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Development of a multiplex real time PCR to differentiate *Sarcocystis* spp. affecting cattle

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ABSTRACT

Cattle are intermediate hosts of *Sarcocystis cruzi*, *Sarcocystis hirsuta* and *Sarcocystis hominis* which use canids, felids or primates as definitive hosts (DH), respectively, and in addition of *Sarcocystis sinensis* from which the DH is unknown. The aims of the present study were to develop and optimize a multiplex real time PCR for a sensitive and specific differentiation of *Sarcocystis* spp. affecting cattle and to estimate the prevalence of *Sarcocystis* spp. in Argentinean cattle. The 18S rRNA genes from individual sarcocysts were amplified and cloned to serve as controls. For the amplification of bovine *Sarcocystis* spp. a total of 3 primers were used in combination with specific individual probes. Each assay was evaluated and optimized individually and subsequently combined in a multiplex assay (BovSarcoMultiplex real time PCR). The analytical specificity of the multiplex assay was assessed using 5 ng of DNA of heterologous *Sarcocystis* spp. and other apicomplexan parasites, and no positive reactions were observed other than for the species the PCR targeted. The analytical sensitivity ranged between 0.0125 and 0.125 fg of plasmid DNA (equivalent to the DNA of 2–20 plasmid DNA copies) or resembling DNA of 0.1–0.3 bradyzoites. A total of 380 DNA loin samples from Argentina were tested and 313, 29, 14 and 2 were positive for *S. cruzi*, *S. sinensis*, *S. hirsuta* and *S. hominis*, respectively. *S. sinensis* was the most prevalent species among thick walled *Sarcocystis* spp. in Argentinean cattle. Mixed infections were detected in 8.9% of all samples. Diagnostic sensitivity and specificity for the BovSarcoMultiplex real time PCR relative to previous microscopic examination for thin and thick-walled cyst were 91.5% and 41.7%, 36.3% and 95.9% respectively. Improved DNA extraction methods may allow to further increase the specific and sensitive detection of *Sarcocystis* spp. in meat samples.

1. Introduction

Infections caused by protozoan apicomplexan parasites of the genus *Sarcocystis* are globally distributed and generally affect a proportion greater than 40% of herbivorous animals (Dubey et al., 1989). *Sarcocystis* spp. have a heteroxenous life cycle, generally with carnivores or omnivores as definitive hosts (DH) and herbivores as

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intermediate hosts (IH). Infections in IH are mainly asymptomatic and chronic producing muscle cysts (sarcocysts). Infections in DH are intestinal and mostly asymptomatic, with reports of clinical symptoms in humans acting as DH of *S. hominis* and *S. suihominis* (Dubey et al., 1989; Fayer, 2004). Cattle are described as IH of different *Sarcocystis* spp.: *S. cruzi*, *S. hirsuta* and *S. hominis*, which use canids, felids or primates as DH, respectively. Most infections in cattle are chronic producing thin-walled (*S. cruzi*) or thick-walled tissue cysts (*S. hominis* and *S. hirsuta*). Recently, *S. sinensis*, another thick-walled *Sarcocystis* sp., which was first described in buffaloes in China, has also been diagnosed in cattle from China (Yang et al., 2001a,b). However there is limited information on its spatial distribution and the prevalence in different regions. The DH of *S. sinensis* is still unknown, but, one study performed with human volunteers in China suggested that humans are not the DH of *S. sinensis* (Chen et al., 2011).

Optical microscopy, transmission electron microscopy (TEM) of the cyst walls and molecular assays have been used for the specific identification of different *Sarcocystis* spp. in cattle (Dubey et al., 1989; Jehle et al., 2009; Moré et al., 2011; Vangeel et al., 2007). However, most of the original descriptions did not consider *S. sinensis*, which is morphological and molecularly similar to *S. hominis* (Chen et al., 2011; Dubey et al., 1989; Yang et al., 2001b). Recently, differences on the cyst wall structure by TEM between *S. hominis* and *S. sinensis* were described for the first time (Chen et al., 2011). The most common target for molecular diagnosis and species differentiation is the small subunit of the ribosomal RNA gene (18S rDNA), which is repetitive and shows considerable variability among *Sarcocystis* species (Dahlgren and Gjerde, 2007; Tenter, 1995; Xiang et al., 2009; Yang et al., 2001b). The copy number of the 18S rDNA for each *Sarcocystis* spp. is unknown; however for the related apicomplexan protozoan *Toxoplasma gondii* it was estimated that about 110 copies per haploid tachyzoite genome exist (Guay et al., 1992).

Identification of *Sarcocystis* spp. from cattle was performed using several molecular methods, but the sensitivity and specificity of some of these were low, and in most of the cases the presence of *S. sinensis* was not analyzed (Domenis et al., 2011; Jehle et al., 2009; Moré et al., 2011; Vangeel et al., 2007; Xiang et al., 2009). Moreover, one of the 18S rDNA sequences reported as *S. hominis* and used as reference for the evaluation of molecular techniques (GenBank AF006470) has been found to be more closely related to sequences of *S. sinensis* than to those of other *S. hominis* (Yang et al., 2001b).

As the presence of *S. hominis* cysts in meat may cause the condemnation of beef (considering its zoonotic potential), specific tools which allow a reliable identification of *Sarcocystis* spp. and especially the differentiation between *S. sinensis* and *S. hominis* are needed. Such tools could help to avoid unjustified rejections of beef. Moreover, a reliable identification of *Sarcocystis* spp. affecting cattle could help to estimate the prevalence, distribution, source of infection and risk factors for these infections.

The aims of the present study were to develop and optimize a multiplex real time 5′-nuclease PCR for a sensitive and specific diagnosis of *Sarcocystis* spp. affecting cattle and to apply it to identify the prevalence of *Sarcocystis* spp. in Argentinean cattle.

2. Materials and methods

2.1. Real time PCR assays development

2.1.1. Primers and probes

Primers and probes were designed for the 18S rDNA as the target. To identify target regions, a multi-alignment for *Sarcocystis* spp. and other apicomplexan parasites sequences, available in GenBank or generated in the present study (Supplementary data, Table S1), was carried out using the GENEIOUS program. Based on the alignment, PCR primers were designed with help of the online tool primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) to amplify short fragments (< 200 bp), targeting conserved sequences flanking variable regions in the 18S rDNA of *Sarcocystis* spp. One forward primer and two reverse primers located in these conserved regions were designed. The second reverse primer became necessary as *S. hirsuta* was different in three nucleotide positions as compared to other bovine *Sarcocystis* spp. in one of the conserved flanking regions (Table 1 and Supplementary data, Table S1). The forward primer matched with almost all *Sarcocystis* spp. sequences, the primer SarcoRTR matched with *S. sinensis* and *S. hominis* sequences and had one or three mismatches with *S. cruzi* or *S. hirsuta*, respectively. The primer ShirsutaRTR matched with all *S. hirsuta* sequences with a maximum of one mismatch but had three or more mismatches with *S. hominis*, *S. sinensis* and *S. cruzi*. For other non-*Sarcocystis* apicomplexan parasites each primer had at least two or more mismatches (Supplementary data, Table S1). The expected sizes of the products were 166 bp for *S. hominis* and *S. cruzi*, 159–166 bp for *S. sinensis* and 176 bp for *S. hirsuta*.

Conventional dual-labeled probes specific for *S. cruzi* and *S. hirsuta* were designed (Table 1). Since the variable region used to differentiate *S. hominis* and *S. sinensis* were AT rich, locked nucleic acid probes (LNA™) were designed (Table 1) with the help of the technical service of Sigma–Aldrich (Sweden), to increase the melting temperature and specificity (Mouritzen et al., 2003; Ugozzoli et al., 2004). Conventional probes were obtained from Eurofins MWG Operon, Germany and LNA™ probes from Sigma–Aldrich, Germany. Sequences amplified with the real time PCR primers and the location of each probe relative to the consensus sequence of each bovine *Sarcocystis* spp. is shown in Fig. 1.

2.1.2. Identification of control samples and preparation of control plasmid DNA samples

DNA was extracted from individual sarcocysts or cyst fragments of *S. cruzi*, *S. hirsuta*, *S. hominis* and *S. sinensis* isolated from bovine tissues from Argentina and Germany with the NucleoSpin®Tissue kit (Macherey–Nagel, Germany) according to the manufacturer's instructions. All primers used to characterize the DNA control samples (Table 2) were purchased from Eurofins MWG Operon, Germany and all conventional PCRs were carried

Table 1
Primers and probes designed to identify *Sarcocystis* spp. from cattle by real time PCR.

Type	Name	Sequence 5′···3′ and modifications (in brackets)
Primer forward	SarcoRTF	TCTGCTGGAAGCAATCAGTC
Primer reverse	SarcoRTR	AGGCAATAAGCCTGCTTCAA
Primer reverse	ShirsutaRTR	GCAACAATAAGCCTGCTTCAAA
CDL Probe	Shirsuta	(FAM)-CCTTCTAATGAGGGTGTGTACTTGATGAA-(BHQ1)
CDL Probe	Scruzi	(RED)-ACCCATCTATATTGGGATAATACCGTTACT-(BHQ1)
LNA™ Probe	Shom-LNA	(Cy5)-TCT(+T)A(+A)TA(+T)AA(+T)GA(+T)TA(+T)TG(+A)A(+T)TGA-(BHQ2)
LNA™ Probe	Ssin-LNA	(HEX)-CTG(+A)TG(+A)CT(+T)TC(+A)GT(+A)GTCAT-(BHQ1)

Reference: CDL = conventional dual labeled; LNA = locked nucleic acids modification; bases marked as (+A) or (+T) indicates a LNA modification. BHQ = Black Hole Quencher. Fluorophores: Cy5 = Cyanine dye 5; FAM = 6-carboxyfluorescein; HEX = hexachloro-6-carboxyfluorescein; RED = Texas Red.

Table 2
Description of primers used for conventional PCR and sequencing of the 18S rRNA gene from *Sarcocystis* spp.

Primer	Orientation	Sequence (5′···3′)	Reference
ERIB1	Forward	ACCTGGTTGATCCTGCCAG	Barta et al. (1997)
Primer B	Reverse	GATCCTTCTGCAGGTTCACCTAC	Fenger et al. (1995)
S5	Forward	GTTCGATTCCGGAGAGGGAGC	Fischer and Odening (1998)
S3	Forward	TTGTTAAAGACGAACTACTGCG	Fischer and Odening (1998)
Primer 3H	Reverse	GGCAAATGCTTTCGCAGTAG	Yang et al. (2001b)
Primer 4H	Reverse	CAGAAACTTGAATGATCTATCG	Yang et al. (2001b)
SarcoFext	Forward	GGTGATTCATAGTAACCGAACG	Present study
SarcoRext	Reverse	GATTTCTCATAAGGTGCAGGAG	Moré et al. (2011) (as Sarco R)
SarcoFint	Forward	CGCAAATTACCCAATCCTGA	Moré et al. (2011) (as Sarco F)
SarcoRint	Reverse	ATCGTCTTCGAGCCCCTAAC	Present study

out with 1 μl of DNA sample in each reaction using a Personal Mastercycler (Eppendorf, Germany).

Fragments of the 18S rDNA with a size of around 900 bp were amplified using the primers SarcoFext and SarcoRext in a 25 μl final reaction volume with 1 unit of Taq DNA polymerase/reaction (Stratec molecular, Germany) under the following conditions (final concentrations): 20 μg/ml BSA, 1x reaction buffer supplied with the polymerase, 1.5 mM $MgCl_2$, 250 μM dNTPs, 0.5 μM of each primer and the following cycler program: 94 °C (4 min), 40 cycles of 94 °C (40 s), 59 °C (1 min), 72 °C (1 min) and a final extension at 72 °C (5 min). Five μl of each product were examined in 1.5% agarose gels stained with ethidium bromide. SarcoFext/SarcoRext amplicons were sequenced using a LI-COR DNA Sequencer 4200 (MWG Biotech, Germany) as previously described (Schares et al., 2002) employing 5′-labeled internal primers DY782-SarcoFint and DY682-SarcoRint. Sequences were analyzed with the GENEIOUS program (free available version 5.5.6)(Drummond et al., 2011) and obtained 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).

Once the identity of a *Sarcocystis* spp. was confirmed, primers ERIB1 and PrimerB were used to amplify the full-length sequence of the 18S rDNA (around 1850 bp). The amplifications were carried out using the concentrations described above, with the following cycler program: 94 °C (4 min), 40 cycles of 94 °C (40 s), 58 °C (1 min), 72 °C (2 min) and final extension of 72 °C (5 min). Amplification products were purified using the QIAQUICK purification kit (QIAGEN, Germany) according to the manufacturer's instructions using 20 μl as the elution volume. In order to obtain a high concentration of high quality control DNA to optimize real time PCR protocols, purified amplicons were cloned into plasmids with a TA Cloning® kit (Invitrogen, USA) using One Shot® TOP10 chemically competent *Escherichia coli* according to the manufacturer's instructions with 3 μl of purified product as the ligation template.

At least three white colonies for each transformation were grown overnight at 37 °C in 4 ml Luria Bertani

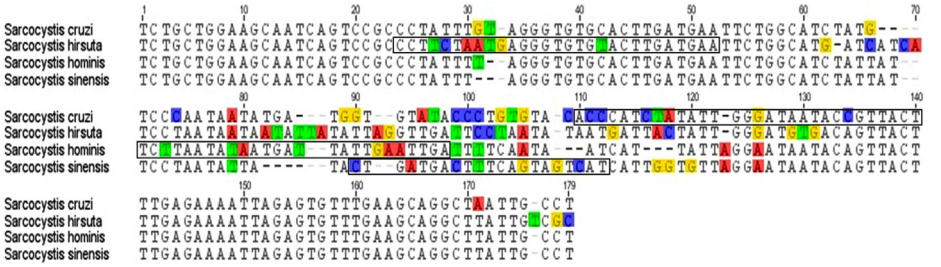

Fig. 1. Alignment of the real time PCR products and probe location for consensus sequences from *Sarcocystis* spp. affecting cattle. Gaps in the sequence alignment are indicate by "-". Colored bases indicate a sequence disagreement between different *Sarcocystis* spp. Probe locations are indicated by boxes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(LB) medium with Ampicillin, and DNA was extracted from 2 ml of LB with the kit Invisorb® Spin Plasmid Mini Two (Stratec Molecular GmbH, Germany), according to the manufacturer's instructions using 100 μl of elution buffer. Each plasmid DNA was amplified with the vector universal primers M13-F (5′-CAGGAAACAGCTATGACC-3′) and T7Prom-R (5′-TAATACGACTCACTATAGGG-3′) to control the size of the fragments cloned (size expected around 2000 bp) according the conditions described above with the following cycler program: 94 °C (4 min), 35 cycles of 94 °C (40 s), 53 °C (1 min), 72 °C (2 min) and a final extension of 72 °C (5 min).

Samples of 5 μl of each plasmid DNA preparation and 5 μl (5 pmol/μl) of each of the primers: M13-F, T7Prom-R, S3, S5, H3 and H4 (Table 2) were mixed in 1.5 ml tubes, labeled with commercial barcodes and submitted for sequencing to the Lightrun service of GATC Biotech (Sanger method), in order to re-confirm species identity. Sequences were aligned and assembled with the GENEIOUS program and the consensus sequences of the 18S rRNA full length gene deposited in the GenBank with the following accession numbers: *S. sinensis* (GenBank JX679466 and JX679469), *S. cruzi* (GenBank JX679467 and JX679468), *S. hominis* (GenBank JX679470 and JX679471) and *S. hirsuta* (GenBank JX855283).

Plasmid DNA concentration was determined by measuring a 1/10 dilution of the plasmid DNA preparation in 0.5% Tris-EDTA nuclease free buffer, pH 8.0 (TE) using the GeneQuant 1300 (Health Care Biosciences, Sweden). Plasmid DNA copy number was estimated by an online tool (Plasmid DNA copy calculator: http://www.uri.edu/research/gsc/resources/cndna.html).

2.1.3. Preparation of bradyzoite DNA and DNA from related apicomplexan parasites

Sarcocysts from samples with *Sarcocystis* spp. identified and confirmed by sequencing were purified as previously described (Moré et al., 2011). Up to 10 cysts for each bovine *Sarcocystis* sp. were digested and bradyzoites purified by Percoll® gradient centrifugation as previously described (Moré et al., 2008) with a volume scale modification. Briefly, sarcocysts were digested in 1.5 ml tubes containing 200 μl of digestion solution (Pepsin–HCl, 30 min, 37 °C) and then centrifuged at 500 × *g*, 5 min. The supernatant was removed, the pellet re-suspended in 200 μl PBS and 200 μl of isotonic Percoll® (Sigma–Aldrich, USA) and centrifuged (4000 × *g*, 10 min). Then the supernatant was discarded and the bradyzoite pellet washed 3 times with 200 μl PBS. Purified bradyzoites were counted in a Neubauer chamber and DNA was extracted as described for the individual cyst samples.

DNA from in vitro cultivated *Besnoitia besnoiti*, *Neospora caninum* and *Toxoplasma gondii* was extracted with the NucleoSpin®Tissue kit (Macherey–Nagel, Germany) (Schares et al., 2011). To obtain DNA from tissue cysts of *Sarcocystis aucheniae* (from *Lama glama*), *S. aucheniae*-like (from *Lama guanicoe*) and *Sarcocystis* sp. from *Cervus elaphus*, a DNeasy Tissue Kit (QIAGEN, Germany) was used according to the manufacturer's instructions. DNA from oocysts of *Hammondia hammondi, H. heydorni* and *Isospora* spp. was extracted as previously described (Schares et al., 2005). DNA samples from *Sarcocystis miescheriana* (from a pig) and *S. gigantea* (from a sheep) were kindly provided by Dr. C. Frey (Bern University, Switzerland) and Dr. A. Pantchev (Chemisches und Veterinäruntersuchungsamt, Stuttgart, Germany), respectively. The routine procedure of DNA extraction included a negative processing control.

2.1.4. Amplification procedure

Initially, different primer concentrations and temperature protocols were tested and optimized using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories GmbH, Germany) with specific plasmid DNA controls (reaction volume 20 μl) in a CFX384 instrument (Bio-Rad Laboratories GmbH, Germany) and further studied by melting curve analysis and by determining the size of the products by gel electrophoresis. Real time amplifications were analyzed using the CFX manager software version 2.1 (Bio-Rad Laboratories GmbH, Germany). Fluorescence emissions were measured automatically and expressed as relative fluorescence units (RFU).

Once a primer combination was selected, reactions with individual probes were tested using IQ™ Supermix (Bio-Rad Laboratories GmbH, Germany) in a reaction volume of 20 μl with different sample volumes (1, 2.5 and 5 μl). Samples that crossed the baseline after cycle 39 were considered as negative.

2.2. Optimization of a multiplex real time PCR assay

Probe concentrations were optimized using standard curves established with triplicates of 6 serial 10-fold dilutions of each plasmid control DNA, starting from 10 pg/reaction. Each probe was tested separately with all four plasmid DNA controls. For each standard curve the values of amplification efficiency (*E*), regression coefficient (R^2), slope (*S*) and *Y*-axis intersection (*Y*-Int) were estimated with the CFX manager software mentioned above.

After individual probe concentrations had been optimized, multiplex reactions were conducted combining the two conventional probes, the two LNA™ probes and all four probes (referred to as BovSarcoMultiplex real time PCR). Multiplex reactions were evaluated performing standard curves as described above, but for the BovSarcoMultiplex real time PCR, triplicates of 10-fold dilutions of premixed control plasmid DNA samples containing the same concentration of the 18S rDNA of *S. cruzi*, *S. hominis*, *S. sinensis* and *S. hirsuta*, starting from 2.5 pg of each plasmid DNA/reaction were used. All reactions were conducted with a negative control (0.5% TE buffer as sample), a specific positive control and a no-template control (NTC). The baseline (fluorescence cut-off) for each probe was estimated based on the best efficiency and optimal regression values for plasmid DNA standard curves.

Optimal cycling conditions were 95 °C (4 min) followed by 40 cycles of 95 °C (15 s), 62 °C (40 s). Optimized reaction mixtures for the BovSarcoMultiplex real time PCR were: total volume 20 μl, containing 2.5 μl of template DNA and 17.5 μl of mastermix (10 μl of IQ™ Supermix) with a final concentration of each primer and the following probes: SarcoRTF (600 nM), SarcoRTR (400 nM) and ShirsutaRTR

(300 nM), Shirsuta-Probe (200 nM), Scruzi-Probe (100 nM), Shom-LNA-Probe (200 nM) and Ssin-LNA-Probe (300 nM).

2.2.1. Analytical sensitivity and specificity

The analytical sensitivity of the BovSarcoMultiplex real time PCR was estimated by preparing 10-fold dilutions of plasmid DNA or DNA obtained from counted bradyzoites in 0.5% TE buffer. The limit of detection (LOD) was determined as the lowest dilution at which all replicates were detected as positives. For the plasmid DNA samples, triplicates of serial dilutions of premixed DNA samples containing the same concentration of the 18S rDNA of *S. cruzi*, *S. hominis*, *S. sinensis* and *S. hirsuta*, starting from 1.25 pg until 1.25 ag of each plasmid DNA/reaction were analyzed. After this experiment, combinations of control DNA were tested using 10-fold dilutions of three plasmid DNA samples at the same concentration (starting from 1.25 pg until 0.0125 fg of each plasmid/reaction) together with 10-fold dilutions of the forth plasmid DNA starting from 0.25 ng until 0.25 fg on each reaction.

Sensitivity based on counts for bradyzoites isolated from tissue cyst fragments was conducted using triplicates of serial dilutions starting on DNA resembling 100, 200 and 300 bradyzoites of each *Sarcocystis* sp., individually and in combination of all four *Sarcocystis* sp. at the same concentration.

Finally, the analytical specificity of the BovSarcoMultiplex real time PCR was estimated using 5 ng DNA of several *Sarcocystis* spp. and from other apicomplexan parasites. A conventional PCR was carried out with primers SarcoFext and SarcoRext, which amplify fragments of the 18 rDNA on Eucoccida parasites, using 2 ng DNA of the same samples as template, in order to confirm that enough parasite DNA was present (specially on samples from oocysts from faces) in each sample used to estimate the specificity of the multiplex real time PCR. All the samples used to estimate analytical sensitivity and specificity were also analyzed by gel electrophoresis to determine whether an amplification product of the expected size was present.

2.3. Examination of tissue samples by the BovSarcoMultiplex real time PCR

A total of 380 cattle loin DNA samples from Argentina were examined by the BovSarcoMultiplex real time PCR. Into each set of samples a positive plasmid control DNA mixture (consisting of 18S rDNA of *S. cruzi*, *S. hominis*, *S. sinensis*, *S. hirsuta*, each at the same concentration), a negative control and a NTC sample were included.

The loin samples had previously been analyzed in fresh condition by microscopical examination and after DNA extraction by PCR-RFLP (Moré et al., 2011). During the microscopical analysis thin and thick-walled cysts were detected and samples had been classified (but the results were not published) according to the observed number of sarcocysts per g of loin as follows: low positive (less than 5 cysts) or moderate-to-high positive (more than 5 cysts). Briefly, 10 g of each muscle were minced in a meat grinder adding 40 ml of PBS. The homogenate was filtered using a strainer with gauze, collected in a 50 ml tube, and centrifuged at 600 × *g* for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 20 ml of PBS and filled into a Petri dish for examination using a stereomicroscope (Moré et al., 2011). Sarcocysts detected in 5 ml of the homogenate (resembling 2 g of beef) were counted and the number of cysts/g was estimated.

Regarding thin-walled cysts 108 of the samples were negative, 209 low positive and 63 moderate-to-high positive. In case of thick-walled cysts, 292 of the samples were negative, 83 low positive and 5 moderate-to-high positive.

2.4. Statistical analysis

The diagnostic sensitivity and specificity were estimated relative to the previous results of sarcocysts detection by microscopical examination in fresh loin samples using Win Episcope 2.0. The analysis was conducted separately for the thin-walled and thick-walled *Sarcocystis* spp.

3. Results

3.1. Real time PCR assays development

A BLAST comparison of 18S rDNA sequences of *S. sinensis* and *S. hominis* (i.e. sequences including the region with highest variability and longer than 1000 bp) showed identity differences of around 3 and 5% between the two species. Among the sequences reported for *S. sinensis* slight differences were observed, but all *S. sinensis* DNA samples we identified by sequencing reacted with the probe selected for *S. sinensis*. Melting curves showed single peaks and primer dimers were detected neither in melting curves nor in gel electrophoresis.

No cross-reactions between the different *Sarcocystis* spp. affecting cattle covered by the newly developed probes were observed.

3.2. Establishment of a multiplex real time PCR protocol for differentiating Sarcocystis spp. in cattle

Standard curves obtained by the BovSarcoMultiplex real time PCR and the evaluation results are shown in Fig. 2A and B. For all probes the linear regression coefficients were equal or higher than 0.94 (Fig. 2B) using the following baseline values for the different probes: 9.34 RFU (*S. cruzi*), 26.5 RFU (*S. hirsuta*), 9.16 RFU (*S. sinensis*) and 12 RFU (*S. hominis*). In addition, a cut-off for the CFX manager software endpoint function was determined using the average of RFU on the last 5 cycles, as follows: 25 (*S. cruzi*), 40 (*S. hirsuta*) and 12 (*S. sinensis* and *S. hominis*).

The amplification efficiency of each PCR with a single probe was similar to the efficiency observed in the multiplex assay with the four probes. In detail the efficiencies were 99.8%, 100%, 102% and 97% in single reactions and 103.3%, 99.9%, 108.9% and 98% in the multiplex assay for *S. cruzi*, *S. hirsuta*, *S. sinensis* and *S. hominis*, respectively (Fig. 2B).

3.2.1. Analytical sensitivity and specificity

The analytical sensitivity or LOD was estimated as 0.0125 fg of plasmid DNA for the *S. cruzi* probe

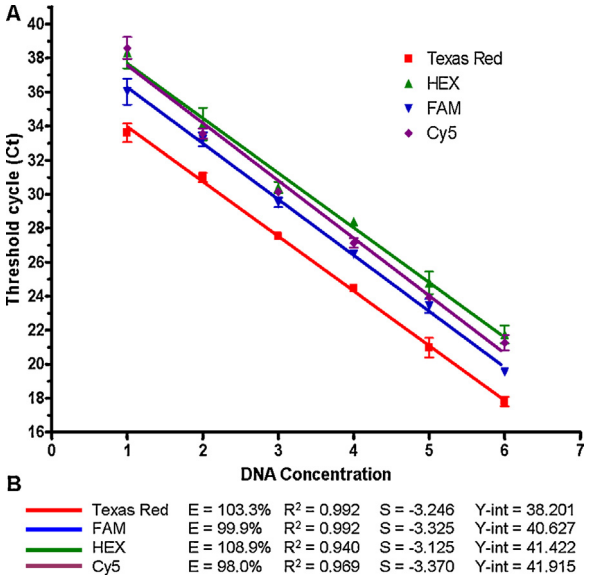

Fig. 2. Standard curves obtained with the BovSarcoMultiplex real time PCR.Standard curves for each *Sarcocystis* spp. probe included in the multiplex real time PCR (A). Amplification of triplicates of 10 fold dilutions of specific plasmid DNA control mixtures (*S. cruzi*, *S. hirsuta*, *S. sinensis* and *S. hominis*) containing the same concentrations of the individual plasmid DNA. Each plasmid DNA concentration/analysis: 1 = 0.025 fg; 2 = 0.25 fg; 3 = 2.5 fg; 4 = 25 fg; 5 = 0.25 pg and 6 = 2.5 pg. Note that the *S. cruzi* probe (red) showed the highest sensitivity. Amplification efficiency (*E*), regression coefficient (R^2), slope (*S*) and *Y*-axis intersection (*Y*-int) estimated for each specific probe as determined by the CFX manager software version 2.1 (B). Color indicate the reactions observed for individual *Sarcocystis* spp. probes: red = *S. cruzi*; blue = *S. hirsuta*; green = *S. sinensis* and violet = *S. hominis*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(corresponding to 2 plasmid DNA copies) and 0.125 fg of specific plasmid DNA samples for the other three probes (corresponding to 20 plasmid DNA copies). Regarding bradyzoite DNA samples, the LOD for the *S. cruzi* probe was the DNA resembling 0.1 bradyzoite and for the other three probes the LOD was the DNA resembling 0.3 bradyzoite. In all cases the *S. cruzi* probe showed the highest sensitivity (Fig. 2A).

When different concentrations of the four species-specific plasmid DNA samples were analyzed in combination, a high concentration (0.25 ng/reaction, equivalent to 4×10^8 DNA copies) of one of the plasmids caused a 10 fold sensitivity loss for the other 3 probes. In this case, the Ct value (i.e., the threshold cycle or the cycle of amplification where the sample crossed the baseline) for the highly concentrated plasmid control DNA was around 10–12.

Samples of DNA from other apicomplexan parasites and several *Sarcocystis* spp. showed products of around 800–900 bp when amplified with primers SarcoFext and SarcoRext using 2 ng DNA as a template (Fig. 3A). Five ng of the same DNA processed by the BovSarcoMultiplex real time PCR showed positive reactions just for the species that the specific probes targeted. However, after real time PCR amplification also products of other *Sarcocystis* spp. (*Sarcocystis* spp. from *Cervus elaphus*, *S. miescheriana*, *S. gigantea* and *S. aucheniae*-like) were detected by gel electrophoresis but not for the remaining cyst forming coccidian parasites (Fig. 3B).

3.3. Examination of bovine tissue samples from Argentina

Out of all loin samples from Argentinean cattle 82.4% (*n* = 313), 7.6% (*n* = 29), 3.7% (*n* = 14) and 0.5% (*n* = 2) were positive by the BovSarcoMultiplex real time PCR for *S. cruzi*,

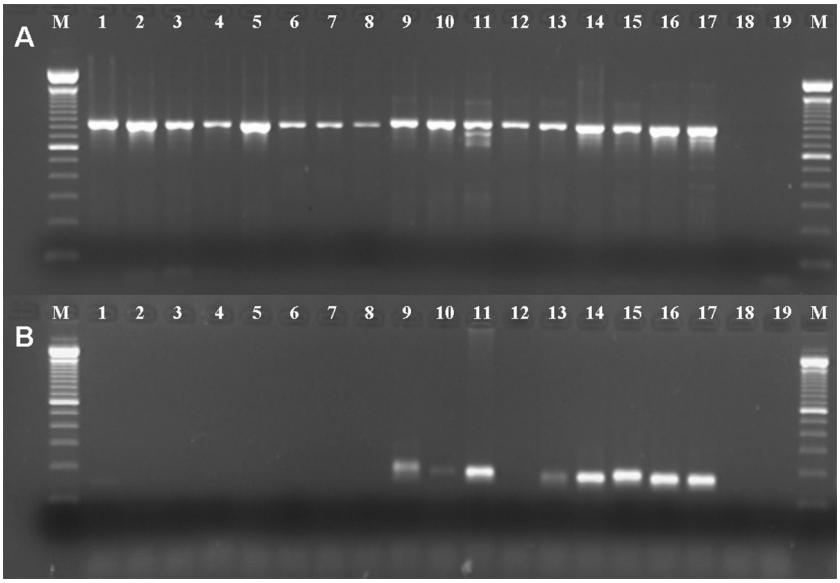

Fig. 3. Gel electrophoresis of samples used for testing the analytical specificity of the BovSarcoMultiplex real time PCR.
Each line contains the amplification by conventional PCR with SarcoFext and SarcoRext primers (A) and by the BovSarcoMultiplex real time PCR (B) from DNA of different apicomplexan parasites in a 2 ng/μl concentration as follows: 1 – *N. caninum*; 2 – *T. gondii*; 3 – *H. hammondi*; 4 – *H. heydorni*; 5 – *B. besnoiti*; 6 – *I. canis*; 7 – *I. rivolta*; 8 – *I. felis*; 9 – *Sarcocystis* spp. from *Cervus elaphus*; 10 – *S. miescheriana*; 11 – *S. gigantea*; 12 – *S. aucheniae*; 13 – *S. aucheniae* like; 14 – *S. cruzi*; 15 – *S. hirsuta*; 16 – *S. sinensis*; 17 – *S. hominis*; 18 – negative control and 19 – no template control (NTC). Ref: M = 100 bp marker.

Table 3
Comparison of the microscopic analysis for thin-walled *Sarcocystis* spp. cysts in loin samples from Argentina with the BovSarcoMultiplex real time PCR for *S. cruzi*.

Microscopy	PCR for *S. cruzi*		
Thin-walled cysts	Positives	Negatives	Total
Moderate-high positive	61	2	63
Low positive	188	21	209
Total positives	249	23	272
Total negatives	64	44	108
Total	313	67	380

Low positive = less than 5 cysts/g of beef observed at microscopy and moderate-to-high positive = more than 5 cysts/g of beef observed at microscopy.Note: Diagnostic sensitivity (91.5% (CI 95%: 88.2–94.8)) and diagnostic specificity (41.7% (CI 95%: 31.4–50.0)) were calculated using total numbers of positive and negative samples.

S. sinensis, *S. hirsuta* or *S. hominis*, respectively. All positive samples showed Ct values between 22 and 39. Out of all samples, mixed infections were detected in 8.9% ($n = 34$) as follows: 23 were positive for both *S. cruzi* and *S. sinensis*, nine for *S. cruzi* and *S. hirsuta*, one for *S. cruzi* and *S. hominis* and one was positive for *S. cruzi*, *S. sinensis* and *S. hirsuta*.

The comparison of microscopical results for thin-walled sarcocysts with those obtained with the BovSarcoMultiplex real time PCR for *S. cruzi* was characterized by a diagnostic sensitivity of 91.5% (95% CI: 88.2–94.8) and a diagnostic specificity of 41.7% (95% CI: 31.4–50.0) (Table 3). Out of the 63 and 209 loin samples classified as microscopically "moderate-to-high positive" and "low-positive" for *S. cruzi* respectively, 61 (97%) and 188 (89.9%) were also *S. cruzi* positive in the multiplex real time PCR, respectively. Comparison between the results of loin sample microscopy for thick-walled sarcocysts and those of the multiplex real time PCR for *S. sinensis*, *S. hominis* and *S. hirsuta* probes revealed a diagnostic sensitivity of 36.3% (IC 95%: 26.3–46.4) and a diagnostic specificity of 95.9% (IC 95%: 93.6–98.1) (Table 4).

4. Discussion

Until recently, *Sarcocystis* spp. infections in cattle were reported to be only caused by *S. cruzi*, *S. hirsuta* and *S. hominis* (Dubey et al., 1989; Moré et al., 2011; Odening, 1998). However, another species that is morphologically and genetically similar to *S. hominis* was detected and named *S. sinensis* a few years ago (Yang et al., 2001b). Experimental results suggested that *S. hominis* and *S. sinensis* are biologically different because only *S. hominis* but not *S. sinensis* uses humans as definitive hosts (Chen et al., 2011). Moreover, morphological and genetic differences between *S. hominis* and *S sinensis* have been described (Chen et al., 2011; Yang et al., 2001b). Considering that simultaneous infections with more than one *Sarcocystis* spp. may occur and that these mixed infections, especially mixed infections with thick-walled cysts (Dubey et al., 1989; Moré et al., 2011), are difficult to identify by morphological examination, DNA from individual sarcocysts or cysts portions was PCR amplified and sequenced. Furthermore the full-length 18S rDNA of the four *Sarcocystis* spp. known to affect cattle were cloned and sequenced to serve as controls in the present study. Sequences obtained in the present study and those deposited in GenBank suggest identity differences between *S. hominis* and *S. sinensis* of around 3–5%, further supporting the suggestion that they represent two different species (Chen et al., 2011; Yang et al., 2001b). Despite of this, many of the differences in the 18S rDNA sequences were concentrated in specific variable regions, allowing to differentiate between species when these were used as a target for molecular assays. The clustering of differences in particular regions was recently also demonstrated for *Sarcocystis* spp. from reindeer (Dahlgren and Gjerde, 2007). Utilizing only size differences of the amplified 18S rDNA fragments may result in misidentifications of *S. hominis* and *S. sinensis* (Domenis et al., 2011; Vangeel et al., 2007).

Many prevalence studies on *Sarcocystis* spp. in cattle around the world were performed using microscopy and molecular techniques to differentiate the species. The presence of thick-walled cyst was usually attributed to *S. hominis*. However, these methods were not suitable to take the presence of *S. sinensis* into account and to identify this parasite (Domenis et al., 2011; Jehle et al., 2009; Moré et al., 2011; Van Knapen et al., 1987; Vangeel et al., 2007; Vercruysse et al., 1989). Moreover, the sequence GenBank AF006470 reported as *S. hominis*, which has been used as a reference for several molecular studies, has a higher degree of similarity with the sequences reported for *S. sinensis* (Yang et al., 2001b) than with those for *S. hominis*. This is also confirmed by sequences for *S. sinensis* reported in the present study (GenBank JX679466 and JX679469). We showed that the newly developed BovSarcoMultiplex real time PCR can differentiate all known *Sarcocystis* spp. from cattle, including also *S. sinensis*. Moreover, the analytical specificity was high, showing no cross-reactions with other

Table 4
Comparison of the microscopic analysis for thick-walled *Sarcocystis* spp. cysts in loin samples from Argentina by microscopy and BovSarcoMultiplex real time PCR.

Microscopy	PCR for thick-walled *Sarcocystis* spp.						
Thick-walled cysts	*S. sinensis*	*S. hominis*	*S. hirsuta*	*S.sin + Shirs*	Total positives	Total negatives	Total
Moderate-high positive	4	0	0	0	5	1	5
Low positive	21	1	6	1	28	55	83
Total positives	25	1	6	0	32	56	88
Total negatives	4	1	7	0	12	280	292
Total	44	29	2	13	44	336	380

Ref. Low positive = less than 5 cysts/gram of beef observed at microscopy and moderate-to-high positive = more than 5 cysts/gram of beef observed at microscopy. *S.sin + Shirs*: mix infection *S. sinensis* and *S. hirsuta* detected. Note: Diagnostic sensitivity (36.3% (CI 95%: 26.3–46.4)) and diagnostic specificity (95.9% (CI 95%:93.6–98.1)) were calculated using total numbers of positive and negative samples.

apicomplexan parasites or with *Sarcocystis* spp. from other hosts. In addition, no cross-reactions between the different *Sarcocystis* spp. covered by the multiplex PCR were observed.

To optimize the multiplex real time PCR, protocols were used which had previously been suggested for the validation of molecular diagnostic techniques (Bustin, 2004; Conraths and Schares, 2006). The amplification efficiency and the regression values obtained for standard curves were excellent, possibly allowing the consideration of the future use of the BovSarcoMultiplex real time PCR as a quantitative technique (qPCR), which was not the aim of the present study.

Comparison of the plasmid DNA copy number and the number of bradyzoites based on counts after isolation from tissue cysts suggest that the copy number of the 18S rRNA gene in a single *Sarcocystis* spp. bradyzoite genome is around 20–60, i.e. slightly lower than the copy number of the 18S RNA gene reported for *T. gondii* (Guay et al., 1992).

The BovSarcoMultiplex real time PCR showed good analytical sensitivity and specificity, even in samples with a mix of specific DNA controls. However, the sensitivity was negatively affected when a high concentration of a single plasmid DNA (equivalent to 4×10^8 DNA copies and revealing Ct values around 10–12) was tested along with plasmid DNA samples of other species at lower concentrations. Yet, samples with such high Ct values do not seem to occurring often in the field as all positive tissue samples showed Ct values higher than 22 and thus were unlikely to be able to interfere with the detection of a lower concentration of other *Sarcocystis* spp. Moreover, other *Sarcocystis* spp. from other hosts produced products with the real time PCR primers, but its presence in beef samples and in a high concentration to affect the sensitivity and efficiency of the BovSarcoMultiplex real time PCR appears to be extremely rare.

The comparison of results obtained for *S. cruzi* by microscopy and multiplex PCR for the loin samples from Argentina showed a good diagnostic sensitivity (91.5%) and a low diagnostic specificity (41.7%). In this case, 64 samples were positive by real time PCR but negative by loin sample microscopy. Considering that almost all animals had been shown to be *S. cruzi* infected by heart samples (Moré et al., 2011), the apparent "false positive" status of these 64 samples with the BovSarcoMultiplex real time PCR could be related to a higher analytical sensitivity of the molecular method, from which the *S. cruzi* probe showed the lowest LOD. The multiplex real time PCR may have detected small cysts, meronts or sarcocysts which may have been over looked by microscopy or destroyed during the homogenization performed prior to microscopy. Nevertheless, the diagnostic sensitivity and specificity of the BovSarcoMultiplex real time PCR were higher than reported previously for a conventional PCR-RFLP using the same DNA samples (Moré et al., 2011). Mixed infections were detected in 8.9% of all samples, and in all cases *S. cruzi* was present, confirming the utility of the multiplex PCR to detect mixed infections and the previous results about the high prevalence of *S. cruzi* (Moré et al., 2011). These results also indicate that *S. cruzi* could be the cause of potential sequencing mistakes or interference when amplification from beef samples DNA is carried out with techniques targeting *Sarcocystis* spp. sequences.

On the other hand, a low diagnostic sensitivity (36.3%) and a good diagnostic specificity (95.9%) were observed when the microscopical detection of thick-walled sarcocysts and the multiplex real time PCR targeting *S. sinensis*, *S. hirsuta* and *S. hominis* were compared. Predominantly samples reported as low-positive by microscopy (i.e. less than 5 thick-walled sarcocysts/gram of tissue) were negative for thick-walled *Sarcocystis* spp. in the BovSarcoMultiplex real time PCR. The low sensitivity can be explained by the fact that the analytical sensitivity for the probes specific for thick-walled *Sarcocystis* spp. was lower than the one for *S. cruzi*, requiring more DNA to reach a positive status; Moreover, the sample volume used for the DNA extraction for these loin samples (50 mg) probably was not large enough to obtain always detectable parasite DNA if the parasite burden was low (Moré et al., 2011).

Moreover, diagnostic sensitivity and specificity were estimated by using microscopical sarcocysts detection as the "reference standard" since the observation of sarcocysts directly confirms infection; however the sensitivity of this test requires at least 1–2 cyst/g and does not allow species identification among *Sarcocystis* spp. with thick-walled cysts or the detection of acute infection (Dubey et al., 1989; Moré et al., 2011).

The higher analytical sensitivity of the multiplex BovSarcoMultiplex real time PCR (0.1–0.3 bradyzoites) in combination with concentrating parasites from several grams of tissue, e.g. by artificial digestion and sedimentation of cysts/bradyzoites, may allow to further increase the specific and sensitive detection of *Sarcocystis* spp. in meat samples (Dubey et al., 1989).

Although a DNA extraction method with potentially low sensitivity was used for the Argentinean loin samples, the BovSarcoMultiplex real time PCR results suggest that *S. sinensis* is the most prevalent species among the thick-walled sarcocysts in Argentinean beef. As discussed above, many samples from Argentina identified previously as *S. hominis* infected turned now out as infected by *S. sinensis*. The prevalence detected suggest that definitive hosts of *S. sinensis* should be carnivores or omnivores that occur in high abundance, at least in Argentina and China, and feed regularly on beef. Further studies are needed to identify the definitive hosts of *S. sinensis*. In this respect the BovSarcoMultiplex real time PCR may be useful for screening fecal samples of carnivores and omnivores to search for potential definitive hosts. Moreover, 24 of 29 loin samples detected as positive for *S. sinensis* were also positive for *S. cruzi* suggesting that risk factors favoring the infection with one of these protozoans may also be advantageous for the other one. In agreement with many other reports around the world on the prevalence of *Sarcocystis* spp. in cattle, *S. cruzi* was detected in a large proportion of the samples from Argentina, indicating frequent contact and efficient transmission of the parasite between canids and cattle in Argentina (Böttner et al., 1987; Dubey et al., 1989; Fukuyo et al., 2002; Moré et al.,2008, 2011; Odening et al., 1995). On the other hand, *S. hominis* was detected only in 2 samples of 380, indicating that this parasite is significantly less often detected in our study than previously

reported (Domenis et al., 2011; Dubey et al., 1989; Jehle et al., 2009; Moré et al., 2011; Vangeel et al., 2007). In many regions, further studies are necessary, especially performing sequencing, multiplex real time PCR and experimental infections in order to confirm that the reported *S. hominis* prevalences are truly attributable to this parasite and not to *S. sinensis*. Moreover, the method developed in the present study could also be useful to identify and differentiate *Sarcocystis* spp. cysts detected by microscopical examinations of cattle tissues as required for the importation of beef into the European Union.

As a conclusion, a multiplex real time PCR method was developed which allows the differentiation of *Sarcocystis* spp. affecting cattle in single and mixed infections with high analytical sensitivity and specificity. Examination of field samples showed that *S. sinensis* is the most prevalent species among thick-walled *Sarcocystis* spp. in Argentinean cattle. However, to increase the diagnostic sensitivity in samples with a low parasite burden of thick-walled cysts, a combination of this new tool with an improved DNA extraction method should be used.

Competing interests

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2013.04.024.

References

Barta, J.R., Martin, D.S., Liberator, P.A., Dashkevicz, M., Anderson, J.W., Feighner, S.D., Elbrecht, A., Perkins-Barrow, A., Jenkins, M.C., Danforth, H.D., et al., 1997. Phylogenetic relationships among eight Eimeria species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. J. Parasitol. 83, 262–271.

Böttner, A., Charleston, W.A., Pomroy, W.E., Rommel, M., 1987. The prevalence and identity of *Sarcocystis* in beef cattle in New Zealand. Vet. Parasitol. 24, 157–168.

Bustin, S.A., 2004. A–Z of quantitative PCR. La Jolla, CA, USA, p. 882.

Chen, X., Zuo, Y., Rosenthal, B.M., He, Y., Cui, L., Yang, Z., 2011. Sarcocystis sinensis is an ultrastructurally distinct parasite of water buffalo that can cause foodborne illness but cannot complete its life-cycle in human beings. Vet. Parasitol. 178, 35–39.

Conraths, F.J., Schares, G., 2006. Validation of molecular–diagnostic techniques in the parasitological laboratory. Vet. Parasitol. 136, 91–98.

Dahlgren, S.S., Gjerde, B., 2007. Genetic characterisation of six *Sarcocystis* species from reindeer (*Rangifer tarandus tarandus*) in Norway based on the small subunit rRNA gene. Vet. Parasitol. 146, 204–213.

Domenis, L., Peletto, S., Sacchi, L., Clementi, E., Genchi, M., Felisari, L., Felisari, C., Mo, P., Modesto, P., Zuccon, F., Campanella, C., Maurella, C., Guidetti, C., Acutis, P.L., 2011. Detection of a morphogenetically novel *Sarcocystis hominis*-like in the context of a prevalence study in semi-intensively bred cattle in Italy. Parasitol. Res. 109, 1677–1687.

Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Heled, J., Kearse, M., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T., Wilson, A., 2011. Geneious v5.5. Available from: http://www.geneious.com/

Dubey, J.P., Speer, C.A., Fayer, R., 1989. Sarcocystosis of Animals and Man. CRC Press, Boca Raton, FL, 215 pp.

Fayer, R., 2004. Sarcocystis spp. in human infections. Clin. Microbiol. Rev. 17, 894–902.

Fenger, C.K., Granstrom, D.E., Langemeier, J.L., Stamper, S., Donahue, J.M., Patterson, J.S., Gajadhar, A.A., Marteniuk, J.V., Xiaomin, Z., Dubey, J.P., 1995. Identification of opossums (*Didelphis virginiana*) as the putative definitive host of *Sarcocystis neurona*. J. Parasitol. 81, 916–919.

Fischer, S., Odening, K., 1998. Characterization of bovine Sarcocystis species by analysis of their 18S ribosomal DNA sequences. J. Parasitol. 84, 50–54.

Fukuyo, M., Battsetseg, G., Byambaa, B., 2002. Prevalence of Sarcocystis infection in meat-producing animals in Mongolia. Southeast Asian. J. Trop. Med. Public Health 33, 490–495.

Guay, J.M., Huot, A., Gagnon, S., Tremblay, A., Levesque, R.C., 1992. Physical and genetic mapping of cloned ribosomal DNA from *Toxoplasma gondii*: primary and secondary structure of the 5S gene. Gene 114, 165–171.

Jehle, C., Dinkel, A., Sander, A., Morent, M., Romig, T., Luc, P.V., De, T.V., Thai, V.V., Mackenstedt, U., 2009. Diagnosis of *Sarcocystis* spp. in cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*) in Northern Vietnam. Vet. Parasitol. 166, 314–320.

Moré, G., Abrahamovich, P., Jurado, S., Bacigalupe, D., Marin, J.C., Rambeaud, M., Venturini, L., Venturini, M.C., 2011. Prevalence of Sarcocystis spp. in Argentinean cattle. Vet. Parasitol. 177, 162–165.

Moré, G., Basso, W., Bacigalupe, D., Venturini, M.C., Venturini, L., 2008. Diagnosis of *Sarcocystis cruzi*, *Neospora caninum*, and *Toxoplasma gondii* infections in cattle. Parasitol. Res. 102, 671–675.

Mouritzen, P., Nielsen, A.T., Pfundheller, H.M., Choleva, Y., Kongsbak, L., Moller, S., 2003. Single nucleotide polymorphism genotyping using locked nucleic acid (LNA). Expert Rev. Mol. Diagn. 3, 27–38.

Odening, K., 1998. The present state of species-systematics in *Sarcocystis* Lankester, 1882 (Protista, Sporozoa, Coccidia). Systematic Parasitology 41, 209–233.

Odening, K., Wesemeier, H.H., Walter, G., Bockhardt, I., 1995. On the morphological diagnostics and host specificity of the *Sarcocystis* species of some domesticated and wild bovini (cattle, banteng and bison). Appl. Parasitol. 36, 161–178.

Schares, G., Heydorn, A.O., Cuppers, A., Mehlhorn, H., Geue, L., Peters, M., Conraths, F.J., 2002. In contrast to dogs, red foxes (*Vulpes vulpes*) did not shed *Neospora caninum* upon feeding of intermediate host tissues. Parasitol. Res. 88, 44–52.

Schares, G., Maksimov, A., Basso, W., Moré, G., Dubey, J.P., Rosenthal, B., Majzoub, M., Rostaher, A., Selmair, J., Langenmayer, M.C., Scharr, J.C., Conraths, F.J., Gollnick, N.S., 2011. Quantitative real time polymerase chain reaction assays for the sensitive detection of *Besnoitia besnoiti* infection in cattle. Vet. Parasitol. 178, 208–216.

Schares, G., Pantchev, N., Barutzki, D., Heydorn, A.O., Bauer, C., Conraths, F.J., 2005. Oocysts of *Neospora caninum*, *Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. Int. J. Parasitol. 35, 1525–1537.

Tenter, A.M., 1995. Current reseach on *Sarcocystis* species of domestic animals. Int. J. Parasitol. 25, 1311–1330.

Ugozzoli, L.A., Latorra, D., Puckett, R., Arar, K., Hamby, K., 2004. Real-time genotyping with oligonucleotide probes containing locked nucleic acids. Anal. Biochem. 324, 143–152.

Van Knapen, F., Bouwman, D., Greve, E., 1987. Study on the incidence of Sarcocystis spp in Dutch cattle using various methods (in Dutch). Tijdschrift voor Diergeneeskunde 112, 1095–1100.

Vangeel, L., Houf, K., Chiers, K., Vercruysse, J., D'Herde, K., Ducatelle, R., 2007. Molecular-based identification of *Sarcocystis hominis* in Belgian minced beef. J. Food Prot. 70, 1523–1526.

Vercruysse, J., Fransen, J., van Goubergen, M., 1989. The prevalence and identity of *Sarcocystis* cysts in cattle in Belgium. Zentralbl Veterinarmed B 36, 148–153.

Xiang, Z, Chen, X, Yang, L, He, Y, Jiang, R, Rosenthal, B.M, Luan, P, Attwood, S.W, Zuo, Y, Zhang, Y.P, Yang, Z, 2009. Non-invasive methods for

identifying oocysts of Sarcocystis spp. from definitive hosts. Parasitol. Int. 58, 206–209.

Yang, Z.Q., Zuo, Y.X., Ding, B., Chen, X.W., Luo, J., Zhang, Y.P., 2001a. Identification of *Sarcocystis hominis*-like (Protozoa: Sarcocystidae) cyst in water buffalo (*Bubalus bubalis*) based on 18S rRNA gene sequences. J. Parasitol. 87, 934–937.

Yang, Z.Q., Zuo, Y.X., Yao, Y.G., Chen, X.W., Yang, G.C., Zhang, Y.P., 2001b. Analysis of the 18S rRNA genes of *Sarcocystis* species suggests that the morphologically similar organisms from cattle and water buffalo should be considered the same species. Mol. Biochem. Parasitol. 115, 283–288.