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Research paper

Molecular identification of *Sarcocystis* spp. in foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) from Germany

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ABSTRACT

More than 200 *Sarcocystis* spp. have been named and most of them appear to be involved in a particular predator-prey cycle. Among canids, the European fox (*Vulpes vulpes*) and the raccoon dog (*Nyctereutes procyonoides*) are widely distributed in Europe and probably play an important role as definitive hosts in the epidemiology of *Sarcocystis* spp. infections. A total of 50 small intestines from foxes and 38 from raccoon dogs were sampled in the Federal State of Brandenburg, Germany. Mucosal scrapings were collected and analyzed by sugar flotation and when oocysts or sporocysts were detected, an overnight sedimentation was performed and DNA extracted with a commercial kit. A PCR was conducted using primers targeting a fragment of the 18S rRNA gene (with a size of approximately 850 bp) and the amplicons were purified and sequenced. Samples with an inconclusive sequencing were cloned into plasmids and ≥ 3 plasmids from each amplicon were sequenced. *Sarcocystis* spp. oocysts/sporocysts were detected in 38% (19/50) of fox and 52.6% (20/38) of raccoon dog samples. Sequencing analysis of amplicons from oocyst DNA revealed mixed infections in 9 fox and 5 raccoon dog samples. In the fox samples, the most often identified *Sarcocystis* spp. were *S. tenella* or *S. capracanis* (10.0%); *S. miescheriana* (8.0%) and *S. gracilis* (8.0%) followed by *Sarcocystis* spp., which use birds as intermediate hosts (6.0%), and *S. capreolicanis* (4.0%). In the raccoon dog samples, sequences with a ≥99% identity with the following species were detected: *S. miescheriana* (18.4%), *S. gracilis* (13.1%), *Sarcocystis* spp. using birds as IH (10.5%), *S. tenella* or *S.capracanis* (2.6%) and *S. capreolicanis* (2.6%). The estimated prevalence of *Sarcocystis* spp. infections determined using mucosal scrapings was higher than in related studies performed by analyzing faecal samples. The methodology of 18S rRNA gene amplification, cloning and sequencing is suitable to identify mixed infections with *Sarcocystis* spp. and to gather information on potential definitive hosts of these parasite species.

1. Introduction

Infections caused by protozoan parasites of the genus *Sarcocystis* are globally distributed and affect a high proportion of animals (Dubey et al., 2015, 1989). *Sarcocystis* spp. are heteroxenous apicomplexan parasites, mostly with carnivores or omnivores as definitive hosts (DH, intestinal sexual development and sporogony) and herbivores as intermediate hosts (IH, asexual development leading to sarcocysts, i.e. muscle cysts). The sexual development regularly occurs in the small intestine of the DH and as a result of the sporogony in the *lamina propria*, oocysts containing two sporocysts (with 4 sporozoites each) are developed. Typically, the thin oocyst wall breaks thus releasing the sporocysts into the intestinal lumen. More than 200 *Sarcocystis* spp. have been named and most of them appear to be involved in a particular predator-prey cycle. A large number of *Sarcocystis* spp. has been described based on the morphology of sarcocysts, but our knowledge on the life cycles of many of them is incomplete (Dubey et al., 2015; Odening, 1998). The sporocysts released by the DH are morphologically indistinguishable among *Sarcocystis* species. Traditionally, the identification of the DH has been conducted feeding potential hosts with meat containing sarcocysts and detecting sporocysts in feces after a prepatent period (around 7 and 14 days) (Dubey et al., 2015). In general, intestinal infections with *Sarcocystis* spp. are asymptomatic with the exception of species that use humans as DH (Fayer et al., 2015). Canids are DH of many *Sarcocystis* spp. and some of them appear in a proportion of more than 90% of the IH populations (Dubey et al., 2015, 1989; Moré et al., 2011). In contrast,

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studies conducted by processing faecal samples of dogs and other canids revealed a lower proportion of animals, in which sporocysts were detected (Barutzki and Schaper, 2003, 2011; Dubey et al., 2015). Experimental infections of canids with *S. cruzi* resulted in an extremely high number of oocysts and sporocysts in the mucosa of the small intestine (Dubey, 1983; Fayer, 1977). Probably, the oocysts remain concentrated in the *lamina propria* and sporocysts are released only sporadically over the course of time (Dubey et al., 2015). Little is known about intestinal infections with *Sarcocystis* spp. in wild carnivores under natural conditions and only few studies have reached to determining the causative agents on the species level (Dahlgren and Gjerde, 2010; Dubey et al., 2015; Prakas et al., 2015). Among canids, the European fox (*Vulpes vulpes*) and the raccoon dog (*Nyctereutes procyonoides*) are widely distributed in Europe and probably play an important role as DH in the epidemiology of *Sarcocystis* spp. infections (Prakas et al., 2015). Most *Sarcocystis* spp. differ in the sequences of 18S rRNA and cytochrome c oxidase subunit I (*cox1*) genes, which allowed differentiation between species (Dubey et al., 2015; Gjerde, 2013; Moré et al., 2013).

The aims of the present study were to detect *Sarcocystis* spp. oocysts and sporocysts in mucosal scrapings of the small intestine of foxes and raccoon dogs sampled in Brandenburg, Germany, to and differentiate infections with a variety of *Sarcocystis* spp. using molecular tools.

2. Material and methods

2.1. Samples

A total of 50 small intestines from foxes and 38 from raccoon dogs were collected as part of an echinococcosis surveillance program and frozen at −80 °C for at least one week. All the animals had been shot by hunters in the Federal State of Brandenburg, Germany, during the year 2012. Samples were thawed and mucosal scrapings from the posterior two thirds of the small intestines were obtained by using an individual glass slide for each sample. The collected material was transferred to a 50 ml tube and conserved at −20 °C until used.

2.2. Coproparasitological studies

Initially, 1.5 g of each mucosal scraping was homogenized with 15 ml of sucrose solution and centrifuged at 500*g* during 10 min. Material from the top of the sugar flotation suspension was transferred to a slide using a wire loop with a diameter of 3 mm, covered with a cover sliver and examined in a microscope at 200× for *Sarcocystis* spp. oocysts and/or sporocysts detection. If oocysts or sporocysts were detected, the remaining mucosal material of the sample was subjected to overnight water sedimentation, followed by sucrose flotation and centrifugation in water as described for the detection and concentration of oocysts of other apicomplexan parasites (Schares et al., 2005). From each positive sample, 400 μl of the overnight sediment and 400 μl of the floated and concentrated material were preserved at −20 °C until used for DNA extraction.

2.3. PCR and sequencing

DNA was extracted from aliquots of the sediment and of the floated and concentrated material using the ZR fecal DNA commercial kit (Zymo, USA) according to the instructions of the manufacturer. PCR was conducted with the primers SarcoFext and SarcoRext targeting a fragment of the 18S rRNA gene (with a size of approximately 850 bp) as previously described (Moré et al., 2013). Positive amplicons were purified and submitted for sequencing in both orientations with primers SarcoFint and SarcoRint following a previously published protocol (Moré et al., 2013). Sequences were aligned and analyzed with the program GENEIOUS (free available version 7.1, http://www.geneious.com). Consensus sequences were compared with data available in GenBank by the megablast function of BLAST (http://blast.ncbi.nlm.nih.gov).

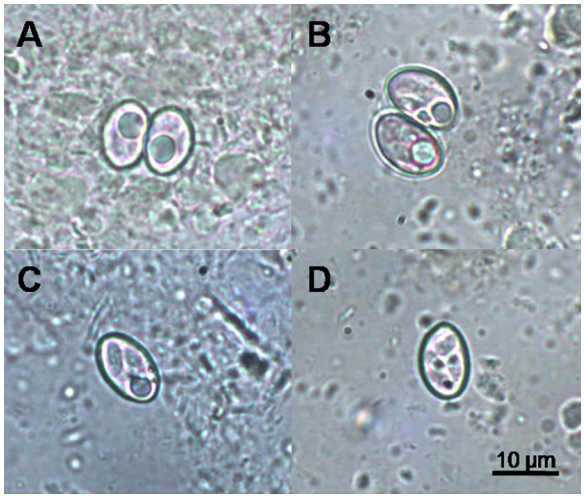

Fig. 1. Microscopic examination of mucosal material after sucrose flotation showing morphologically intact oocysts (A and B) and sporocysts (C and D) from frozen samples from the small intestine of foxes (A and C) and raccoon dogs (B and D). Scale bar apply for all images.

Samples with inconclusive amplicon sequencing results were considered as potential mixed infections of different protozoan species (Moré et al., 2014b). The purified amplicons (18S rRNA gene fragments) from such samples were cloned into plasmids and ≥3 plasmids from each amplicon sequenced with universal plasmid primers as previously described (Moré et al., 2013).

All the *Sarcocystis* spp. sequences obtained in the present study were submitted to GenBank (accessions numbers listed in Tables 1 and 2). Sequences with a BLASTn identity ≥99% were recorded with a specific species name. All samples with ≤98% of sequence identity to sequences of known species deposited in GenBank as well as sequences that showed similar identity with more than one previously reported species were registered as *Sarcocystis* sp.

3. Results

Mucosal infections with *Sarcocystis* spp. were detected in 38.0% (19/50) of fox and 52.6% (20/38) of raccoon dog samples by the initial sugar flotation. The microscopic structure of oocysts and sporocysts had remained conserved in the frozen tissues (Fig. 1).

All samples, in which oocysts or sporocysts of *Sarcocystis* spp. had been observed after initial flotation of mucosal material, were positive by PCR, both in DNA extracted from the overnight sediment and in DNA extracted from floated material. The amplicons obtained from DNA extracted from floated and concentrated material showed a higher DNA concentration in agarose gels (with the exception of one sample) and were further purified for sequencing. Table 1 shows the results of sequencing and BLASTn comparisons as well as the GenBank accession numbers of *Sarcocystis* spp. 18S rRNA sequences obtained from fox and raccoon dog samples. According to the ambiguities observed in chromatograms, mixed infections were recorded in 9 and 5 of fox and raccoon dog samples, respectively (Table 1). Amplicons obtained from samples with mixed infections were cloned into plasmids and sequenced. Table 2 shows the sequencing and BLASTn comparison results as well as the

Table 1
Consensus sequences of *Sarcocystis* spp. 18S rRNA gene fragments amplified from DNA extracted from mucosal scrapings of the small intestine of fox and raccoon dogs containing microscopically visible *Sarcocystis* spp. oocysts/sporocysts.

Sample ID	Sequence base pairs	BLASTn identity (#)	*Sarcocystis* sp. (GenBank accession number, this study)
F93	619	99% *S. tenella* (KC209734, KC209737) and *S. capracanis* (L76472)	*Sarcocystis* sp. (KT873734)
F102	626	100% *S. gracilis* (FJ196261, JN226126)	*S. gracilis* (KT873735)
F127	626	100% *S. gracilis* (FJ196261, JN226126)	*S. gracilis* (KT873736)
F129	343	99% *S. tenella* (KC209734, KC209737) and *S. capracanis* (L76472)	*Sarcocystis* sp. (KT873737)
F132	Mixed	–	NR
F712	Mixed	–	NR
F718	322	99% *Sarcocystis* sp. JQ733511, *S. columbae* (HM125054, GU253883), *S. albifronsi* (EU502868) and *S. anasi* (EU553477)	*Sarcocystis* sp. (KT873738)
F722	Mixed	–	NR
F732	598	99% *S. tenella* (KC209734 and KC209737) and *S. capracanis* (L76472)	*Sarcocystis* sp. (KT873739)
F740	Mixed	–	NR
F755	626	100% *S. gracilis* (FJ196261 and JN226126)	*S. gracilis* (KT873740)
F756	Mixed	–	NR
F772	614	100% *S. miescheriana* (JN256123)	*S. miescheriana* (KT873741)
F777	Mixed	–	NR
F786	Mixed	–	NR
F792	Mixed	–	NR
F793	540	99% *S. tenella* (KC209734 y KC209737) and *S. capracanis* (L76472)	*Sarcocystis* sp. (KT873742)
F794	573	100% *S. albifronsi* (EU502868) and *S. anasi* (EU553477)	*Sarcocystis* sp. (KT873743)
F795	Mixed	–	NR
RD15	577	96% *S. cruzi* (JX679467, AB682779, AF176933)	*Sarcocystis* sp. (KT873744)
RD17	217	100% *S. miescheriana* (KT120004-KT120022, JN256123, GU395554)	*S. miescheriana* (KT873745)
RD19	224	100% *S. rileyi* (KM233682, KJ396583, JQ733511, GU120092)	*S. rileyi* (KT873746)
RD21	883	99-100% *S. miescheriana* (JN256123, GU395554, EU327974)	*S. miescheriana* (KT873747)
RD23	598	99-100% *S. tenella* (KC209734, KC209737) and 99% *S. capracanis* (L76472)	*Sarcocystis* sp. (KT873748)
RD24	Mixed	–	NR
RD25	626	100% *S. gracilis* (FJ196261 and JN226126)	*S. gracilis* (KT873749)
RD28	614	99-100% *S. miescheriana* (JN256123, JX840464-JX8404647)	*S. miescheriana* (KT873750)
RD32	890	99-100% *S. miescheriana* (JN256123, JX8404664-JX8404667)	*S. miescheriana* (KT873751)
RD35	Mixed	–	NR
RD41	614	99-100% *S. miescheriana* (JN256123, JX840464-JX8404647)	*S. miescheriana* (KT873752)
RD43	626	100% *S. gracilis* (FJ196261 and JN226126)	*S. gracilis* (KT873753)
RD44	614	99-100% *S. miescheriana* (JN256123, JX840464-JX8404647)	*S. miescheriana* (KT873754)
RD46	557	100% *S. albifronsi* (EU502868) and *S. anasi* (EU553477)	*Sarcocystis* sp. (KT873755)
RD51	Mixed	–	NR
RD52	626	100% *S. gracilis* (FJ196261 and JN226126)	*S. gracilis* (KT873756)
RD53	626	100% *S. gracilis* (FJ196261 and JN226126)	*S. gracilis* (KT873757)
RD55	Mixed	–	NR
RD56	Mixed	–	NR
RD57	626	100% *S. gracilis* (FJ196261 and JN226126)	*S. gracilis* (KT873758)

References: F (Fox); RD (Racoon dog); (#) = accession numbers of sequences reported to GenBank. Mixed = no consensus sequence obtained or mixed chromatograms. (–) = sequences with ambiguities were not compared by BLASTn. NR: not reported.

GenBank accession numbers for these plasmids obtained from fox and raccoon dog samples with mixed infections. A total of 61 (25 from oocysts/sporocysts sample amplicons and 36 from plasmids) *Sarcocystis* spp. 18S rRNA gene sequences were registered in the GenBank under the accession numbers: KT873734- KT873794 (Tables 1 and 2 for detailed accession numbers). Fig. 2 summarizes the number of fox and raccoon dog samples with *Sarcocystis* spp. molecular identification.

Identification of *Sarcocystis* spp. infection (≥99% sequence identity in BLAST comparisons) by sequencing fox samples yielded the following results: 10.0% *S. tenella*, *S. capracanis* (5/50; 4 single and 1 mixed infection), 8.0% *S. miescheriana* (4/50; 1 single and 3 mixed), 8.0% *S. gracilis* (4/50; 3 single infections and 1 mixed infection), 6.0% *Sarcocystis* spp. using birds as IH (3/50; 2 single infections and 1 mixed infection) and 4.0% *S. capreolicanis* (2/50, both from mixed infections) (Tables 1 and 2). In addition, cloned samples showed 98% identity with *S. capreolicanis* (n = 3 samples, one clone each), *S. tenella or S. capracanis* (n = 1 sample one clone) and *S. gracilis* (n = 1 sample, 2 clones); 3 clones from 1 fox sample showed 96–97% identity with *S. cruzi* and one clone from another sample was only 94% identical with the sequence of *S. capreolicanis*. Two fox samples (one clone each) showed a sequence identity of ≥99% with *Cystoisospora* spp. sequences (Table 2).

Out of all raccoon dog samples, sequences with a ≥99% identity were identified as follows: 18.4% *S. miescheriana* in (7/38; 6 single infections and 1 mixed infection), 13.1% *S. gracilis* (5/38; 5 single infections), 10.5% *Sarcocystis* spp. using birds as IH (4/38; 2 single and 2 mixed infections), 2.6% *S. tenella, S. capracanis* (1/38; single infection) and 2.6% *S. capreolicanis* (1/38; mixed infection). One raccoon dog sample showed a sequence of 96% identity with sequences reported for *S. cruzi* (Table 1). Plasmid sequences obtained from raccoon dog samples showed homology with other organisms, which were different from *Sarcocystis* spp. in two samples (RD24 and RD55). The remaining three samples (identified as mixed

Table 2
Description of *Sarcocystis* spp. 18S rRNA gene fragments cloned into plasmids after amplification from DNA extracted from the intestinal mucosa of foxes and raccoon dogs. A conventional direct sequencing had revealed sequence ambiguities, which suggested mixed protozoan infections.

Original sample ID	(Number of sequences), base pairs (bp)	BLASTn identity (#)	*Sarcocystis* sp. (GenBank accession number, this study)
F132	(1), 809 bp (1), 909 bp (1), 892 bp	99% *S. miescheriana* (JN256123, JX840464, JX840466, JX840467) 99-100% *S. miescheriana* (JN256123, JX840464-JX840467) 98% *S. tenella* (KC209734, KC209737) and *S. capracanis* (L76472)	*S. miescheriana* (KT873759) *S. miescheriana* (KT873760) *Sarcocystis* sp. (KT873761)
F712	(2), 909 bp (1), 850 bp	98% *S. gracilis* (FJ196261, KF880741) 99% *Cystoisospora belli* (JX025649, JX025650, JX025652) *Cystoisospora* sp. (AB519674)	*Sarcocystis* sp. (KT873762, KT873763) NR
F722	(2); 914 and 911 bp (1), 914 bp	99% *S. capreolicanis* (JN226117, JN226118) 99% *S. gracilis* (FJ196261, KF880741)	*S. capreolicanis* (KT873765, KT873766) *S. gracilis* (KT873764)
F740	(3), 909 bp	99% *S. miescheriana* (JN256123, JX840464, JX840466, JX840467)	*S. miescheriana* (KT873767-KT873769)
F756	(1), 912 bp (1), 904 bp (1), 851 bp	98% *S. capreolicanis* (JN226117-JN226119) 94% *S. capreolicanis* (JN226117-JN226119) 99% *Cystoisospora* sp. (AB519675), *C. timoni* (EU200792) and *C. ohioensis* (AY618555)	*Sarcocystis* sp. (KT873770) *Sarcocystis* sp. (KT873771) NR
F777	(2), 904 bp (1), 914 bp	99% *S. capreolicanis* (JN226117 and JN226119) 98% *S. capreolicanis* (JN226117 and JN226119)	*S. capreolicanis* (KT873772, KT873773) *Sarcocystis* sp. (KT873774)
F786	(1), 662 bp (1), 822 bp (1), 668 bp (1), 867 bp	99% *S. albifronsi* (EU502868) and *S. anasi* (EU553477) 97% *S. cruzi* (JX679467, AB682779 and AF176933) 96% *S. cruzi* (JX679467, AB682779 and AF176933) 97% *S. cruzi* (JX679467, AB682779 and AF176933)	*Sarcocystis* sp. (KT873777) *Sarcocystis* sp. (KT873775) *Sarcocystis* sp. (KT873776) *Sarcocystis* sp. (KT873778)
F792	(2), 909 bp (1), 393 bp	99-100% *S. miescheriana* (JN256123, JX840464-JX840467) 98% *S. capreolicanis* (JN226118 y JN226119)	*S. miescheriana* (KT873779, KT873780) *Sarcocystis* sp. (KT873781)
F795	(2), 892 bp (1), 598 bp	99% *S. tenella* (KC209734 and KC209737) and 99% *S. capracanis* (L76472) 99% *S. tenella* (KC209734 and KC209737) and 99% *S. capracanis* (L76472)	*Sarcocystis* sp. (KT873782, KT873783) *Sarcocystis* sp. (KT873784)
RD24	(4); 900 bp	99% *Monocystis agilis* (AF457127)	NR
RD35	(2); 904 bp (1); 858 bp	99% *S. capreolicanis* (JN226117, JN226119) 98% *S. albifronsi* (EU502868) and *S. anasi* (EU553477)	*S. capreolicanis* (KT873785, KT873786) *Sarcocystis* sp. (KT873787)
RD51	(2); 906 and 908 bp (1); 856 bp	99% *S. miescheriana* (JN256123, JX840464, JX840466, JX840467) 99% *S. albifronsi* (EU502868) and *S. anasi* (EU553477)	*S. miescheriana* (KT873788, KT873789) *Sarcocystis* sp. (KT873790)
RD55	(4); 851 bp	99% *Eimeria nafuko* (JQ993665), *Eimeria* sp. (JQ993657), *Eimeria chinchillae* (JQ993650)	NR
RD56	(2); 911 and 912 bp (1); 858 bp (1); 856bp	98% *S. gracilis* (FJ196261, KF880741) 98% *S. albifronsi* (EU502868) and *S. anasi* (EU553477) 100% *S. albifronsi* (EU502868) and *S. anasi* (EU553477)	*Sarcocystis* sp. (KT873791, KT873794) *Sarcocystis* sp. (KT873793) *Sarcocystis* sp. (KT873792)

References: F (Fox); RD (Racoon dog); (#) = accession numbers of sequences reported to GenBank. NR: not reported.

infections) contained *Sarcocystis* spp. sequences (2 or more on each sample); two samples (a total of 4 plasmids) evidenced sequences with 98% sequence identity (Table 2).

4. Discussion

The prevalence of *Sarcocystis* spp. infections in foxes and raccoon dogs observed in this study using small intestine mucosal scrapings was much higher than in related studies performed by analyzing fecal samples from canids (Barutzki and Schaper, 2003, 2011; Dubey et al., 2015). In addition, *Sarcocystis* spp. showed a higher prevalence in raccoon dogs (50%) than foxes (38%). The high proportion of *Sarcocystis* spp. positive mucosal samples suggests – as expected – frequent natural carnivorism in these canid species. Previous comparative studies performed with faecal and intestinal mucosa samples from infected DH revealed a higher proportion of oocysts or sporocysts in mucosa-derived material than in faecal samples (Dubey et al., 2015; Murphy and Mansfield, 1999). These results suggest that oocysts are concentrated in the *lamina propria* of the small intestine and that sporocysts are released only sporadically during the course of the infection (Dubey et al., 2015). In addition, the results of the present study suggest that frozen preservation of fresh intestinal samples at low temperature (−20 °C or less) do not alter the morphology of oocysts or sporocysts of *Sarcocystis* spp. Probably, freezing effects. Our study did not address the viability of oocysts and sporocysts, however, the intact morphology of these stages may suggest that at least some parasites may have remained viable after freezing. This fact could explain the presence of *Sarcocystis* spp. in geographical areas with temperature below 0 °C (Bergler et al., 1980; Dubey et al., 2015;

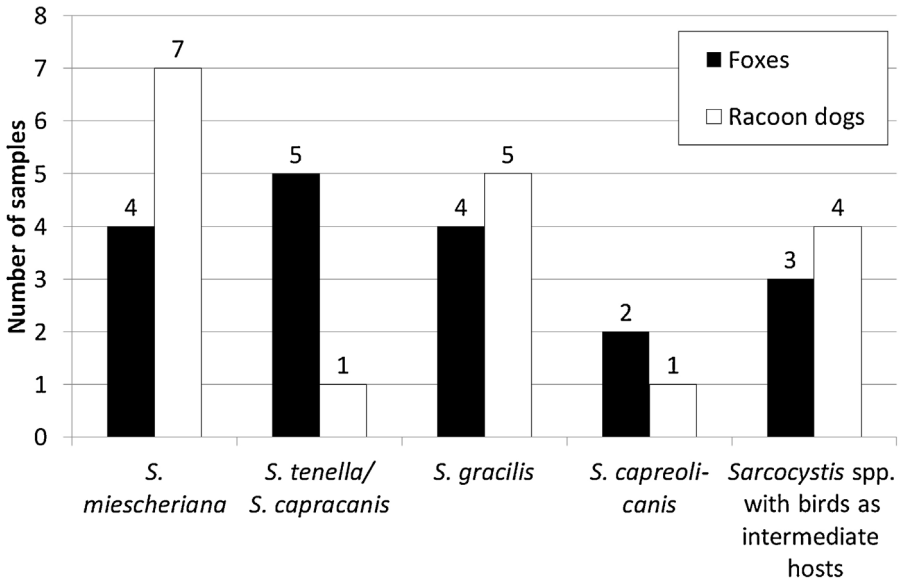

Fig. 2. Number of samples with *Sarcocystis* spp. identified by using molecular tools in mucosal material isolated from the small intestines of foxes and raccoon dogs. Results are shown for 18S rRNA gene sequences with an identity ≥ 99% with species-specific sequences deposited in GenBank.

Gjerde and Schulze, 2014). However, further studies are needed to characterize the viability of *Sarcocystis* spp. obtained from frozen intestines or stool samples.

Most *Sarcocystis* spp. can be identified based on sarcocysts morphology and molecular characterization of the 18S rRNA or *cox*1 genes (Dubey et al., 2015; Gjerde, 2013; Moré et al., 2013). In the present study, molecular identification of the observed *Sarcocystis* spp. sporocysts/oocysts was performed targeting a fragment of the 18S rRNA gene. In order to avoid misinterpretations, only sequences with a BLASTn identity ≥99% were considered as species-specific and the respective sequence entered into GenBank along with the respective species name. Sequences with lower identity as well as sequences that showed similar identity with more than one previously reported species were considered as '*Sarcocystis* sp.' and submitted to GenBank with this taxonomic description. On the other hand, samples that had shown inconclusive results in sequencing were considered as putative mixed sequences as in previous studies (Moré et al., 2014a,b). These mixed infections were detected in 9/50 and 5/38 of fox and raccoon dog samples. After cloning and clone sequencing, specific *Sarcocystis* sp. sequences were detected in all mixed infection samples from foxes and in 3/5 of those obtained from raccoon dogs. Therefore, this approach was appropriate to identify most mixed infections with *Sarcocystis* spp. The exceptions were two raccoon dog samples including one sample with all clones identified as *Monocystis agilis* (99% sequence identity; AF457127) and another with all clones identified as *Eimeria* spp. from rodents (99% sequence identity; JQ993665, JQ993657, JQ993650). The primers we used amplified a 18S rRNA gene fragment from several protozoans, which is advantageous when screening for Sarcocystidae species (Moré et al., 2014a,b, 2013). However, in samples where other oocysts/sporocysts than *Sarcocystis* spp. are present, direct sequencing and cloning from unspecific amplicons may be difficult. Genus-specific primers could be tested to analyze such samples.

Out of all fox samples, the most often identified *Sarcocystis* spp. were *S. tenella* or *S. capracanis* (10.0%); *S. miescheriana* and *S. gracilis* (8.0% of samples each) followed by *Sarcocystis* spp. using birds as IH (6.0%) and *S. capreolicanis* (4.0%). The sequences of *S. tenella* (KC209734, KC209737) obtained from microscopically visible cysts from sheep muscles in Norway (Gjerde, 2013) and the sequence of *S. capracanis* (L76472) (Jeffries et al., 1997) are highly similar and share 99% sequence identity. Further descriptive and molecular studies on *Sarcocystis* spp. from sheep and goats are thus needed to obtain well defined reference sequences. Our results may suggest that a number of foxes analyzed in our study had fed on tissues that originated from small domestic ruminants, or that *S. tenella* or *S. capracanis* or related *Sarcocystis* spp. had parasitized the prey of these foxes. *Sarcocystis miescheriana* (which uses wild boar and domestic pigs as IH) and *S. gracilis* (which uses roe deer, i.e. *Capreolus capreolus* as IH) were both quite often detected (8.0%) in fox intestinal samples. The species *S. capreolicanis*, which induces sarcocysts in roe deer muscles (*Capreolus capreolus*), was also detected in fox samples (4.0%). Therefore, considering the frequency of identification of *S. gracilis* and *S. capreolicanis*, both *Sarcocystis* spp. affecting roe deer muscles, our findings indicate that foxes consume these cervids in the German Federal State of Brandenburg. Both *Sarcocystis* spp. from cervids, *S. miescheriana* and the two species that seem to occur in small ruminants (*S. tenella* or *S. capracanis*), are known to use canids (including *Vulpes vulpes*) as definitive hosts (Dubey et al., 2015). *Sarcocystis* spp. that use birds as IH were also detected in three fox samples. A reliable molecular identification is generally difficult for these species, since a highly conserved 18S rRNA gene sequence has been reported among *Sarcocystis* spp. producing cysts in different avian species and a high identity (≥98%) with *S. neurona*, *S. canis* and *S. mucosa* has been described by BLAST comparison (Dubey et al., 2015, 2006; Jenkins et al., 1999; Kutkiene et al., 2011). Other gene targets like ITS1, *cox*1 or microsatellite markers should be applied in samples suspected to contain DNA of such *Sarcocystis* spp. for a reliable specific identification (Dubey et al., 2015; Gjerde, 2013; Prakas et al., 2015; Wendte et al., 2010).

In the raccoon dog samples, the *Sarcocystis* spp. sequences often found were sequences with a ≥99% identity with *S. miescheriana*, *S. gracilis*, *Sarcocystis* spp. that use birds as IH, *S. tenella, S.capracanis* and *S. capreolicanis*. As in the fox samples, *S. miescheriana* was among the species most frequently identified (18.4% out all samples). This result confirmed that the raccoon dog is a natural DH of *S. miescheriana* (Saito and Itagaki, 1994). Considering the two species using roe deer as IH (*S. gracilis* and *S. capreolicanis*), the frequency of detection was particularly high (15.7% of samples). To our knowledge, this is the first report suggesting that the raccoon dog is a DH for *S. gracilis* and *S. capreolicanis* (Dubey et al., 2015). The raccoon dog has been described as DH of *Sarcocystis* spp. from

reindeer (*Rangifer tarandus*) (Gjerde, 1984). It is likely that raccoon dogs act as DH for further *Sarcocystis* spp. parasitizing in cervids and for which canids are confirmed or suspected as DH. In contrast to fox samples, only one specimen was identified as *S. tenella* or *S. capracanis* in the raccoon dog samples. Altogether our results suggest that raccoon dogs harbor primarily *Sarcocystis* spp. infections contracted by consumption of tissues originating from wild boar or pigs and cervids. As stated for fox samples, a reliable identification of *Sarcocystis* spp. affecting avian species as IH was difficult, however sequences of such species were recorded in 10.5% of raccoon dog samples. Only one short sequence was obtained from a raccoon dog sample which was 100% identical with sequences specific for *S. rileyi*. This result is in agreement with a study that suggests that raccoon dogs and foxes could act as DH for *S. rileyi* in Europe (Prakas et al., 2015).

A total of nine sequences obtained from plasmids from fox samples, one from oocyst sequences and three plasmid sequences from raccoon dog samples showed identities lower than suggested for species identification (between 94 and 98%) (Gjerde, 2013; Moré et al., 2013). These sequences were reported as *Sarcocystis* sp. to GenBank, as they might either belong to a *Sarcocystis* sp. already described, but for which no 18S rRNA sequence is available, or could represent new taxa.

Our study presents a molecular approach to identify *Sarcocystis* spp. infections in the intestinal mucosa from foxes and raccoon dogs collected in Brandenburg, Germany. The results show that a method comprising of 18S rRNA gene amplification, cloning and sequencing is suitable to identify *Sarcocystis* spp. mixed infections and to identify potential DH of these parasites. Moreover, the application of this approach could help to identify potential natural DH of various *Sarcocystis* spp. and to complement findings obtained by experimental infection studies.

Competing interests

The authors declare that they have no competing financial or non-financial interests.

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