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ORIGINAL PAPER

Sarcocystis sinensis is the most prevalent thick-walled *Sarcocystis* species in beef on sale for consumers in Germany

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Abstract Bovines are intermediate hosts of *Sarcocystis cruzi*, *Sarcocystis hirsuta*, and *Sarcocystis hominis*, which use canids, felids, or primates as definitive hosts, respectively. Cattle represent also intermediate hosts of *Sarcocystis sinensis*, but the definitive hosts of this parasite are not yet known. Sarcocystosis in cattle is frequently asymptomatic. The infection is characterized by the presence of thin-walled (*S. cruzi*) or thick-walled muscle cysts or sarcocysts (*S. hominis*, *S. sinensis*, and *S. hirsuta*). Recent reports suggest high prevalence of the zoonotic *S. hominis* in beef in Europe. We therefore aimed at differentiating *Sarcocystis* spp. in beef offered to consumers in Germany using molecular and microscopical methods, focusing on those species producing thick-walled sarcocysts. A total of 257 beef samples were obtained from different butcheries and supermarkets in Germany and processed by conventional and multiplex real-time PCR. In addition, 130 of these samples were processed by light microscopy and in 24.6 % thick-walled cysts were detected. Transmission electron microscopical analysis of six of these samples revealed an ultrastructural cyst wall pattern compatible with *S. sinensis* in five samples and with *S. hominis* in one sample. PCR-amplified 18S ribosomal DNA (rDNA) fragments of 28 individual thick-walled cysts were sequenced, and sequence identities of ≥98 % with *S. sinensis* (n=22), *S. hominis* (n=5) and *S. hirsuta* (n=1) were observed. Moreover, nine *Sarcocystis* sp. 18S rDNA full length gene sequences were obtained, five of *S. sinensis*, three of *S. hominis*, and one of *S. hirsuta*. Out of all samples (n=257), 174 (67.7 %) tested positive by conventional PCR and 179 (69.6 %) by multiplex real-time PCR for *Sarcocystis* spp. Regarding individual species, 134 (52 %), 95 (37 %), 17 (6.6 %), and 16 (6.2 %) were positive for *S. cruzi*, *S. sinensis*, *S. hirsuta*, and *S. hominis*, respectively. In conclusion, *S. sinensis* is the most prevalent thick-walled *Sarcocystis* species in beef offered for consumption in Germany. Further studies are needed to identify the final host of *S. sinensis* as well as the potential role of this protozoan as a differential diagnosis to the zoonotic species *S. hominis*.

Keywords *Sarcocystis* spp. · Multiplex real-time PCR · Sequencing · Electron microscopy · Cattle · Beef

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Introduction

Infections caused by protozoan parasites of the genus *Sarcocystis* are globally distributed and affect a high

proportion of animals (Dubey et al. 1989). *Sarcocystis* spp. are heteroxenous apicomplexan parasites, mostly with carnivores or omnivores as definitive hosts (DHs, intestinal sexual reproduction) and herbivores as intermediate hosts (IH, asexual reproduction ending in sarcocysts, i.e., muscle cysts). Cattle represent the IH of a variety of *Sarcocystis* spp.: *Sarcocystis cruzi* (syn. *Sarcocystis bovicanis*), *Sarcocystis hirsuta* (syn. *Sarcocystis bovifelis*), and *S. hominis* (syn. *Sarcocystis bovihominis*), which use canids, felids, or primates as DHs, respectively (Dubey et al. 1989; Fayer 2004; Heydorn et al. 1975; Heydorn et al. 1976), and *Sarcocystis sinensis*, for which the identity of its DH is still unknown. *S. sinensis* has been found in buffaloes and cattle from China and Argentina (Gjerde 2013; Moré et al. 2013; Yang et al. 2001a; Yang et al. 2001b). A recent study showed that human volunteers did not excrete sporocysts upon ingestion of buffalo meat containing *S. sinensis* cysts (Chen et al. 2011); this suggests that humans are not DHs of *S. sinensis*. Sarcocystosis in cattle is frequently asymptomatic. Cattle sarcocystosis is characterized as a mainly chronic infection producing thin-walled (*Sarcocystis cruzi*) or thick-walled sarcocysts (*S. hominis*, *S. sinensis*, and *S. hirsuta*) (Chen et al. 2011; Dubey et al. 1989; Heydorn et al. 1975; Moré et al. 2011). As the detection of *S. hominis* sarcocysts in beef during meat inspection may lead to the condemnation or rejection of carcasses or beef portions due to the zoonotic potential of this parasite, specific tools are needed, which allow a reliable identification among *Sarcocystis* spp. developing thick-walled sarcocysts. Despite of this, some rejections have been reported via the European Rapid Alert System for Food and Feed (RASFF), which were only based on the detection of thick-walled cysts arguing that they might represent *S. hominis*, but no further analyzes were conducted to determine the species of the parasites (http://ec.europa.eu/food/food/rapidalert/reports/). Moreover, the authors of a study recently performed in Italy suggest that any thick-walled cyst detected in beef should be considered as *S. hominis* (Domenis et al. 2011).

Co-infections with more than one *Sarcocystis* spp. in cattle are frequent; therefore, molecular approaches using tissue DNA instead of individual cyst DNA may reveal inconclusive results (Moré et al. 2011). The reliable identification of *Sarcocystis* spp. by molecular methods may thus require the processing of individual cysts (Fischer and Odening 1998; Yang et al. 2001b). Transmission electron microscopy (TEM) of cyst walls, sequencing of DNA obtained from individual cysts and species-specific molecular assays allow a reliable identification of different *Sarcocystis* spp. Recently, a multiplex real-time PCR with specific probes for cattle *Sarcocystis* species has been developed and applied to analyze the occurrence of *Sarcocystis* spp. in Argentinean cattle (Moré et al. 2013). The results of this study suggest a wide distribution of *S. sinensis* in this South American country.

Bovine *Sarcocystis* spp. infections in Europe and particularly in Germany have been extensively analyzed; however, most examinations were conducted mainly making use of microscopical techniques (Boch et al. 1978; Drost 1982; Dubey et al. 1989; Fischer and Odening 1998; Odening 1998; Odening et al. 1995; Tenter 1995; Van Knapen et al. 1987; Vercruysse et al. 1989). These studies in the past did not take *S. sinensis* into account, which has recently been reported to be morphologically and molecularly similar but not identical with *S. hominis* (Chen et al. 2011; Moré et al. 2013).

Recently, an apparently high percentage of *S. hominis* infections has been reported in cattle from Belgium and Italy (Domenis et al. 2011; Vangeel et al. 2007) and was further correlated with the occurrence of bovine eosinophilic myositis (Vangeel et al. 2013). However, the analytical tools applied in these studies were not able to exclude the presence of *S. sinensis* (Domenis et al. 2011; Vangeel et al. 2007; Vangeel et al. 2013).

Since the morphological differences among *Sarcocystis* spp. with thick-walled cysts are minor and molecular differentiation between *S. hominis* and *S. sinensis* is difficult, it is not unlikely that previous studies have underestimated the prevalence of *S. sinensis*, while overestimating the proportion of *S. hominis* infections in cattle (which are of zoonotic concern). The objective of the present study was therefore to differentiate *Sarcocystis* spp. in beef offered to consumers in Germany using molecular and microscopical methods, focusing on the thick-walled sarcocysts.

Materials and methods

Samples

A total of 257 beef samples (around 100 g each) were collected from retail in Germany. From these samples, 130 were minced beef collected by members of the working group at Friedrich-Loeffler-Institute (FLI) in butcheries and supermarkets located in the northeast (n=49, i.e., samples from the federal states of Mecklenburg-Western-Pomerania, Berlin, Brandenburg, Saxony-Anhalt, and Saxony), in the northwest (n=43, i.e., samples from the federal states of Schleswig-Holstein, Hamburg, Bremen, Lower Saxony, and North Rhine-Westphalia), and in the south of Germany (n=38, i.e., samples from the federal states of Thuringia, Bavaria, Hesse, Rhineland-Palatinate, Saarland, and Baden-Württemberg).

Sixty three samples of minced beef (n=9) and different beef portions (n=54) were collected from butcheries close to Stuttgart (federal state of Baden-Württemberg, South Germany) by the Chemisches und Veterinäruntersuchungsamt Stuttgart (CVUA S). The remaining 64 samples of minced beef (n=23) and different portions of meat (n=41) were collected by the

Chemisches und Veterinäruntersuchungsamt Karlsruhe (CVUA KA), in the South of Germany.

The origin of the slaughtered animals was not known for most of the samples (n=200); for the remaining 57 samples, it was documented that they had originated from cattle raised in Germany (n=50), The Netherlands (n=2), France (n=2), Austria (n=1), Belgium (n=1), and Italy (n=1).

Light microscopy and TEM

The 130 minced beef samples collected by FLI were processed fresh and examined by light microscopy essentially as previously described (Moré et al. 2011) but using an inverted microscope for observation (TMS Nikon, Japan). The sarcocysts observed were classified as thin or thick walled, and individual thick-walled cysts or cyst fragments were collected into 1.5-ml nuclease-free tubes and preserved at −20 °C until further analyzed.

If several thick-walled cysts were observed by light microscopy in a sample, at least 10 cysts or cyst fragments were recovered, fixed in 2.5 % glutardialdehyde for 4 h and maintained in PBS at 4 °C until further processed. For TEM, the samples were fixed in 1 % osmium tetroxide for 24 h and embedded in epoxy resin using standard procedures (Hayat 1986). Ultrathin sections were examined in a transmission electron microscope (EM 10, Carl Zeiss AG, Oberkochen, Germany).

Conventional PCR and multiplex real-time PCR

Around 5 g beef from all 257 samples were processed by artificial digestion in individual tubes as previously described to isolate *Toxoplasma gondii* from animal tissues (Dubey 1998). From the resulting pellet, DNA was extracted using the NucleoSpin® Tissue kit (Macherey-Nagel, Germany) for samples collected at FLI or High Pure PCR Template Preparation Kit (Roche, Germany) for samples collected at CVUA S and CVUA KA, according to the manufacturer's instructions.

The DNA was also isolated from samples of individual thick-walled cysts or cyst fragments obtained during microscopical examination using the NucleoSpin® Tissue kit as mentioned above. In addition, 20 individual cyst fragments (18 containing thick-walled and 2 containing thin-walled cysts) were subjected to four freezing-thawing cycles in 50 μl of 0.1 % TE buffer, at −20 and 94 °C for 10 min each and the resulting solution used as PCR templates.

All DNA samples were analyzed by conventional PCR to detect Eucoccida parasite DNA using SarcoFext/SarcoRext primers and by a recently published multiplex real-time PCR using a combination of bovine *Sarcocystis* species-specific probes (Moré et al. 2013).

Agreement between both test results was analyzed estimating kappa value by using Win Episcope 2.0 software.

Sequencing

Initially, the SarcoFext/SarcoRext amplicons obtained from individual cysts were sequenced using a LI-COR DNA Sequencer 4200 (MWG Biotech, Germany) by a protocol previously described (Moré et al. 2013). From selected cyst DNA samples, the full length sequence of the *Sarcocystis* 18S ribosomal DNA (rDNA) gene was amplified, purified, cloned into plasmid, and sequenced as previously described (Moré et al. 2013). In addition, conventional PCR products were purified from beef DNA samples that had tested positive in the SarcoFext/SarcoRext PCR but yielded inconclusive or negative results in the multiplex real-time PCR, mixed with SarcoFint/SarcoRint primers (Moré et al. 2013) and submitted for sequencing to the Lightrun service of GATC Biotech, Konstanz, Germany.

Sequences were analyzed with the GENEIOUS program (freely available version 6.1, http://www.geneious.com) and the resulting consensus sequences compared with others reported in GenBank using the megablast alignment of the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov).

Results

Light microscopy and TEM

Of the 130 examined minced beef samples, 40.0 % (52/130) were positive for *Sarcocystis* spp. cysts by light microscopy, 28.5 % (37/130) containing thin-walled (*S. cruzi*) and 24.6 % (32/130) thick-walled cysts. In 13.1 % of the samples (17/130), both thin- and thick-walled cysts were detected. From six samples, thick-walled cyst or cyst fragments were examined by TEM. In one sample, the ultrastructural cyst wall pattern was compatible with that of *S. hominis* and in the remaining five samples, the observed pattern resembled that of *S. sinensis* (Fig. 1a, b). Both patterns were characterized by finger-like protrusions, but these were upright and with a thick ground substance (>1 μm) in case of *S. hominis* cysts (Fig. 1a) and angled with a thin ground substance (approximately 0.5 μm) in *S. sinensis* cysts (Fig. 1b). Moreover, *S. sinensis* cyst walls showed vesicle-like structures at the basis of the protrusions (Fig. 1b). DNA was isolated from cysts or cyst fragments of the same beef samples, and by sequencing, by BLAST comparisons, and by the multiplex real-time PCR, the electron-microscopical results were confirmed.

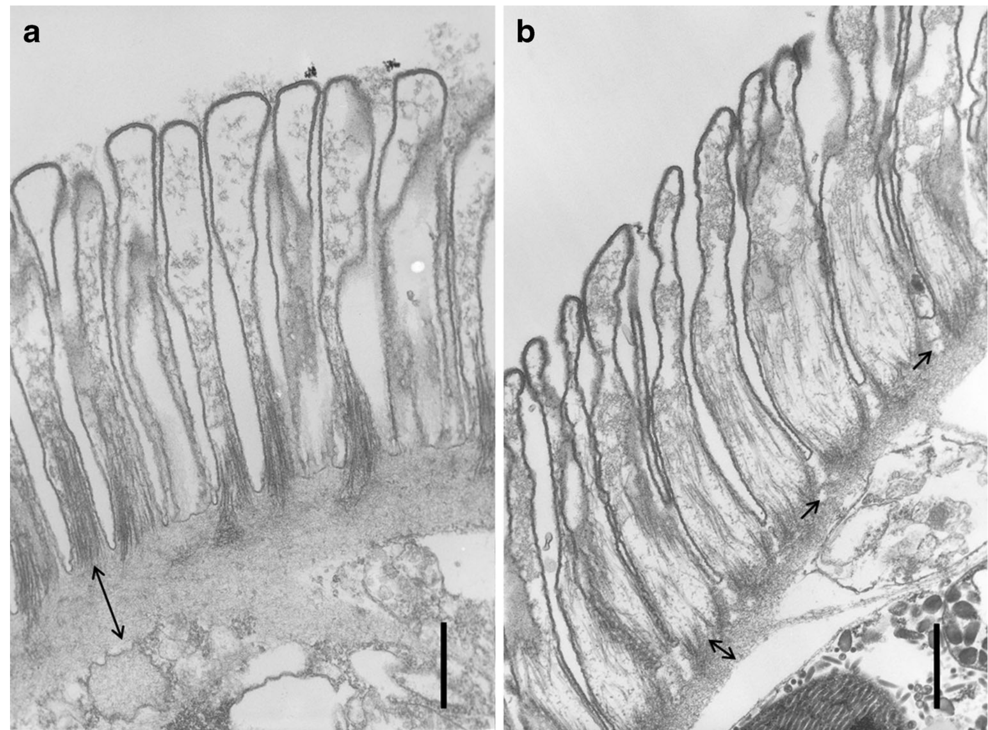

Fig. 1 Transmission electron microscopical (TEM) examination of the cyst walls of *S. hominis* (**a**) and *S. sinensis* (**b**) sarcocysts from beef samples collected in butcheries and supermarkets in Germany. Note the thicker ground substance (*double-head arrows*) in *S. hominis*. The *S. sinensis* cyst wall showed vesicle-like structures at the basis of the protrusions (*arrows*). *Scale bar*= 1 μm

Conventional PCR and multiplex real-time PCR results

Out of all samples (*n*=257), 174 (67.7 %) tested positive by conventional PCR and 179 samples (69.6 %) by multiplex real-time PCR. Agreement beyond chance between both tests was good (kappa value 0.738 [CI 95 % 0.616–0.860]; Table 1). Results of conventional PCR and multiplex real-time PCR according to meat store location are shown in Table 2.

Mixed infections were detected in 72 samples (28 %) by multiplex real-time PCR. Most of them tested positive for *S. cruzi* and *S. sinensis* (*n*=46; 17.9 % of all samples) at the same time, followed by *S. cruzi* and *S. hominis* (*n*=8; 3.1 %), *S. cruzi* and *S. hirsuta* (*n*=5, 1.9 %), *S. cruzi*, *S. sinensis*, and *S. hirsuta* (*n*=5; 1.9 %), *S. cruzi*, *S. sinensis*, and *S. hominis* (*n*=4; 1.5 %), and *S. sinensis* and *S. hirsuta* (*n*=3; 1.2 %). One sample (0.4 %) reacted positive for *S. sinensis*, *S. hirsuta*, and *S. hominis*, and another sample (0.4 %) reacted with all four probes. Considering the individual *Sarcocystis* species, 134 (52 %), 95 (37 %), 17 (6.6 %), and 16 (6.2 %) of all samples were positive for *S. cruzi*, *S. sinensis*, *S. hirsuta*, or *S. hominis*, respectively.

Table 1 Comparison of conventional PCR (Sarcoext; using SarcoFext/SarcoRext primers) and multiplex real-time PCR results for all beef DNA samples

Sarcoext	Multiplex real-time PCR (+)	(−)	Total
(+)	162	12	174
(−)	17	66	83
Total	179	78	257

(+) and (–) indicate positive and negative results, respectively. Kappa value 0.738 (CI 95 % 0.616–0.860)

From those samples whose origin from German cattle was known, 74 % (37/50) were positive in the SarcoFext/SarcoRext PCR, while 33 (66 %), 23 (46 %), 2 (4 %), and 1 (2 %) of the samples were positive for *S. cruzi*, *S. sinensis*, *S. hirsuta*, or *S. hominis*, respectively, by multiplex real-time PCR. Mixed infections were identified in 34 % (17/50) of the samples of German origin.

According to the multiplex real-time PCR results, the two beef samples from France tested both positive for *S. cruzi* and one also for *S. hominis*; the samples from The Netherlands, Belgium, and Italy were negative, and the sample from Austria was positive for *S. sinensis*.

Out of the 20 samples from individual cysts or cyst fragments processed after freezing-thawing instead other DNA extraction, 13 were positive in the SarcoFext/SarcoRext PCR. By multiplex real-time PCR, both samples containing thin-walled cysts were identified as *S. cruzi*, and from the remaining 18 thick-walled cyst samples, 12 reacted positive with the *S. sinensis* probe and 2 with the *S. hominis* probe, and 4 tested negative.

Table 2 Results of conventional and multiplex real-time PCR from beef DNA samples, according to meat store (butcheries/supermarkets) location in Germany

Conventional PCR				Multiplex real-time PCR				
Meat store location	Samples (*n*)	(+)	(−)	*S. cruzi*	*S. sinensis*	*S. hominis*	*S. hirsuta*	(−)
Northeast	43	33	10	19	18	2	3	12
Northwest	49	26	23	22	6	4	2	23
South	165	115	50	93	71	10	12	43
Total	257	174	83	134	95	16	17	78

Meat store location within Germany where the beef samples were obtained, Northeast=federal states of Mecklenburg-Western-Pomerania, Berlin, Brandenburg, Saxony-Anhalt, and Saxony; Northwest=federal states of Schleswig-Holstein, Hamburg, Bremen, Lower Saxony, and North Rhine-Westphalia; South=federal states of Thuringia, Bavaria, Hesse, Rhineland-Palatinate, Saarland, and Baden-Württemberg. (+) and (–) indicate positive and negative results, respectively

Sequencing

Out of the 32 minced beef samples showing thick-walled sarcocysts, 28 individual cysts/cyst fragments were collected from 27 beef samples (one single sample contained morphologically different cysts which were analyzed separately). DNA was extracted and the 18S rDNA fragment was PCR-amplified and sequenced. Consensus sequences of about 400-bp length showed identities of ≥98 % with *S. sinensis* (22 samples), *S. hominis* (5 samples), and *S. hirsuta* (1 sample) when the sequences were compared to those deposited in GenBank. Of two morphologically different cysts collected from one beef sample, one resulted as *S. sinensis* and one *S. hominis* (sequence identities of ≥98 %). The identities were also confirmed by multiplex real-time PCR of the single cyst DNA samples using species-specific probes.

From these individual cyst/cyst fragment DNA samples, nine *Sarcocystis* sp. 18S rDNA full gene sequences were obtained, five of *S. sinensis*, three of *S. hominis*, and one of *S. hirsuta*. Five sequences had previously been reported in GenBank (two of *S. hominis* JX679470 and JX679471, two of *S. sinensis* JX679466 and JX679469, and one of *S. hirsuta* JX 855283; Moré et al. 2013). The remaining four sequences obtained in the present study were also deposited in GenBank (three *S. sinensis* KF954728, KF954729, and KF954730, and one *S. hominis* KF954731). The origin of the animals was known for all these nine beef samples from which single cysts or cysts fragments had been obtained; all originated from Germany with a single exception which had its origin in Austria (*S. sinensis* GenBank KF954730).

Sequencing was performed on 18S rDNA fragments from 9 out of 12 samples that were positive in the conventional PCR but negative by multiplex real-time PCR, resulting in 5 samples with 99 % identity with *Sarcocystis miescheriana*, 2 samples with 99 % identity with *Sarcocystis capracanis*, and 2 samples with an inconclusive sequencing result (i.e., several double peaks in the chromatograms).

Discussion

Sarcocystis spp. infections in cattle are widely distributed and affect a large proportion of animals including livestock such as cattle. In particular, bovine *S. cruzi* infections have been detected in several countries with prevalence >80 % (Dubey et al. 1989). Due to the high prevalence of *S. cruzi* infections, mixed *Sarcocystis* spp. infections are often observed in cattle if additional species are present, as reported by several investigators (Boch et al. 1978; Böttner et al. 1987; Domenis et al. 2011; Dubey et al. 1989; Moré et al. 2008; Moré et al. 2011; Van Knapen et al. 1987; Vercruysse et al. 1989). This was also observed in the present study.

The high number of mixed infections with various *Sarcocystis* spp. in cattle complicates the species identification by molecular means and the sequencing of PCR products targeting conserved regions among *Sarcocystis* spp. (Fischer and Odening 1998; Pritt et al 2008; Yang et al. 2001b). To avoid problems associated with the potential presence of several *Sarcocystis* spp. in a single sample, we conducted microscopical studies followed by PCR amplification and sequencing of individual cysts or cyst fragments. However, this approach is laborious and time consuming. Therefore, a recently developed real-time PCR (Moré et al. 2013) was also applied to analyze a higher number of samples and to identify mixed infections properly.

All beef samples used in the present study were obtained in different butcheries or supermarkets widely distributed over Germany. However, the origin of the cattle was recorded only from a small proportion (around 22 %) of the samples. It is therefore possible that some samples analyzed in the present study originated not from Germany but from other European countries or even from other continents.

As in many other studies, *S. cruzi* was the most frequently observed *Sarcocystis* spp. in beef samples collected in Germany. By multiplex real-time PCR, 28 % of the samples (72/257) reacted with at least 2 of the applied *Sarcocystis* spp.-

specific probes, and in most of them (95.8 %; 69/72), *S. cruzi* was present. In spite of this, the estimated prevalence of *S. cruzi* observed in the present study was lower than those reported in previous studies where beef from Germany or other European countries had been tested (Boch et al. 1978; Drost 1982; Domenis et al. 2011; Van Knapen et al. 1987; Vercruysse et al. 1989) and also lower than in Argentina where the same diagnostic protocols had been applied (Moré et al. 2011; Moré et al. 2013). Possible explanations for the lower prevalence of *S. cruzi* infections observed in our study could be differences in management practices applied in the production of beef for the German market not favoring infections with *S. cruzi*. These may include feeding practices of cattle, sources of feed or other potential risk factors such as the number of dogs kept on or close to beef farms or the feeding practices of these dogs.

Thick-walled cysts were observed by light microscopy in 24.6 % of 130 minced beef samples obtained in German butcheries and supermarkets; this proportion is similar to that detected using the same techniques in Argentinean loin samples (Moré et al. 2011). Previous studies performed in other European countries showed a higher proportion of beef samples positive for thick-walled cysts, for example, 56 % (Vercruysse et al. 1989) and 91 % (Vangeel et al. 2007) in Belgium or around 57 % in Italy (Domenis et al. 2011). A previous light-microscopical study performed in southern Germany observed a proportion of 63.3 % beef samples positive for thick-walled cysts, which were diagnosed as *S. hominis* cysts at that time (Boch et al. 1978). Moreover, work from New Zealand demonstrated a proportion of 79.8 % of thick-walled cysts in beef samples (Böttner et al. 1987). Differences in the results between studies may be related to a varying sensitivity of the diagnostic techniques applied and to the type of tissue sample used, but they may also reflect true differences in the prevalence of infections with thick-walled tissue cysts in these countries. However, future comparisons between countries or production systems should be based on a proper identification of *Sarcocystis* species, as comparisons are further complicated by the fact that the observation of "thick-walled cysts" may be explained by the presence of at least three *Sarcocystis* species (Chen et al. 2011; Dubey et al. 1989; Heydorn et al. 1975; Moré et al. 2013).

The ultrastructure of the cyst wall has been considered an important tool to identify various *Sarcocystis* spp., especially to differentiate among *Sarcocystis* spp. developing thick-walled cysts (Chen et al. 2011; Dubey et al. 1989; Heydorn et al. 1975; Odening et al. 1995). We observed by TEM the same differential cyst wall patterns previously described for *S. sinensis* from buffaloes and *S. hominis* from cattle in China (Chen et al. 2011), but both from cattle samples. The finding that the *S. sinensis* cyst wall patterns we observed in cattle resembled that previously observed in buffaloes further supports the view that differences in cysts wall assemblage are only related to the *Sarcocystis* species, but not to the intermediate host species the samples were obtained from.

Sequencing of amplified 18S rDNA fragments from 28 individual thick-walled cyst DNA samples showed that the majority of the positive minced beef samples (n=32) harbored *S. sinensis* cysts (22 *S. sinensis* versus 5 *S. hominis* and 1 *S. hirsuta*). In addition, we reported full length *S. sinensis* 18S rDNA sequences amplified from DNA extracted from minced beef from cattle of German (n=4) and Austrian (n=1) origin. These *S. sinensis* sequences represent the first ones reported from cattle from European countries and represent to the best of our knowledge, the first report of a detection of *S. sinensis* in cattle originating from European countries. As judged by multiplex real-time PCR, *S. sinensis* was the most prevalent among thick-walled species. It was detected more frequently than those in cattle from Argentina, where the same technique had been applied in a similar study using loin samples (Moré et al. 2013). Moreover, samples from cattle of known German origin also showed a high proportion of *S. sinensis* (46 %; 23/50). These results suggest that *S. sinensis* has a wider distribution than previously thought, as the parasite had previously only been described in cattle from China and Argentina (Gjerde 2013; Moré et al. 2013; Yang et al. 2001b) as well as in buffaloes from China (Chen et al. 2011) and probably from India (*S. sinensis* sequence GenBank JQ713823, unpublished). Consequently, it can be predicted that the as yet unknown definitive hosts of *S. sinensis* feed on beef frequently and have a wide geographic distribution. Further studies are needed in order to identify the definitive host of *S. sinensis* as well as the involvement of this protozoan as a differential diagnosis to the zoonotic species *S. hominis*. These achievements will help to given full validation to the species name (Frenkel et al. 1979; Odening 1998).

A previous study conducted in China suggests that human volunteers suffered abdominal symptoms after intake of raw buffalo meat containing *S. sinensis* cysts (Chen et al. 2011). However, as no other pathogens causing food-born illness were evaluated on the mentioned raw meat (Chen et al. 2011), further studies on *S. sinensis* pathogenicity should be conducted using purified sarcocysts, in order to avoid misinterpretations.

As regards to the mixed infections detected, out of all samples positive to *S. sinensis* (n=95), only six reacted also with the *S. hominis* probe, suggesting that the risk factors for cattle to become infected with these two species could be different from or not associated with each other.

In a previous study conducted in Belgium, thick-walled cysts were observed in 61/67 (91 %) of minced beef samples (Vangeel et al. 2007). For 38 18S rDNA gene fragments amplified from these samples (which represents the 56.7 % of the examined beef samples), a sequence identity of >95 % with *S. hominis* 18S rDNA was observed. In these analyses, the possible presence of *S. sinensis* was not taken into

consideration, although the level of sequence identity might also by explained by the presence of this parasite in the tested samples (Gjerde 2013; Moré et al. 2013). It seems therefore possible that the prevalence of *S. hominis* in Belgium is lower than previously assumed and that some of the observed thick-walled cysts could have been *S. sinensis*.

In our study, *S. hominis* was detected in only 6.2 % of all samples by multiplex real-time PCR and the identity of the parasite confirmed by DNA sequencing and TEM. In samples of known German origin (n=50), only one tested positive for *S. hominis*. As previously mentioned, this prevalence is lower than that detected by other investigators and higher than that reported for loin samples of Argentinean cattle (Boch et al. 1978; Domenis et al. 2011; Dubey et al. 1989; Moré et al. 2013; Vangeel et al. 2007). Although *S. hominis* had the lowest prevalence among thick-walled *Sarcocystis* spp., concern about its potential zoonotic impact seems to be justified. Hygienic conditions should be improved at the farm level, especially in areas where the consumption of raw or undercooked beef is frequent to reduce the risk of human and cattle infections (Domenis et al. 2011; Vangeel et al. 2007).

S. hirsuta was detected by multiplex real-time PCR in 6.6 % of beef DNA samples and was confirmed in one sample by sequencing. This proportion of positive samples is slightly higher than that reported for Argentinean beef samples, which were tested by the same multiplex real-time PCR (Moré et al. 2013). The prevalence reported in the present study is also higher than that reported in work from Italy and Belgium (Domenis et al. 2011; Vangeel et al. 2007). Among samples of known origin from German cattle, 4 % (2/50) were positive for *S. hirsuta*. Interestingly, a previous study conducted in southern Germany found *S. hirsuta* cysts in 34.5 % of beef samples as judged by morphological criteria (Boch et al. 1978). The prevalence and the economical impact of this species may therefore be declining also because recent reports of macroscopical cyst detection are scarce and carcass condemnations due to the presence of macroscopically visible cysts are rare (Dubey et al. 1990). In addition, *S. hirsuta* was detected only rarely in cases of bovine eosinophilic myositis (Vangeel et al. 2013). Reasons for a declining prevalence of *S. hirsuta* in Germany might be related to improved hygienic conditions on farms and meat inspections at slaughter together with an increased use of commercial, i.e., canned and heated cat food.

The agreement between the results of the conventional SarcoFext/SarcoRext PCR and multiplex real-time PCR was good as judged by the kappa value determined in this study; differences may be related to a higher sensitivity of the multiplex real-time PCR (i.e, 17 samples were positive only by real-time PCR). The multiplex real-time PCR also showed a better specificity, as in 7 of 12 samples that were negative by the multiplex real-time PCR but positive by the SarcoFext/SarcoRext PCR; other *Sarcocystis* species were identified by sequencing as *S. miescheriana* (n=5) and *S. capracanis* (n=2). As pork is on sale in most German butcheries and supermarkets and since some of them also sell meat of small and wild ruminants, it is not unlikely that a number of beef samples we analyzed could have been contaminated with meat or sarcocysts of other vertebrate species. This has to be taken into account in particular when studies are only based on light microscopy as contamination with material from other species may prompt false conclusions with regard to the presence of thick-walled cysts, which are in fact not sarcocysts of cattle, but contaminants.

Additionally, we showed that in 80 % (16/20) of single cysts or cyst portions treated only by freezing-thawing cycles, a specific identification was possible by using multiplex real-time PCR. This approach could be potentially applied for a fast identification of single cysts after microscopical detection.

Based on the results obtained by microscopical examinations, multiplex real-time PCR, and sequencing, we conclude that *S. sinensis* is the most prevalent thick-walled *Sarcocystis* species in beef on sale for consumers in Germany.

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