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Molecular identification targeting *cox1* and 18S genes confirms the high prevalence of *Sarcocystis* spp. in cattle in the Netherlands ☆

B.J.A. Hoeve-Bakker [a,b], J.W.B van der Giessen [b], F.F.J. Franssen [b,*]

[a] Centre for Infectious Diseases Research, Diagnostics and Laboratory Surveillance, National Institute for Public Health and the Environment (RIVM) Bilthoven, The Netherlands
[b] Centre for Zoonoses and Environmental Microbiology, National Institute for Public Health and the Environment (RIVM) Bilthoven, The Netherlands

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ABSTRACT

The reported prevalence of *Sarcocystis* infection in cattle in Europe ranges between 66 and 94%. Although in the Netherlands a prevalence of 100% was reported in 1993, this study aimed to develop a method for sensitive and specific molecular detection and species identification of *Sarcocystis* spp., in order to provide more recent data on the prevalence and identification of these protozoa in cattle meat intended for human consumption in the Netherlands. For this purpose, 104 cattle samples were obtained from Dutch slaughterhouses. Genomic DNA was extracted, and analysed by 18S and *cox1* PCR. Magnetic capture was used to extract and amplify 18S-specific DNA. *Sarcocystis* DNA was detected in 82.7% of the samples. PCR amplicons of both targets were sequenced, and sequence identities of ≥97% were observed for *Sarcocystis cruzi* (65.4%), *Sarcocystis hominis* (12.5%), *Sarcocystis bovifelis* (8.7%), *Sarcocystis hirsuta* and *Sarcocystis heydorni* (both 1.0%). Mixed infections were observed in 17.3% of the samples. The magnetic capture was not significantly more sensitive compared with standard DNA extraction, but magnetic capture did add to the overall sensitivity. Using *cox1* sequencing, all species are clearly distinguished, whereas for 18S the variation between species is limited, which particularly hampers reliable identification of thick walled *Sarcocystis* spp. Furthermore, the detection of 12.5% *S. hominis* and 1% *S. heydorni* points towards an established transmission route between cattle and humans in the Netherlands. The availability of four additional well-identified and well-referenced *S. hominis cox1* sequences in public databases enables development of species-specific diagnostic PCRs targeting *cox1*, which in combination with magnetic capture could provide the means to determine the prevalence of human sarcocystosis.

1. Introduction

The protozoan genus *Sarcocystis* is a member of the apicomplexan family, and consists of at least 196 species (Dubey et al., 2015a). *Sarcocystis* spp. need two hosts to complete their life cycle, typically in a predator–prey relationship. *Sarcocystis* spp. can infect a wide range of hosts, among which are two important meat producing animals: cattle and pigs. Cattle serve as intermediate hosts for six species of *Sarcocystis*, with canids (*Sarcocystis cruzi*), felids (*Sarcocystis bovifelis*, *Sarcocystis bovini* and *Sarcocystis hirsuta*) and humans (*Sarcocystis hominis* and *Sarcocystis heydorni*) as final hosts (Dubey et al., 2015b; Gjerde, 2016).

In cattle, infections are generally asymptomatic (sarcosporidiosis), but can lead to chronic infection, resulting in reduced milk production, weight loss and decreased meat quality (Vangeel et al., 2014). Cattle may develop bovine eosinophilic myositis (BEM): pathological lesions due to inflammatory reactions to sarcocysts (Dubey et al., 2015a). Upon meat inspection or during meat cutting, focal to diffuse lesions that appear greenish to grey may be observed (Vangeel et al., 2014; Dubey et al., 2015a). Detection of lesions during meat inspection might lead to removal of visible lesions or condemnation of the carcass depending on the generalisation of the lesions (European Union directive 804/2004). Globally reported prevalence in slaughtered cattle ranges from 0.002 to 0.011% (Vangeel et al., 2013) and Belgium reported a prevalence of 0.012% to the European Food Safety Authority (EFSA, Parma, Italy) in 2016. The Netherlands have not reported to EFSA, although sarcocysts were detected by microscopy in a limited sample of 91 examined Dutch cattle in 1993 (van Knapen et al., 1993). Studies that are more recent combine microscopy with molecular techniques, and report prevalence between 52 and 100% worldwide

☆ *Note:* Nucleotide sequence data reported in this paper are available in GenBank under accession numbers: **MK482391**, **MK497840–MK497843**, **MK962347–MK962351**, **MK981198–MK981208**.

* Corresponding author.
E-mail address: Frits.Franssen@rivm.nl (F.F.J. Franssen).

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(Savini et al., 1992; Moré et al., 2011; Mirzaei and Rezaei, 2016), and 66 to 94% in Europe (Vangeel et al., 2007; Moré et al., 2013; Hornok et al., 2015). Additionally, these studies report 6.2–91.6% *S. hominis* prevalence, and 23–60% mixed infections involving *S. hominis*. In 2015, a pilot study was performed on 17 beef samples of Dutch origin using molecular techniques on the 18S gene target. Of these samples, 12% (2/17) showed greyish focal lesions suspected to be BEM and *Sarcocystis* DNA was detected in 70.6% (12/17) of the samples. *S. hominis* was detected in 17.6% (3/17) of the samples and one mixed infection was reported (unpublished data).

The presence of *S. hominis* cysts in cattle is apparent evidence for a complete transmission cycle, since cattle cannot become infected without the infected human final host. Intestinal sarcocystosis in humans has been reported from several European countries between 1960 and 1982, including the Netherlands, and more recently from Asia (China, Tibet, Laos and Thailand) and from Australia, Argentina and Brasil (reviewed in Fayer et al., 2015). In light of a lack of recent and systematic infection data for humans in Europe, *Sarcocystis* detection must be both sensitive and specific to reliably identify *S. hominis*. Molecular techniques for the identification of *Sarcocystis* spp. in cattle generally target the nuclear small subunit (18S) rRNA gene (Ho et al., 1996; Yang et al., 2001; Moré et al., 2013); hence, many 18S sequences are available in public databases for reliable identification. However, interspecific variations between strains isolated from different hosts have been reported (Yang et al., 2001), as well as intraspecific variations among *Sarcocystis* spp. isolated from the same host (Gjerde, 2013). These variations were successfully clarified by targeting the mitochondrial cytochrome c oxidase subunit I (*cox1*) gene (Gjerde, 2013). However, only one unreferenced sequence of the *S. hominis cox1* gene is thus far available in public databases, which hampers the development of *cox1*-based diagnostic methods to investigate human intestinal sarcocystosis (Poulsen and Stensvold, 2014). Moreover, the relevance of this target for *Sarcocystis* spp. identification in cattle remains to be determined.

Sarcocysts are unevenly distributed in muscle tissue, and therefore examining larger amounts of meat will increase the probability of detection. For PCR, DNA extraction from meat is restricted to 25–100 mg using commercially available DNA extraction kits (Chiesa et al., 2013; Hornok et al., 2015). Pepsin digestion of 50 g of meat prior to DNA extraction increases the probability of detection (Moré et al., 2014), although the high concentration of host DNA may inhibit the detection (Bellete et al., 2003). To exclude the effect of abundant host DNA, Opsteegh et al. (2010) combined tissue homogenization of 100 g of meat with sequence-specific magnetic capture of target DNA for the detection and genotyping of *Toxoplasma gondii*. Application of this technique could further increase the sensitivity of *Sarcocystis* spp. detection in meat samples.

The aim of this study was to develop a method for sensitive and specific molecular detection and species identification of *Sarcocystis* spp. in order to determine the prevalence of *Sarcocystis* spp. in Dutch slaughter cattle.

2. Materials and methods

2.1. Samples and controls

Based on an expected prevalence of 94%, assuming a similar prevalence as reported from Belgium, 100 fresh cattle diaphragms were obtained from Dutch slaughterhouses via The Netherlands Food and Consumer Product Safety Authority (NVWA). Four fresh beef samples of condemned carcasses, which were submitted for *Sarcocystis* confirmation, were also included.

DNA from individual tissue cysts of *S. hominis* (sample HRF115), *S. cruzi* (sample D170269) and *S. bovifelis* (sample HRF3) – as diagnosed by real-time PCR (Moré et al., 2013) – was kindly provided by Dr. G. Schares (Friedrich Loeffler Institute, Germany).

2.2. Pepsin digestion

Fifty grams of clean meat (central tendon, fat and connective tissue removed) was digested using a modification of the pepsin digestion protocol described previously (Dubey, 1998). Briefly, 50 g of clean meat with 75 ml of PBS were ground in a blender for 10 intervals of 1 s at high speed. The blender was rinsed with 75 ml of PBS, which was added to the tissue homogenate. Two hundred and fifty ml of freshly prepared, prewarmed (37 °C) pepsin solution, containing 15 g of pepsin (0.7 U/mg, Pharmacopoeia Europaea), 8.5 ml of HCl (9.5% vol/vol) and 2.5 g of NaCl, was added. The homogenate was incubated on a magnetic stirrer for 1 h at 37 °C, subsequently filtered through a 180 µm sieve and centrifuged at 1800*g* for 10 min at room temperature. The supernatant was removed and the pellet resuspended in 10 ml of PBS. To neutralize the pH of the homogenate, 16–17 ml of 1.2% (w/v) $NaHCO_3$, pH 8.3 was added. The suspension was centrifuged at 3000*g* for 10 min (room temperature) and the supernatant was removed. The pellet was stored at −20 °C until further analysis.

2.3. Cell lysis

To further lyse the digested tissue homogenate, the pellet was resuspended in a 1.25 fold volume of cell lysis buffer, which contained 200 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.4% SDS, 400 mM NaCl, 40 mg/ml of Proteinase K (>600 mAnson Unit/mL; Qiagen, Hilden, Germany). Cell lysis was performed overnight at 56 °C in a shaking water bath. Subsequently, total genomic DNA (gDNA) was extracted or target specific DNA was captured by magnetic capture.

2.4. Extraction of total gDNA

Total gDNA was extracted from 100 µl of cell lysate with the DNeasy Blood and Tissue Kit (Qiagen) using the 'Purification of Total DNA from Animal Tissues' protocol provided with the kit. Cell lysis buffer was used as a negative control. The DNA was eluted with 200 µl of elution buffer. The DNA extracts were stored at −20 °C until further analysis.

2.5. Extraction of 18S-specific DNA using magnetic capture

Oligonucleotides for magnetic capture were designed to be complementary to the *S. cruzi* isolate B2.1 18S rDNA sequence (GenBank accession number KT901169) using Primer3Plus Version 2.4.2 (Untergasser et al., 2012). The capture oligonucleotides were designed to capture both strands of the 18S rRNA gene upstream of the 5′ end of the primer binding sites (Table 1). A biotin-triethylene-glycol (biotin-TEG) spacer arm was added to the 5′ end of each oligonucleotide to enable Dynabeads™ M-270 Streptavidin-mediated purification of the captured DNA.

Extraction of target-specific DNA using magnetic capture was performed as previously described (Opsteegh et al., 2010). To capture the 18S rDNA from the cell lysate, 10 pmol of each capture oligonucleotide (Sarc 18S CapF and Sarc 18S CapRs, Table 1) were added per sample, and hybridized at room temperature for 45 min while rotating at 10 rpm. The DNA was stored at −20 °C until further analysis, and is further referred to as mcDNA.

Table 1
Primers and capture oligonucleotides used in this study.

Name	Sequence 5′–3′	5′ label	Position[a]	Reference
Sarc 18S Fext	GGT GAT TCA TAG TAA CCG AAC G		275–296	Moré et al. (2013)
Sarc 18S Rext	GAT TTC TCA TAA GGT GCA GGA G		1148–1127	Moré et al. (2013)
Sarc 18S CapF	CGG CGA AAC TGC GAA TGG CTC ATT AAA ACA GTT ATA GTT TAT TTG ATA GTC ATA TCA GAT GAA AAT CTA CTA CAT GGA TAA CCG TGG TAA	Biotin-TEG	78–167	This study
Sarc 18S CapRs	CTC CCC CCA GAA CCC AAA GAC T	Biotin-TEG	1151–1172	This study
Sarc cox1 SF1	ATG GCG TAC AAC AAT CAT AAA GAA		−24 to −1	Gjerde (2016)
Sarc cox1 SR9	ATA TCC ATA CCR CCA TTG CCC AT		1061–1039	Gjerde (2016)

[a] Relative to *Sarcocystis cruzi* isolate B2.1 (GenBank accession number **KT901169**) for 18S oligonucleotides, and to *S. cruzi* isolate B1.2, (**KC209597**) for *cox1* oligonucleotides.

2.6. Examination of condemned carcasses

Meat samples from condemned carcasses were macroscopically evaluated for the presence of lesions typical for *Sarcocystis* infection: focal or diffuse lesions with a grey to greenish discoloration. Total genomic DNA was extracted from excised lesions as described and DNA was eluted in 50 µl of elution buffer (see Section 2.4). Additionally, 50 grams of lesion-free meat were processed as described for the cattle diaphragms (see Section 2.2).

2.7. Molecular detection

For the identification of *Sarcocystis* spp., both the 18S rRNA gene and *cox1* gene were targeted using PCR. Genomic DNA extracts were analysed on both loci; in mcDNA only the 18S locus could be targeted.

A ~900 bp fragment of the 18S rRNA gene was amplified using primers Sarc 18S Fext and Sarc 18S Rext (Table 1), slightly adapted from the previously described protocol by Moré et al. (2013). PCR was performed in a 50 µl reaction mix containing 25 µl of HotStarTaq Master Mix (Qiagen), 25 picomoles of each primer, 4 µl of template DNA and RNase-free water.

A PCR targeting an ~1100 bp fragment of the *cox1* gene was developed using previously described primers Sarc cox1 SF1 and Sarc cox1 SR9 (Table 1) (Gjerde, 2016). PCRs were performed in a 50 µl reaction mix containing 25 µl of HotStarTaq Master Mix, 40 picomoles of each primer, 4 µl of template DNA and RNase-free water. The touch down protocol for the *cox1* PCR included an initial denaturation step for 15 min at 95 °C, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s with a decrement of 0.1 °C per cycle, and elongation at 72 °C for 90 s. Subsequently, the protocol continued with 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s, and a final extension for 10 min at 72 °C.

Both PCRs were performed in a C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, USA). Amplicons were visualised by electrophoresis on 1.5 % agarose gels, staining with SYBR® Gold Nucleic Acid Gel Stain (Invitrogen, Carlsbad, USA), and UV illumination on a Gel Doc™ XR+ System (Bio-Rad). In each PCR run, 4 µl of 100-fold diluted reference DNA and 4 µl of RNase-free water were included as positive and negative controls, respectively.

2.8. Species identification

To remove excess primers and nucleotides, PCR amplicons were treated with ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. Sequence reactions (total volume of 20 µl) containing purified PCR product and 25 pmol of PCR primer according to company instructions (Table 1) were sent to BaseClear (Leiden, The Netherlands) for bidirectional Sanger sequencing. Obtained sequences were assembled and edited manually, using BioNumerics (version 7.6.3, Applied Maths, Sint-Martens-Latem, Belgium). For species identification, obtained consensus sequences were queried in GenBank using the Nucleotide Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (blast.ncbi.nlm.nih.gov). A sequence homology of ≥97% was considered suitable for reliable species identification. Lacking a documented consensus cut-off value for species definition in parasitology, this cut-off was adopted from the species definition used in bacteriology (Stackebrandt and Goebel, 1994). Finally, BioNumerics was used for phylogenetic analysis of the sequences. A multiple alignment was made using the program's default settings. Neighbour Joining cluster analysis of the 18S and *cox1* sequences was performed using the Jukes and Cantor correction setting, with bootstrap values calculated from 2500 simulations. *Sarcocystis* spp. reference sequences were included for 18S rDNA and *cox1* (Table 2); *Toxoplasma gondii* was used as the outgroup.

2.9. Statistical analysis

Fisher's exact test was used to compare proportions and 95% confidence intervals (CIs) of positive samples obtained with magnetic capture with default DNA extraction, and to compare *cox1* PCR with 18S PCR. $P > 0.05$ was considered significant.

2.10. Nucleotide sequence accession numbers

Nucleotide sequences generated in this study were deposited in GenBank under the accession numbers **MK497840** (*S. hominis* HRF115 cox1), **MK962349** (*S. cruzi* D170269 cox1), **MK962348** (*S. bovifelis* HRF3 cox1), **MK497841** (*S. hominis* S026 cox1), **MK497842** (*S. hominis* S027 cox1), **MK962351** (*S. cruzi* S037 cox1), **MK962350** (*S. cruzi* S054 cox1), **MK497843** (*S. hominis*

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

Species	Strain	18S rDNA	*cox1*
Sarcocystis bovifelis	B1.13	KT901117	KT900964
	B4.5	KT901135	KT900971
	B12.57	NA	KT900998
Sarcocystis bovini	B7.2	KT901150 (clone 1)	KT901005
	B4.7	KT901139 (clone 1)	KT901000
Sarcocystis cruzi	3AS.cruziArg	JX679467	NA
	B1.2	KC209738	KC209597
	ID1305	KP640133	NA
	B2.1	KT901169	KT901087
Sarcocystis heydorni	Isolate 1	KX057996	KX057994
	Isolate 2	KX057997	KX057995
Sarcocystis hirsuta	Clone 6B HRF30	JX855283	NA
	B12.1	KT901166	KT901057
	B5.1	KC209741	KC209634
Sarcocystis hominis	Bt2hom	AF006471	NA
	clone 1B HRF93A	JX679470	NA
	Unknown*	NA	MH021119
Toxoplasma gondii	RH	NA	JX473253
	ME49	L37415	NA

NA: sequences not available GenBank; *unpublished strain from human stool.

S046 cox1), **MK962347** (*S. bovifelis* S072 cox1), **MK482391** (*S. hominis* HRF115), **MK981201** (*S. cruzi* D170269 18S), **MK981200** (*S. bovifelis* HRF3 18S), **MK981198** (*S. bovifelis* S006 18S), **MK981206** (*S. hirsuta* S010 18S), **MK981207** (*S. hominis* S011 18S), **MK981199** (*S. bovifelis* S056 18S), **MK981208** (*S. hominis* S073 18S), **MK981204** (*S. cruzi* S089 18S), **MK981205** (*S. heydorni* S093 18S), **MK981202** (*S. cruzi* S100 18S) and **MK981203** (*S. cruzi* S103 18S).

3. Results

3.1. Prevalence of Sarcocystis spp. in Dutch cattle samples

The prevalence of *Sarcocystis* spp. in Dutch cattle differed depending on the extraction method used. With total gDNA extraction, *Sarcocystis* DNA was detected in 76.9% (80/104; 95% CI: 67.6–84.6%) of the examined cattle muscle samples using 18S primers. Two of these were doubtful: a weak band was detected on a gel; the PCR result was not reproducible, and in some cases, no DNA sequence was obtained. With the magnetic capture (mcDNA) method 79.8% (83/104; 95% CI: 70.8–87.0%) samples were positive (of which four were doubtful), which was not significantly higher (Fisher's exact test two-tailed, $P = 0.7365$).

The results for both DNA extraction methods were not in agreement: for three cattle samples the identification using gDNA was positive, whereas this was negative using mcDNA; for six samples this was vice versa (Table 3). When both methods were combined, *Sarcocystis* DNA was detected in 82.7% (86/104; 95% CI: 74.0–89.4) of all cattle samples, which is not significantly different from either method (Fisher's exact test two-tailed, *P*-values 0.3880 and 0.7227 for gDNA extraction and magnetic capture, respectively).

Out of 86 18S positive samples, 80 were found positive by *cox1* PCR; however, this was not significantly lower than for 18S (Fisher's exact test two-tailed, $P = 0.3880$).

3.2. Species identification

The 18S rDNA sequences obtained from mcDNA and gDNA per sample were in agreement for all samples, except for one: *S. cruzi* was identified from mcDNA, whereas gDNA revealed *S. hominis*. This resulted in 86 consensus sequences out of 85 positive samples.

Eighty consensus sequences of the 18S rDNA fragment were 424 to 891 bp in length, and showed ≥97% sequence homology with GenBank entries of *S. cruzi* (59, three haplotypes), *S. hominis* (13, two haplotypes), *S. bovifelis* (six, two haplotypes), *S. hirsuta* (one) and *S. heydorni* (one). The remaining six sequences had ≥90% homology to the genus *Sarcocystis*, however those were of poor quality (too short and/or ambiguous bases) despite sufficient amounts of amplicon and were excluded from further analyses. Despite sufficient amounts of 18S amplicon of *S. bovifelis* HRF3 reference DNA and repeated attempts at sequencing, obtained sequences were too short for further sequencing analysis.

Sixty-nine out of 78 obtained *cox1* consensus sequences were 591–1021 bp in length and were ≥97% homologous with GenBank entries of *S. cruzi* (60, two haplotypes) and *S. bovifelis* (nine, one haplotype). Based on phylogenetic analysis, HRF115 (*S. hominis* reference DNA), three of our isolates and the *S. hominis* GenBank sequence (MH021119) formed a cluster, which clearly separated from the other species clusters (Fig. 2). These three isolates were therefore considered *S. hominis* (three haplotypes). Four sequences were appointed *Sarcocystis* spp. as they were ≥80% homologous to the genus *Sarcocystis*. However, due to poor quality, these sequences were excluded from further analyses. Two additional sequences with <97% sequence homology to *S. cruzi* were also excluded.

Table 3
Comparison of 18S PCR results from magnetically captured DNA (mcDNA) and genomic DNA extracts (gDNA) for all cattle samples.

	mcDNA +	mcDNA −	Total
gDNA +	77	3	80
gDNA −	6	18	24
Total	83	21	104

+ and − indicate positive and negative results, respectively. Fisher's exact two-tailed test, *P* value equals 0.7365.

3.3. Cluster analysis of 18S and cox1 sequences

The interspecies and intraspecies similarities for both targets were calculated based on Neighbour Joining cluster analysis (Figs. 1 and 2). For 18S rDNA sequences, similarity between species varied from 94.1 to 97.8%, and the intraspecific homology ranged from 98.6 to 100% (Table 4), which corresponds with 16–42 single nucleotide polymorphisms (SNPs) among species, and 0–10 SNPs within species clusters. For *cox1* sequences, these ranges were 65.9–94.9% (50–302 SNPs) between species and 96.8–100% (0–28 SNPs) within species (Table 5).

3.4. Mixed infections

The observed overall prevalence of *Sarcocystis* spp. in Dutch cattle was 82.7%. For 60 samples, the identification of 18S and *cox1* were identical: 51 *S. cruzi*, six *S. bovifelis* and three *S. hominis* (Table 6). Disagreement between both markers, which was considered indicative of a mixed infection, was detected in 17.3% of the samples (18/104; 95% CI: 10.6–26.0%).

Sarcocystis cruzi was detected in 65.4% (68/104; 95% CI: 55.4–74.4%) of the samples: for 51 samples, both targets were in agreement, in eight (18S) and 11 (*cox1*) additional samples, one of both targets was detected. Moreover, 12.5% *S. hominis* (13/104; 95% CI: 6.8–20.4%), 8.7% *S. bovifelis* (9/104; 95% CI: 4.0–15.8%), 1.0% of *S. hirsuta* (1/104; 95% CI: 0.02–5.2%), and 1.0% of *S. heydorni* (1/104; 95% CI: 0.02–5.2%) were detected (Table 6).

3.5. Sarcocystis in condemned carcasses

Muscle samples from four condemned carcasses were submitted to our laboratory for confirmation and identification of *Sarcocystis* spp. Macroscopic examination revealed suspected lesions in three out of four samples, from which *S. cruzi* was the most frequently detected species. In the fourth sample a greenish shade on the meat surface and a lesion in the fat tissue were observed, from which no *Sarcocystis* DNA was detected. The tissue homogenates contained *S. cruzi*, *S. hominis*, and in one case both species (Table 7).

4. Discussion

The aim of this study was to develop a method for sensitive and specific molecular detection and species identification of *Sarcocystis* spp. in order to determine the prevalence of *Sarcocystis* spp. in Dutch slaughter cattle.

Examination of condemned carcasses revealed *Sarcocystis* DNA both inside and outside lesions in the meat. These findings confirm that *Sarcocystis* infection is not exclusively associated with lesions typical for bovine eosinophilic myositis, nor do these lesions appear to be induced by one particular *Sarcocystis* sp. (Vangeel et al., 2013). In Belgium, the reported incidence of bovine eosinophilic myositis in 0.012% of the inspected carcasses (EFSA, 2016) contrasts with 94% prevalence of *Sarcocystis* spp. in beef (Vangeel

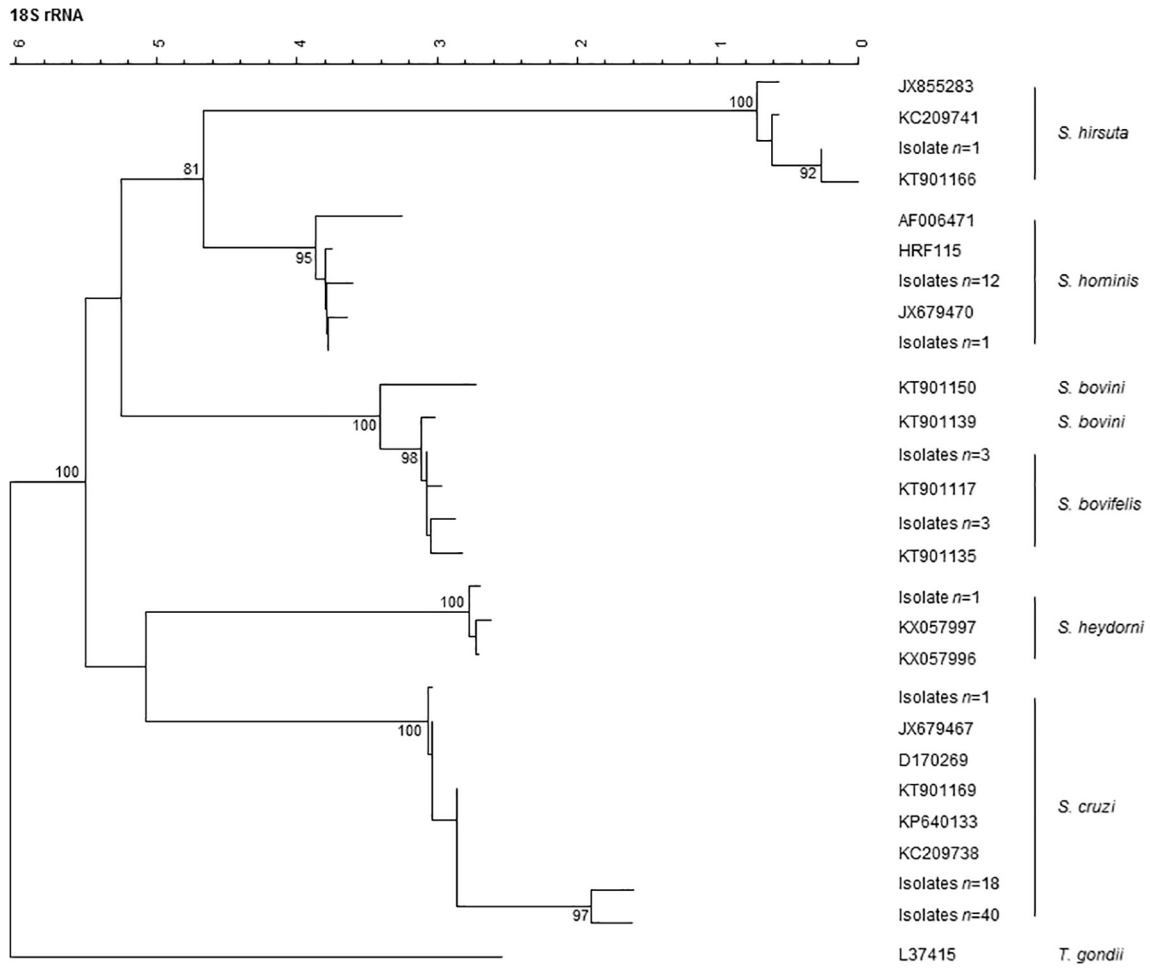

Fig. 1. Neighbour joining tree inferred from *Sarcocystis* 18S rDNA sequences isolated in this study and sequences from GenBank. Except for *Sarcocystis bovifelis* and *Sarcocystis bovini*, *Sarcocystis* spp. are well separated, and our isolates clustered with GenBank reference sequences. *Toxoplasma gondii* serves as the outgroup. Bootstrap values were inferred from 2500 replicates; values below 75% are not shown.

et al., 2007). Lesions are thus only observed in a small minority of *Sarcocystis* infections; hence, most *Sarcocystis* infections are missed throughout visual meat inspection. Moreover, greenish lesions can be related to other infections, caused by a wide range of bacteria and parasites (Jensen and Turbain, 1936; Alemneh, 2017). Therefore, lesions are not a good indicator for *Sarcocystis* infection, emphasising the importance of other means of detection.

In the current study, 100-fold more cell lysate was used for magnetic capture compared with standard DNA extraction, and the DNA was eluted in a four-fold smaller volume, thus resulting in 400-fold more target DNA. However, the magnetic capture method was not significantly more sensitive compared with standard DNA extraction. In contrast, magnetic capture significantly improved sensitivity of *Toxoplasma gondii* detection from meat samples using real-time PCR (Opsteegh et al., 2010). This may be due to the fact that *Toxoplasma* tissue cysts are far less prevalent in cattle and therefore depend on a concentration method such as magnetic capture to find positives, which affects recorded prevalence. The prevalence of *Sarcocystis* tissue cysts is probably much higher in muscle samples of cattle and therefore the extraction method is less critical. However the exact tissue cyst density of *Sarcocystis* in cattle is unknown, but in sheep, a density of approximately 20 sarcocysts per square centimetre has been reported (Dong et al., 2018). Taking into consideration that the magnetic capture procedure is relatively laborious and expensive compared with gDNA extraction, it is not advantageous to replace the latter. It should be noted, however, that positive samples were missed with both methods individually. Therefore, screening samples using gDNA extracts, supplemented with magnetic capture of the negative samples, provides a more time and cost effective approach to enhance detection.

A smear of aspecific PCR products was observed from gDNA extracts, which is likely caused by aspecific binding of primers to host DNA. Moreover, the band intensities of products obtained from gDNA extracts were less clear compared to mcDNA, which suggests less efficient PCRs when gDNA is used. This was confirmed by a 100-fold reduced sensitivity when serially diluted reference DNA was amplified in a background of *Sarcocystis*-negative bovine gDNA (data not shown), and is in agreement with previous observations that a high background of host DNA in gDNA extracts inhibits the detection of target DNA (Bellete et al., 2003). Since the load of target DNA in the cattle samples was apparently high, standard DNA extraction was not less sensitive than the magnetic capture procedure. However, detection from samples with low parasite loads in high backgrounds of host DNA, such as that observed in human faeces (Dubey et al., 2015a), may indeed benefit from the magnetic capture method.

The detection of 13.5% *S. hominis* and *S. heydorni*, the zoonotic *Sarcocystis* spp. in Dutch cattle, points towards an established transmission cycle between cattle and humans. Human intestinal sarcocystosis is mostly asymptomatic and self-limiting, and is not considered a major public health threat. Therefore, the most

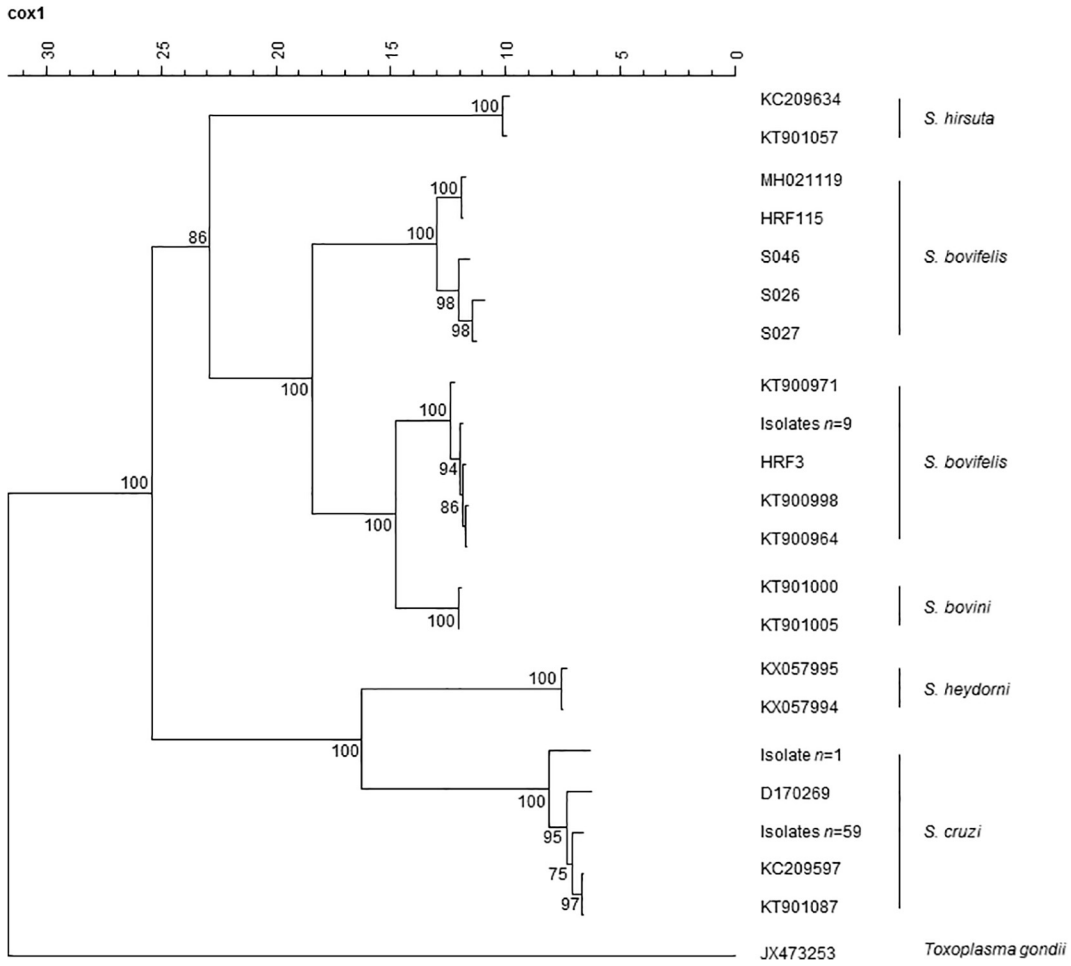

Fig. 2. Neighbour joining tree inferred from *Sarcocystis cox1* sequences isolated in this study and sequences from GenBank. Different *Sarcocystis* spp. are well separated, and our isolates clustered with GenBank reference sequences. *Toxoplasma gondii* serves as the outgroup. Bootstrap values were inferred from 2500 replicates; values below 75% are not shown.

Table 4
Inter- and intraspecies similarities between *Sarcocystis* spp. 18S rDNA sequences.

	Sarcocystis hirsuta	*Sarcocystis hominis*	*Sarcocystis bovifelis/S. bovini*	*Sarcocystis heydorni*	*Sarcocystis cruzi*
S. hirsuta (*n* = 4)	99.0–99.7				
S. hominis (*n* = 16)	95.3	98.7–100			
S. bovifelis/S. bovini (*n* = 10)	94.7	97.8	98.6–100		
S. heydorni (*n* = 3)	94.1	95.8	95.2	99.7–99.9	
S. cruzi (*n* = 63)	94.8	96.5	95.9	96.3	98.0–100

The numbers in the table indicate the percentage of similarity between species. The ranges refer to similarity between strains within the species. The number of strains is indicated in parentheses.

Table 5
Inter- and intraspecies similarities between *Sarcocystis* spp. *cox1* sequences.

	Sarcocystis hirsuta	*Sarcocystis hominis*	*Sarcocystis bovifelis*	*Sarcocystis bovini*	*Sarcocystis heydorni*	*Sarcocystis cruzi*
S. hirsuta (*n* = 2)	99.5					
S. hominis (*n* = 5)	77.8	97.6–99.9				
S. bovifelis (*n* = 12)	77.4	87.2	99.4–100			
S. bovini (*n* = 2)	77.5	88.3	94.9	99.9		
S. heydorni (*n* = 2)	65.9	68.7	68.3	69.4	99.7	
S. cruzi (*n* = 63)	66.6	69.4	69.0	70.1	83.1	96.8–100

The numbers in the table indicate the percentage of similarity between species. The ranges refer to similarity between strains within the same species. The number of strains is indicated in parentheses.

recent epidemiological data from the Netherlands date from 1968, when sporocysts were detected in 8.0% of healthy army recruits and 8.6% of patients with intestinal complaints (Manschot et al., 1968). To corroborate and explain our current findings, as well as to update knowledge on transmission and disease burden in humans, further research into the epidemiology of human intesti-

Table 6
Sarcocystis spp. identity according to 18S (horizontal) and *cox1* (vertical) sequences.

	cox1					
	Sarcocystis cruzi	*Sarcocystis hominis*	*Sarcocystis bovifelis*	*Sarcocystis* sp.	No sequence	Total
18S						
S. cruzi	51		**2**	**1**	5	59
S. hominis	**7**	3		**2**	1	13
S. bovifelis			6			6
S. hirsuta					1	1
S. heydorni	**1**					1
S. sp.	**3**		**1**	**1**	1	6
Total	62	3	9	4	8	86

Bold numbers indicate mixed infections.

Table 7
The identities of *Sarcocystis* spp. in tissue homogenates and lesions of condemned carcasses.

Sample ID	Tissue homogenate	Lesion 1	Lesion 2	Lesion 3
S046	*Sarcocystis hominis*	*Sarcocystis bovifelis*	*Sarcocystis* sp.	NA
S047	*Sarcocystis cruzi* + *S. hominis*	*S. cruzi*	*S. cruzi*	*S. cruzi*
S062	*Sarcocystis cruzi*	*S. cruzi*	*S. cruzi*	*S. cruzi*
S063	*S. cruzi*	–	–	NA

NA, not applicable; third lesion not detected. –, no *Sarcocystis* sp. was detected.

nal sarcocystosis is required. Mining of human gut microbiome datasets for *Sarcocystis* DNA may provide insight into the occurrence of intestinal sarcocystosis (Delmont et al., 2011). However, screening of human faeces is a more accurate approach.

Targeting the 18S gene, we observed a maximum number of 43 SNPs among all isolates and reference sequences. *Sarcocystis bovini* and *S. bovifelis* sequences differed by at most 10 SNPs and formed a single cluster that was merely 16 SNPs removed from the *S. hominis* cluster. This confirms the previously observed difficulties in distinguishing *S. hominis* from other thick-walled sarcocysts using 18S as a marker (Yang et al., 2001; Gjerde, 2013). In contrast, *cox1* sequences showed a much larger variation of up to 302 SNPs, and all *Sarcocystis* spp. formed separate clusters that were at least 50 SNPs apart. This clear distinction between *Sarcocystis* spp. is in line with previous observations (Gjerde, 2013), and confirms that *cox1* is a more reliable marker for differentiation of closely related thick-walled *Sarcocystis* spp. compared with 18S.

To improve species identification, we implemented a PCR protocol targeting the *cox1* gene, based on the previous work of Gjerde et al. (2016), and provided evidence that *cox1* is a more reliable marker for differentiation of *Sarcocystis* spp. However, our results suggest different amplification preferences for different *Sarcocystis* spp. of both markers. Compared with 18S, we were able to detect *S. cruzi* and *S. bovifelis* more frequently using cox1 primers, but at the same time, *S. hominis* was identified less frequently and *S. hirsuta* and *S. heydorni* were not detected at all. Improvement of the reverse primer could further increase the sensitivity. The forward primer (SF1) is universal to *Sarcocystis* spp., since the 3′ part of the primer targets a highly conserved region of 11 bp at the beginning of the *Sarcocystis cox1* gene. Thus far, such a region has not been identified at the end of the gene, or further downstream of the gene (Gjerde, 2013). As a result, Gjerde et al. have developed several reverse primers in the course of their research (Gjerde, 2016), of which SR9 was used in the current study. Adding additional reverse primers could improve the sensitivity of the protocol. However, rolling circle amplification and subsequent whole genome sequencing of the mitochondrial genome (Simison et al., 2006) could aid the identification of a highly conserved region downstream of the gene, suitable for development of a universal reverse primer.

To our knowledge, only one partial sequence of *S. hominis cox1* is present in GenBank, for which the current study confirms its identity. The submission of four additional *S. hominis cox1* sequences described in the present study strengthens the collection, and the availability of well-identified and well-referenced sequences in public databases aids the development of species-specific diagnostic PCRs targeting *cox1* (Poulsen and Stensvold, 2014). Real-time PCR targeting *cox1*, in combination with magnetic capture, may clarify *Sarcocystis* epidemiology in humans.

The 82.7% prevalence of *Sarcocystis* infection among Dutch cattle reported here is comparable to the high prevalence (66–94%) reported in surrounding European countries (Vangeel et al., 2007; Moré et al., 2014; Hornok et al., 2015; Meistro et al., 2015). Considering that we detected *S. cruzi* most frequently, followed by human and feline *Sarcocystis* spp., the epidemiological situation in the Netherlands shows most resemblance to that of Italy and Hungary (Hornok et al., 2015; Meistro et al., 2015). By comparison, in Germany, feline *Sarcocystis* spp. are the second most prevalent and only 6% *S. hominis* was detected (Moré et al., 2014). In contrast, in Belgium *S. hominis* is the most prevalent *Sarcocystis* spp. (Vangeel et al., 2007). This may be related to the experimental setup, since the former performed detection on DNA extracts directly from meat samples, whereas in Belgium cysts were isolated and microscopically identified prior to molecular detection.

Mixed infections complicate species identification by Sanger sequencing of PCR amplicons (Moré et al., 2014; Meistro et al., 2015; Gjerde and Hilali, 2016). In the current study, we also observed this phenomenon. Because we used two different markers, we were able to identify different species in the same host. Moreover, Gjerde et al. (2016) describe non-functional nuclear copies of the mitochondrial *cox1* gene complicating Sanger sequencing. In previous studies, these sequencing complications were circumvented by performing PCR on microscopically isolated cysts or cyst fragments, or cloning of PCR amplicons prior to sequencing analysis (Moré et al., 2014; Meistro et al., 2015; Gjerde and Hilali, 2016). Both these workarounds are laborious and time consuming, but next generation sequencing (NGS) could provide a promising alternative. Sanger sequencing compiles one sequence (read) from multiple DNA strands in a sample, whereas NGS platforms – such as Illumina or nanopore sequencing – enable simultaneous sequencing of each individual DNA molecule. Zahedi et al. (2017) compared Sanger sequencing and NGS to study the within-host *Cryptosporidium gp60* subtype diversity in faeces of

several mammals. While Sanger sequencing could only detect the most abundant subtype per sample, NGS was capable of identifying multiple additional subtypes (Zahedi et al., 2017). Moreover, Kaupke et al. successfully applied NGS by targeting two overlapping 18S rDNA fragments to study mixed infections of *Cryptosporidium* spp. in pig faeces (Kaupke et al., 2017). Additionally, Illumina MiSeq sequencing of 18S rDNA amplicons was applied to study waterborne parasites. Using this approach, the presence of *Giardia intestinalis*, *Acanthamoeba castellanii*, *T. gondii*, *Entamoeba histolytica* and *Blastocystis* spp. were detected in irrigation water samples (Moreno et al., 2018). Although compared with bacteriology, the application of NGS in parasitology is still lagging, the results of these studies show that NGS is being embraced by parasitologists worldwide, and could further enhance research into *Sarcocystis* spp., and foodborne parasites in general.

In conclusion, sequence-specific DNA extraction using magnetic capture is a valuable supplement to the existing method for molecular detection of *Sarcocystis* spp. Moreover, PCR and Sanger sequencing targeting *cox1* provides more distinct species identification compared with 18S. The availability of the *S. hominis cox1* sequence opens the door to development of sensitive and specific real-time PCR methods, by which *Sarcocystis* spp. prevalence in cattle and humans may be determined more effectively. Additionally, the application of magnetic capture could further improve detection, especially in sample types in which low amounts of target DNA in high backgrounds can be anticipated. Therefore, the results of the current study could enhance research into the epidemiology and prevalence of human intestinal sarcocystosis.

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