativeness of the sample towards the presence of *Sarcocystis* species in the entire carcass, a method of artificial digestion and bradizoytes concentration from 10 g of muscle has been optimised, according to the protocol described by Heckeroth and Tenter (2007). After trypsin digestion of 10 g of muscle in 50 mL of digestion solution (2.5 g trypsin 1:250/1l 1x PBS) for 2 h at 37°C in a water bath thermostat with stirring, the suspension was centrifuged for 5 min at 7000 rpm. The pellet was resuspended in 2 mL of PBS and 300 µL of the suspension was subjected to DNA extraction as reported above. To demonstrate the aliquots representative of the total volume of the digestion, a test has been performed, where 10 aliquots of 200 µL of the total DNA extracted from the sample were analysed.

Table 1. Sequences accession numbers in GenBank.

Species	Reference sequences accession no. in GenBank
S. hominis	AF176945, AF176944, AF176943, AF176942, AF006471, AF006470, AF006473
S. cruzi	AF017120, AB682779, AB682780, AB682781, AF176934, AF176933, AF176935
S. hirsuta	AF006469, AF176938, AF017122, AF176940, AF176941
N. caninum	GQ899206, U16159, U17346, U03069, L24380
T. gondii	L37415, U03070, L24381

Table 2. Forward and reverse primers based on alignment results.

Forward primer GenBank accession no.	Sequence	Position	T (°C)
S. cruzi AF017120	ATCAGATGAAAATCTACTACATGG	110-133	57
S. hominis AF006470	ACAGAACCAACACGCTC	143-159	57
S. hirsuta AF017122	CATTTCGGTGATTATTGG	313-330	56
Reverse primer GenBank accession no.			
S. cruzi AF017120	AACCCTAATTCCCCGTTA	378-395	58
S. hominis AF006470		306-323	
S. hirsuta AF017122		403-420	

Results

The amplification of the 18S rDNA with the species-specific primers pairs resulted in bands of 108, 182 and 284 bp, for *S. hirsuta*, *S. hominis* and *S. cruzi*, respectively (Figure 1). Each primer pair did not was proved to be specific for the target *Sarcocystis* species and did not amplify the two non-target species.

Aliquots of the total DNA extracted by the sample gave the same result (positive), demonstrating that it was possible to analyse one aliquot and that this would be representative of the total volume.

The results of analyses on the samples used for the development of the protocol, showed a prevalence for the presence of *Sarcocystis* spp. in 91% (40/44) of the samples. In Figure 2 the results related to the presence of individual species are reported. *S. cruzi* is the most present, with 68% of positive animals (30/44), followed by *S. hominis*, with 43% (20/44), while *S. hirsuta* was detected only in one animal (2%). The 17 animals with eosinophilic myositis lesions showed a higher positivity for *S. hominis* (52%) compared to the other samples (37%).

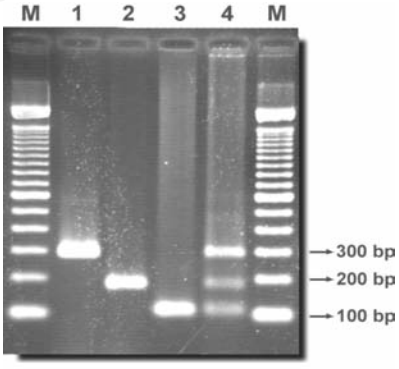

Figure 1. Bands resulting from amplification of the 18S rDNA with the species-specific primers pairs. M=100 bp ladder; 1=*S. cruzi*; 2=*S. hominis*; 3=*S. hirsuta*; 4=mix of the three species.

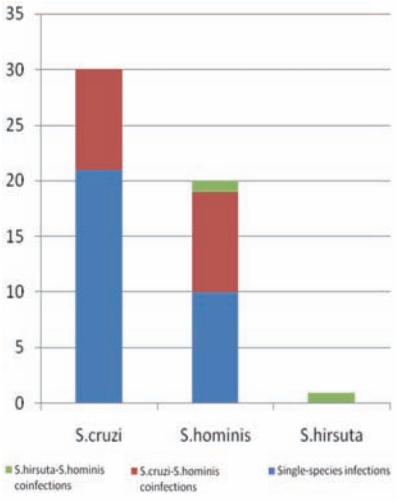

Figure 2. Results of analyses on the samples related to the presence of individual species.

Discussion

Based on the EC Regulation 854/2004 on official controls on meat intended for human consumption, the presence of *Sarcocysts* in the carcass of slaughtered animals is not covered by routine meat inspection. However, the high prevalence rate of infection, the presence of *Sarcocysts* species considered potentially zoonotic and the growing habit, in European countries, to eat raw or undercooked meat, brought some authors to consider the genus *Sarcocystis* as a possible zoonotic risk for consumers (Bucca *et al.*, 2010).

Cattle acts as intermediate host for three different species, of which only *S. hominis* is considered potentially zoonotic to humans; the infestation is usually macroscopically unapparent. (Dubey *et al.*, 1989; Fayer, 2004). Although light microscopy can be used for the diagnosis of sarcocystosis from histological section, species identification require the application of electron microscopy. Alternatively, molecular methods have been implemented to facilitate the identification of bovine *Sarcocystis*. Most of these techniques are based on the analysis of the 18S ribosomal RNA gene (Fischer and Odening, 1998).

Given the massive presence of coinfections in bovine muscles and the absence of a current molecular fast and effective method for discriminating between the species, the aim of our work was the development of a multiplex-PCR, capable of amplify and separate gene fragments of different lengths belonging to *S. cruzi*, *S. hirsuta* and *S. hominis* species.

Although the aim of the study was not oriented towards an epidemiological investigation and, therefore, no representative sampling of the bovine population was planned, the data obtained on the presence of *Sarcocystis* spp. in cattle in this study (91%) is in line with the data found in literature. Several studies, in fact, conducted in different countries and with different methods, provide prevalence data between 80 and 95% (Cama and Ortega, 2006; Dubey and Lindsay, 2006; Vangeel *et al.*, 2007; Bucca *et al.*, 2010; Moré *et al.*, 2011). Also the prevalence of the single species are similar to those reported by other authors: the prevalence for *S. cruzi* reported in the literature, between 65 (Bucca *et al.*, 2010) and 98% (Cama and Ortega, 2006), is compatible with that obtained in the present work (68%), as well as that of *S. hirsuta* (2%) and *S. hominis* (43%) (Dubey and Lindsay, 2006; Domenis *et al.*, 2011). For these latter species, unlike *S. cruzi*, the range of positivity reported in the various countries is very wide, ranging, respectively, from 1 to 49% and from 20 to 70% (Cama and Ortega, 2006; Dubey and Lindsay, 2006; Vangeel *et al.*, 2007; Bucca *et al.*, 2010; Moré *et al.*, 2011). The variability of these data is due, in part, to differences in the sensitivity and specificity of the diagnostic methods used, but also to the different contributions from the definitive hosts to the maintenance of the life cycle of the parasite in different countries.

The difference between the prevalence for *S. hominis* in the group of samples from cattle with eosinophilic myositis lesions at slaughter (52%), compared to other samples (37%), is a matter worthy of note: the reduced number of samples and the type of sampling did not give the possibility for statistical inferences, let alone to reach conclusions, but, nevertheless, the data suggest the use of new molecular protocol to clarify the role of various *Sarcocystis* species in the etiology of eosinophilic myositis. The presence of *S. hominis*, considered a potential pathogen in almost half of the animals tested, it would seem to call into question the zoonotic characteristics of this species: some of the studies that demonstrate the pathogenicity of *S. hominis* for humans are dated (Fayer, 2004) or not entirely reliable (Chen *et al.*, 1999). The absence of reporting, by the network of surveillance for foodborne diseases, of cases referable to sarcocystosis in the Piedmont region area, characterised by a high consumption of raw beef, puts further doubt on these studies. In particular, it may advance two sets of assumptions: first, the pathogenicity of *S. hominis* it occurs only under certain conditions, probably related to the immune status of the consumer or in the presence of a synergistic effect with other food pathogens. The second hypothesis considers the possibility that many cases of intestinal sarcocystosis, following the spontaneous resolution within a few onset of symptoms, remain undiagnosed. It should also be considered how, in the rare case of hospitalization, following episode of foodborne illness, research by the national health system tend to move towards other pathogens. The time of onset of the gastrointestinal syndrome caused by *Sarcocystis* is of the order of a few hours (4-6 on average) and spontaneous resolution occurs in 24-36 h. These parameters and the type of food involved (raw or undercooked meat) make easy to understand why the diagnosis tent often to focus towards other etiological agents. To the probability of an ususpected diagnosis for sarcocystosis, it has to be added the troublesome detection of sporocysts in faeces (14-18 days for *S. hominis*). The discovery of the latter is currently the only way to diagnose sarcocystosis in humans (Fayer, 2004). It remains, however, to prove the causal link between the presence of the parasite and the onset of symptoms. The lack of certain scientific evidences on this topic determined the request, by the European Food Safety Authority (EFSA), of a method of monitoring for the presence of *Sarcocystis* spp. in meat.

Conclusions

The results of this study demonstrate how the multiplex PCR, developed in this study, represents an effective and rapid method for an unbiased identification of *Sarcocystis* species in cattle. In addition, the trypsin digestion protocol carried out on pooled tissues, obtained from a single carcass, could be proposed as a reliable screening method for the detection of the parasite in meat. This protocol will be of the utmost importance for further studies on the prevalence of *S. hominis* in meat and humans and on the role of *Sarcocystis* species in the aetiology of eosinophilic myositis.

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