The Journal of Veterinary Medical Science

FULL PAPER

Parasitology

Development of a new quantification method of *Sarcocystis cruzi* through detection of the acetyl-CoA synthetase gene

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ABSTRACT. *Sarcocystis cruzi* is a member of the genus *Sarcocystis*, infecting bovine animals such as cattle and bison as intermediate hosts, and canids such as dogs and raccoon dogs as definitive hosts. Acute sarcocystosis of *S. cruzi* causes occasional symptoms in cattle, including weight loss, reduced milk production, abortions, and death, and similar to other *Sarcocystis* species can potentially cause food poisoning in humans when raw or undercooked infected cattle meat is consumed. Despite these issues, genetic information on *S. cruzi* is scarce, and there is no specific quantitative method for the detection and quantification of the parasite in infected cattle. In this study, we aimed to develop a method based on high-throughput sequencing of *S. cruzi* genome and transcriptome that specifically and quantitatively detects the *S. cruzi* acetyl-CoA synthetase gene (*ScACS*). Cardiac muscles were collected from slaughterhouses in Saitama Prefecture to obtain sarcocysts from which DNA and RNA were extracted for the high-throughput sequencing. Using the sequences, we developed a specific quantitative PCR assay which could distinguish *S. cruzi* ACS from that of *Toxoplasma gondii* by taking advantage of the differences in their exon/intron organizations and validated the assay with the microscopic counting of the *S. cruzi* bradyzoites. Thus, this assay will be useful for future studies of *S. cruzi* pathogenesis in cattle and for the surveillance of infected animals, thereby easing public health concerns.

KEYWORDS: acetyl-CoA synthetase, bradyzoite, quantitative real-time PCR, *Sarcocystis cruzi*

J. Vet. Med. Sci.
85(1): 105–110, 2023
doi: 10.1292/jvms.22-0481

Received: 24 October 2022
Accepted: 21 November 2022
Advanced Epub:
1 December 2022

Members of the genus *Sarcocystis,* classified in the family Sarcocystidiae of the phylum Apicomplexa, are protozoan parasites that have been reported to infect various vertebrates, including mammals, birds, amphibians, and fish [8, 11]. *Sarcocystis* species require intermediate and definitive hosts to complete their life cycles. In intermediate hosts, *Sarcocystis* parasites propagate asexually in cysts formed in the muscle and nerve tissues of infected animals, whereas in definitive hosts, they reproduce sexually in the small intestine, releasing oocysts and sporocysts in the host's feces [8]. More than 200 species of *Sarcocystis* have been described, but the hosts and exact life cycles are known for only some species [8]. Most *Sarcocystis* species have specific animals as their intermediate hosts, except for *S. neurona* and *S. canis,* which infect multiple animals as their intermediate hosts [8].

Sarcocystis cruzi infects bovine animals such as cattle and bison as intermediate hosts, whereas canine animals such as dogs, foxes, and raccoon dogs as definitive hosts [5, 7, 8, 28]. Notably, *S. cruzi* is the most prevalent parasitic species in cattle worldwide, with infection rates as high as 64.0% to 82.4% [4, 15, 23, 29]. Although the majority of *S. cruzi* infections in cattle are asymptomatic, the parasite occasionally causes acute sarcocystosis with a variety of symptoms, including weight loss, muscle cramps, decreased milk production, abortion, neurological symptoms, and death, depending on the number of sporocysts ingested [8]. In addition, *Sarcocystis* infections cause also chronic sarcocystosis with symptoms of bovine eosinophilic myositis (BEM) and eosinophilic myocarditis in cattle, which are often identified in slaughterhouses [10, 13, 32, 33].

Apart from affecting the health of infected cattle, *S. cruzi* is a potentially important cause of food poisoning. For instance, *Sarcocystis*-associated food poisoning cases have been reported in Japan following the consumption of raw *Sarcocystis*-infected Shika-deer meat

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[2, 3, 16, 25, 34]. In addition, the rabbit ileal loop test, a standard method for the measurement of enterotoxicity, has been reported to demonstrate the enterotoxicity of *S. cruzi* cyst extracts [30]. Moreover, a 15-kDa potential enterotoxin isolated from *S. fayeri* cysts [17, 18] was found to antigenically cross-react with homologues present in *S. cruzi* and other *Sarcocystis* spp. [2, 14, 20, 22, 34]. These evidence suggest the potential importance of *S. cruzi* as a cause of food poisoning following the consumption of raw or undercooked cyst-containing meat from parasite-infected cattle.

In this study, we developed a specific quantitative assay using quantitative PCR with primers based on the *S. cruzi* acetyl-CoA synthetase gene sequence (*ScACS*) obtained via high-throughput sequencing of mRNA and genomic DNA isolated from *S. cruzi* cysts. The assay was shown to be useful in the detection and quantification of parasites in cysts of affected cattle and can facilitate future research on the pathogenesis of parasitic infections in cattle and enable the surveillance of *S. cruzi*-infected cattle, easing public health concerns.

MATERIALS AND METHODS

Cyst collection and morphological identification of S. cruzi

Cardiac muscles were collected from slaughtered cattle at slaughterhouses in Saitama Prefecture. Each muscle was cut into approximately 2 cm (length) × 5 cm (width) × 0.5 cm (thickness) sections. Sarcocysts were picked up using a needle under a stereomicroscope, and morphological observations were made under an optical microscope. Samples of 30–50 cysts with septa within each cyst, thin cyst walls (<1 µm thickness), and hair-like villar protrusions were collected in 1.5 mL tubes containing 200 µL sterile phosphate buffered saline (PBS) (TAKARA Bio Inc., Kusatsu, Japan). The samples were then centrifuged at 400 × *g*, washed three times with sterile PBS, and stored at 4°C until nucleic acid extraction.

DNA extraction and identification using mitochondrial cytochrome c oxidase (cox1)

DNA was extracted from 30 collected cysts using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's protocol for cultured cells. The primers used were SF1 (5′-ATGGCGTACAACAATCATAAAGAA-3′) and SR9 (5′-ATATCCATACCRCCATTGCCCAT-3′) [12]. Reaction solutions were prepared using 0.2 µL TaKaRa Ex Taq (Takara Bio Inc.), 2.5 µL 10x Ex Taq Buffer, 2 µL dNTP mixture (2.5 mM each), 1 µL of each primer (final concentration 0.8 µM), and 2.5 µL DNA template. PCR was performed for 40 cycles of amplification (94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min). PCR products were electrophoresed, and the expected amplified product was confirmed by the size of the observed band (approximately 1,100 bp). Direct sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific K. K., Tokyo, Japan), according to the manufacturer's instructions, using 3.3 pM of each primer and 1.5 µL of amplified DNA. Samples were sequenced using an Applied Biosystems 3500 (Thermo Fisher Scientific K. K.), and after sequencing, the reaction products were purified using a BigDye XTerminator Purification Kit (Thermo Fisher Scientific K. K.). The obtained gene sequences were analyzed using the CLC Genomic Workbench 12.0.3 (CLC bio., Aarhus, Denmark) and the blastn suite (https:// blast.ncbi.nlm.nih.gov/).

mRNA extraction, library preparation, and sequencing

Cysts were digested with pepsin to collect mature bradyzoites. Briefly, 500 µL digestion solution [1% HCl, 0.25% pepsin (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan)] was added to 50 cysts of *S. cruzi* and digested in a heat block at 37°C for 15 min with mixing every 5 min, followed by centrifugation at 400 × *g* at 4°C for 10 min. The pellet was washed three times with sterile PBS and suspended in 100 µL sterile PBS. Bradyzoites were counted on a cell-counting plate and stored at −30°C until RNA extraction.

For the extraction of total RNA from bradyzoites, the QIA shredder (QIAGEN) and RNeasy Mini Kit (QIAGEN) were used according to the manufacturer's instructions. Subsequently, the NEBNext Poly (A) mRNA Magnetic Isolation Module (New England BioLabs Inc., Rawley, MA, USA) was used to purify mRNA from total RNA samples. Libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs). Sequencing was performed on a MiSeq benchtop sequencer (Illumina, Inc., San Diego, CA, USA) using the MiSeq v3 Reagent Kit (150 cycles) (Illumina, Inc.).

De novo assembly and homology search

Each read was obtained as a FASTAQ format file using the MiSeq reporter and analyzed using the CLC Genomic Workbench 12.0.3 software (CLC bio.). For each read, a command was executed to trim low-quality sequences, with a limit of 0.05. Contigs were created from trimmed reads with a length fraction of 80%, similarity of 80%, and minimum contig length of 500.

Sequences of contigs generated by RNAseq were searched by tblastx (Bioinformatics Resources Center's Eukaryotic Pathogen, Vector & Host Informatics Resources (VeuPathDB): https://veupathdb.org/veupathdb/app/), using the dataset. The following reference datasets were used: *Cystoisospora suis* strain Wien I (accession no. PRJNA341953) [26], *Hammondia hammondi* strain H.H.34 (accession no. PRJNA80807), *Neospora caninum* Liverpool (source version: GCA_000208865.2, February 27, 2015, n/a) [27], *Sarcocystis neurona* SN3 (source version: GCA_000727475.1, April 13, 2015, n/a), and *Toxoplasma gondii* ME49 (strain ATCC 50611; source version: GCF_000006565.2, March 22, 2015, n/a). The RNA sequence corresponding to acetyl-CoA synthetase (ACS) from *S. cruzi* (*ScACS*) was deposited in GenBank under accession number ICSQ01000001.

Determination of genomic ScACS sequence

Nucleic acids from *S. cruzi* bradyzoites were extracted using the GeneLEAD VIII system (Precision System Science Co., Ltd., Chiba, Japan). Bradyzoites (3.7×10^6) were added to 200 µL Prep Buffer A to apply the gene LEAD VIII, and nucleic acids were eluted in

50 µL buffer. PCR was performed in 50 µL reactions containing 25 µL 2x PCR buffer for KOD FX Neo, 10 µL 2 mM dNTPs, 0.3 µM of each primer, and 1 U of KOD FX Neo DNA polymerase (ToYoBo, Osaka, Japan). The primers were designed based on the *ScACS* coding sequence described above. The primer sequences were as follows: *ScACS*-F, 5′-ATGGACCCGGAGCCGATAAAAGCCGA-3′ (the putative initiation codon is underlined), *ScACS*-R, 5′-CTAAGTGTCCCTTGTATTGATGGCGCT-3′ (the putative terminator codon is underlined). The PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification (98°C for 10 sec and 68°C for 5 min). An amplified fragment of approximately 7 kb was used for DNA library preparation for high-throughput sequencing. A DNA library was generated using the Nextera XT DNA Sample Preparation Kit (New England BioLabs) according to the manufacturer's instructions. Sequencing was performed on a MiSeq benchtop sequencer (Illumina, Inc.) using the MiSeq v3 Reagent Kit (150 cycles). The obtained contig sequence was aligned to the RNA sequence of *ScACS*, and exons and introns were determined. The genomic *ScACS* sequence was deposited in GenBank under the accession number LC729541.

Quantification of S. cruzi genomic DNA extracted from cysts using real-time PCR

Primers and probes were designed based on the mRNA sequence of *ScACS* in the region of exon 1; a PCR product would not be amplified when *T. gondii* genomic DNA is used as template owing to the presence of the intron in the *T. gondii* (KR013277) genome. Primers and probes used were as follows: primer F, 3′-TTCCTGAACGGAAAACTCAATGTGTG-5′, primer R, 5′-TTGCAAACTGACAGACTCTTTTGAG-3′, and probe, 5′-56-FAM/GTACCCCGA/ZEN/CGCTGTGGCAC/3 IABkFQ/-3′.

Twelve *S. cruzi* cyst samples, each comprising 20–50 cysts, from 11 bovine hearts were collected under a stereomicroscope and examined to identify *S. cruzi* morphology. Bradyzoites collected from cysts in each specimen were counted on a cell-counting plate, and specimens were stored at −30°C. Real-time PCR was performed and validated using DNA extracted from 12 cryopreserved *S. cruzi* bradyzoites and a DNA sample extracted from *T. gondii* ME49 tachyzoites. To construct a copy number standard curve, *S. cruzi* genomic DNA was extracted from bradyzoites, and PCR was performed using 25 µL Taqman Environmental Master Mix 2.0 (Life Technologies Japan Ltd., Tokyo, Japan) according to the manufacturer's instructions. The reaction solution was adjusted to a final primer concentration of 0.5 pmol and final probe concentration of 0.2 pmol; subsequently, 2 µL template DNA was added, and real-time PCR was performed with 40 cycles of amplification (94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min) using the Quant Studio 5 system (Thermo Fisher Scientific K. K.). After confirming the linearity of the standards, the copy number per µL of each sample was calculated and compared with the number of bradyzoites per µL. All samples were quantified twice, and the average values were used.

Statistical analysis

Correlation value was used to compare microscopic counts of bradyzoites and real-time PCR copy number measurements of DNA extracted from bradyzoites. All statistical analyses were performed using the Excel 2016 software (Microsoft Corp., Redmond, WA, USA).

RESULTS

Species identification of S. cruzi via morphological observation of cysts and detection of the mitochondrial cytochrome c oxidase (cox1) gene

We identified *S. cruzi* in all cysts collected from bovine hearts by microscopically observing the unique characteristics of *S. cruzi*, including septa in the cyst, hair-like villar protrusions on the cyst wall, and a relatively thin cyst wall (<1 µm) (Fig. 1). In addition to microscopic identification, we PCR-amplified and sequenced the mitochondrial cytochrome c oxidase (*cox1*) gene to confirm *S. cruzi* identification in all samples.

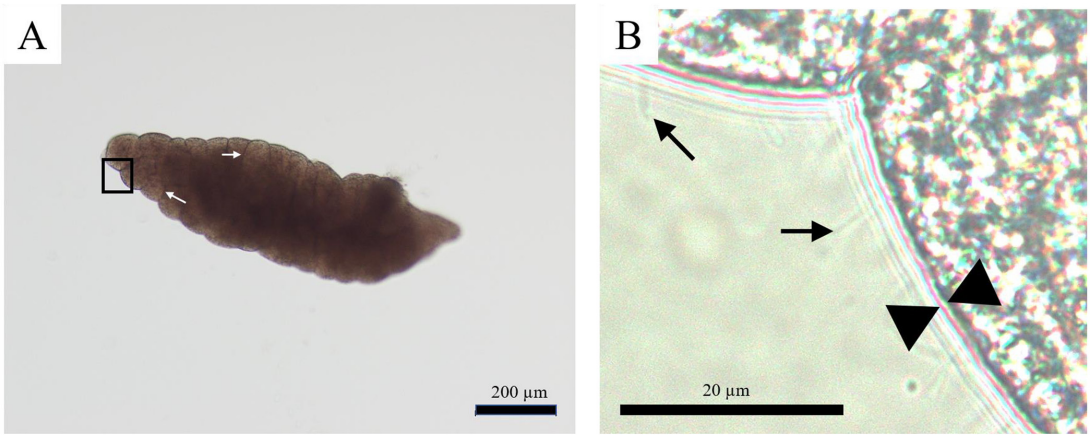

Fig. 1. A cyst of *Sarcocystis cruzi* under an optical microscope. **A**: Overview of an *S. cruzi* cyst (×100). White arrows indicate the septum in the cyst (bar=200 µm). **B**: The wall of an *S. cruzi* cyst (×1,000). The cyst wall was thin (<1 µm, black arrowheads). Black arrow indicates the hair-like protrusions in the cyst (bar=20 µm).

mRNA and genomic sequence of ScACS

We detected that a 3,859 bp contig sequence obtained from the RNAseq of *S. cruzi* bradyzoites was highly similar to the ACS of the *S. neurona* SN3 reference strain (SN3_00700215) based on blastn analysis on the ToxoDB website (https://toxodb.org/toxo/app). In particular, we found that the length of the putative coding sequence (CDS) of *ScACS* was 2,175 bp. The CDS of *ScACS* shared 80% and 69% sequence identity with the *ACS* of *S. neurona* and *ACS* of the *T. gondii* ME49 reference strain (TGME49_266640), respectively. Both *ACS* genes from *S. neurona* and *T. gondii* were registered as single-copy genes in each genome on ToxoDB.

We amplified the *ScACS* genomic fragments from *S. cruzi* bradyzoite genomic DNA using PCR with a primer pair containing putative initiation and termination codons. The length of the amplified fragment was 6,970 bp, and exons were determined by comparing the mRNA and genomic sequences of *ScACS*. We found that the exon/intron composition of *ScACS* was similar to that of *S. neurona ACS* (Fig. 2). In contrast, the exon/intron structure of *ScACS* was different from that of *T. gondii ACS* (Fig. 2).

Development and validation of a real-time PCR assay for quantification of S. cruzi

To obtain a genetic assay for the quantification of *S. cruzi*, we developed a quantitative PCR system based on the sequence of *ScACS*. We designed the primer pair and probe such that the forward primer was complementary to the *ScACS* sequence that corresponds to the exon–intron border in *T. gondii ACS* (Fig. 3) to avoid PCR amplification when the *T. gondii* genome is used as template. To validate the assay, we compared the detection results of 12 cyst samples obtained from separate cows with the bradyzoite numbers obtained via microscopic counting (Fig. 4) and found that they demonstrated strong correlations based on Pearson's regression analysis (R^2=0.92). To confirm the specificity of the assay in terms of distinguishing between *ScACS* and *T. gondii ACS,* we performed the assay using *T. gondii* genomic DNA. Accordingly, we observed that no signal was detected when we used 1.3×10^2–1.3×10^4 copies of *T. gondii* DNA (data not shown), whereas the assay detected up to 10 copies of *S. cruzi* genomic DNA (Fig. 4).

DISCUSSION

Prior to this study, only a quantitative PCR assay for *S. cruzi* using the 18S ribosomal gene has been reported [23]. However, neither that assay nor the assay using the *cox1* gene to identify *Sarcocystis* species [1, 12] are suitable for the quantification of this parasitic species because *S. cruzi* is known to contain multiple copies of these genes, which might vary depending on the parasite strain [6, 24]. In contrast, *ACS* is known to be a single-copy gene in the genomes of *T. gondii* [9, 31], and the genomic sequences adjacent to *ScACS,* which were obtained via high-throughput sequencing (data not shown), suggested that *ScACS* was also a single-copy gene, making this gene suitable for qPCR-based quantification of the parasite.

Similar to *Sarcocystis* species, cysts of *T. gondii,* which are difficult to be distinguished morphologically from those of *Sarcocystis* species, are also observed in the muscles of various animals. Comparison of the *ACS* sequences in the genomes of *S. cruzi* and *T. gondii* revealed significant differences in their exon/intron organizations. Based on these differences, the qPCR assay developed in this study effectively distinguishes *S. cruzi ACS* from that of *T. gondii*, as the primer pair was designed to amplify a part of the first exon of *ScACS*, in which the corresponding sequence in *T. gondii ACS* contains an intron, thus hindering the amplification of the *T. gondii* gene (Fig. 3). In Japan, *T. gondii* infections have been reported in cattle and swine, and as such, the distinction between these parasites is particularly important [21, 35].

In *T. gondii*, ACS synthesizes acetyl-CoA from acetic acid imported from the extracellular environment, and acetyl-CoA can also

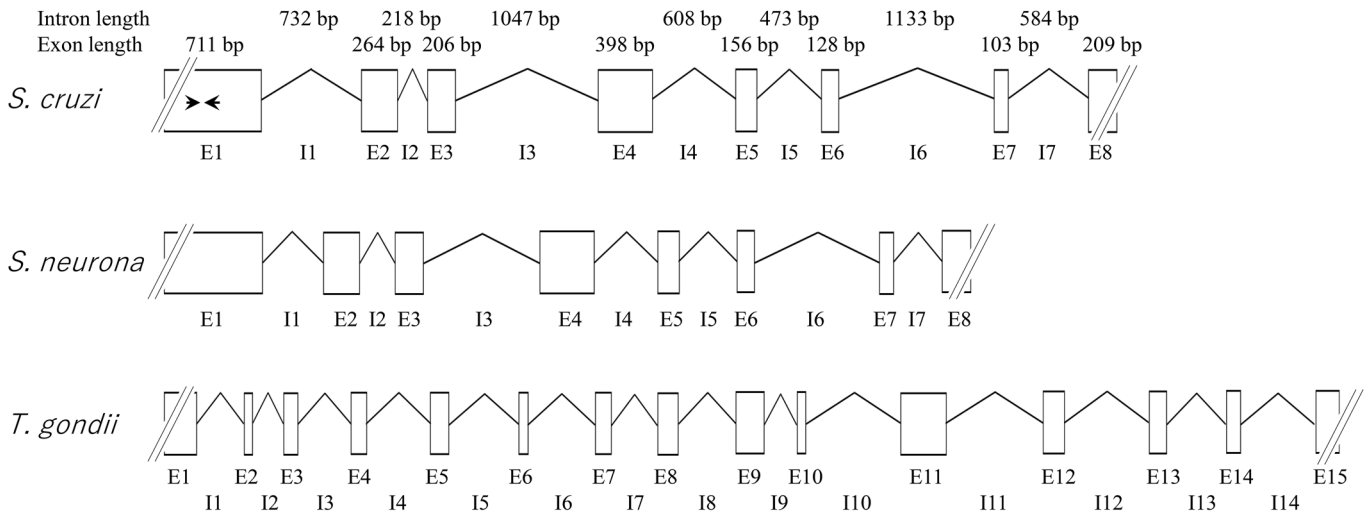

Fig. 2. Genomic structure of *ScACS* and comparison with *S. neurona ACS* and *T. gondii ACS*. Numbers above boxes indicate the lengths of *ScACS* exons/introns. Exons composed of UTRs are not included in this figure and are indicated with double slashes. Primers used for qPCR are indicated by arrows.

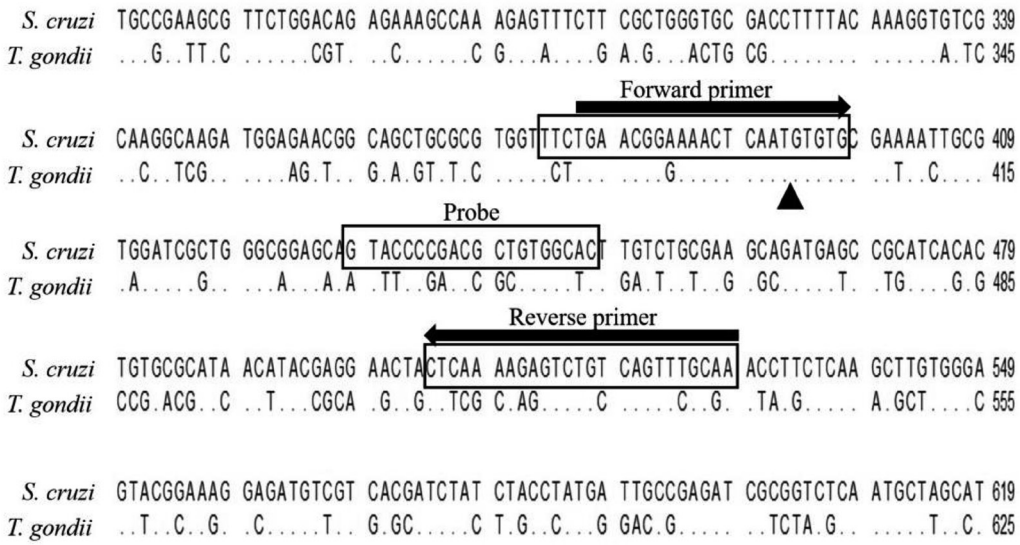

Fig. 3. Partial alignment of the *ACS* sequences of *S. cruzi* and *Toxoplasma gondii*. Boxes indicate the primers and probe. Black triangle indicates the splicing site (exon 4/intron 4) in the *T. gondii* gene.

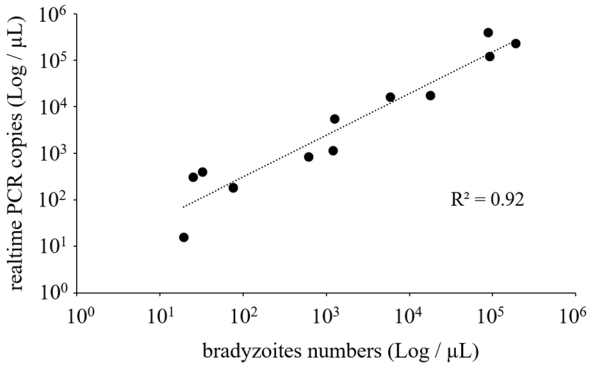

Fig. 4. Scatterplot of the microscopic counts and copy number values measured using the real-time PCR of DNA extracted from the bradyzoites in each cyst specimen (Log/µL). R^2 indicates correlation coefficient of the Pearson's regression analysis.

be made via a cytosolic ATP-citrate lyase (ACL) using a by-product of the TCA cycle, oxaloacetate. [9], Acetyl-CoA is mainly involved in synthesis of long-chain fatty acids, elongation in the endoplasmic reticulum, and synthesis of fatty acid synthetase I (FAS I) in the cytosol [9, 19, 31]. Although knockdown of either ACS or ACL did not affect the parasite's growth, the simultaneous knockdown of both genes affected the parasite's fatty acid synthesis and stopped essential fatty acid elongation for nuclear and plasma membrane biosynthesis, leading to the death of the parasite [19, 31]. Hence, the identified *ScACS* CDS in this study will facilitate the study of fatty acid metabolism pathways in *S. cruzi* and other *Sarcocystis* parasites.

Although *S. cruzi* infections in cattle, bison, and buffalo have been reported worldwide, only very limited gene sequences such as ribosomal RNA and *cox1* are known [5, 8, 12]. In this study, transcriptome analysis of *S. cruzi* using high-throughput sequencing revealed the *ACS* genome sequence for the first time. Furthermore, a new quantification method using qPCR for *S. cruzi* was established, enabling the number of parasites in muscle to be determined genetically, whereas previously only visual quantification methods were available. Although most Japanese cattle are infected only with *S. cruzi* [20, 29], cattle have been reported to be infected with various *Sarcocystis* species other than *S. cruzi*, such as *S. hominis* and *S. hirsuta* [8, 12, 15]. Therefore, it will be necessary to investigate the genes carried by these species in order to clarify the pathogenesis of BEM and acidophilic myositis in cattle and the risk of food poisoning from beef.

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

ACKNOWLEDGMENTS. We would like to thank Dr. Tatsuki Sugi (The International Research Institute for Zoonosis Control, Hokkaido University) for generously providing us with *T. gondii* genomic DNA. We would also like to thank Director Mitsuhiro Yoshinaga and the Saitama Meat Inspection Center for generously providing cattle samples.

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