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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

# Preliminary findings and molecular characterization of thin-walled *Sarcocystis* species in hearts of cattle and buffaloes in Thailand, Lao PDR, and Cambodia

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## Abstract

Cattle and buffaloes, popular protein sources worldwide, are intermediate hosts for several *Sarcocystis* species. These coccidian protozoans cause sarcocystosis resulting in subclinical and chronic infections in striated muscles by forming macrocysts or microcysts. In Thailand, Lao People's Democratic Republic, and Cambodia, *Sarcocystis* species have been reported, but molecular identification has been lacking. This study investigates the prevalence of infection, histo-morphology, and molecular identification of *Sarcocystis* species in hearts of cattle and buffalo sold in local markets. A phylogenetic tree inferred from a portion of the 18S ribosomal (r) RNA gene was used to identify the genus and species of *Sarcocystis*. The mitochondrial cytochrome c oxidase subunit 1 (*cox-1* gene) was sequenced to confirm the species of host tissue. In Thailand, *Sarcocystis* was detected in 66.7% (14/21) of samples. In Lao People's Democratic Republic, 90% (9/10) of samples were infected and in Cambodia 100% (8/8). For the first time from these countries, we report *Sarcocystis cruzi*, *Sarcocystis heydorni*, and *Sarcocystis levinei* found in taurine cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*). Zoonotic protozoan transmission needs to be controlled by inspection activities by local health inspectors, and appropriate action is required at all points in the food chain by competent authorities to protect consumer health and prevent sarcocystosis in cattle and water buffaloes.

**Keywords** *Sarcocystis cruzi* · *Sarcocystis heydorni* · *Sarcocystis levinei* · Taurine cattle · Water buffalo · Molecular analysis

## Introduction

*Sarcocystis* is a foodborne zoonotic pathogen classified in the phylum Apicomplexa and can infect animals and humans (Dubey 2015; Fayer et al. 2015). These parasites require two hosts in a simple predator–prey relationship to complete their life cycle (Fayer 2004). The intermediate host is infected by ingesting sporocysts, and then merogony occurs in the endothelial cells. Merozoites are released into the blood circulation and develop into sarcocysts in the muscle cells (Fayer et al. 2015; Lindsay et al. 1995). Cattle are intermediate hosts of five *Sarcocystis* species (*Sarcocystis cruzi*, *S. hirsuta*, *S. hominis*, *S. rommeli*, and *S. heydorni*), with the first three of these causing disease (Decker Franco et al. 2018; Dubey 2015). *Sarcocystis hominis* (syn. *S. bovihominis*) and *S. heydorni* are zoonotic: Humans can act as definitive hosts of these species (Fayer et al. 2015; Dubey 2015). Human infections are generally asymptomatic, but intestinal symptoms, such as nausea, abdominal discomfort, watery diarrhea, and dizziness, can occur (Fayer 2004; Dubey 2015). Four species of *Sarcocystis* are known from water buffalo (*Bubalus bubalis*): *S. fusiformis*, *S. buffalonis*, *S. levinei*, and *S. dubeyi* (Dissanaike and Kan 1978; Claveria and Cruz 2000; Dubey et al. 2014; Gjerde et al. 2016).

Infected livestock, such as cattle, buffaloes, sheep, and horses, can experience fever, lethargy, poor growth, low

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feed use, reduced milk production, lameness, wool and hair loss, abortion, and death (Fayer et al. 2015). Sarcocysts in muscle samples can be detected by microscopy via histological sections or by transmission electron microscopy (TEM). Species are differentiated by morphological features of sarcocysts such as size, presence or absence of septa, and cyst-wall thickness (thick > 3 μm and thin < 1 μm). A problem for morphological identification is that fresh meat samples with a high parasite burden are required (Fayer 2004; Xue et al. 2019). Nowadays, molecular methods have become popular. These are more efficient and sensitive than TEM for species identification of *Sarcocystis* in meat samples (Xue et al. 2019). The 18S ribosomal (r) RNA gene (18S rRNA) is an appropriate genetic marker able to distinguish between the five known species infecting cattle (Xue et al. 2019).

In Thailand, *Sarcocystis* screening has been reported during meat inspection (Nateeworanart et al. 2004; Dokmaikaw et al. 2017); however, the results to date have been limited to only morphological identification: There have been no molecular studies in Thailand. *Sarcocystis* has never been reported from Lao People's Democratic Republic or Cambodia.

This study aimed to investigate the prevalence, histomorphology, molecular characterization, and genetic diversity of *Sarcocystis* in hearts of cattle and buffaloes sold as meat products in local markets in Thailand, Lao PDR, and Cambodia.

## Materials and methods

### Sample collection

Heart muscle samples were collected from retail meat booths at local markets in Thailand, Lao PDR, and Cambodia from June 2018 to Dec 2019. A total of 39 samples were examined using a modified muscle-squashing method (Latif et al. 1999). Briefly, about 1 g of tissue cut into small pieces, approximately 3–5 mm thick, was crushed firmly between two glass slides, washed with 0.85% normal saline, and examined under a stereomicroscope for the presence of sarcocysts. The sarcocyst-positive samples were preserved with RNAlater® (QIAGEN, Germantown, MD) and then kept at -70 °C for molecular analysis. Samples found to be negative by the squashing method were subjected to a squeezing process (Latif et al. 1999), and the resulting fluid was observed microscopically for the presence of bradyzoites. The presence of the bradyzoites, and also absence was collect using for molecular analysis. Each individual sample was tested using the molecular method.

### Histological examination

For the morphological study, all tissue samples that were positive by the squashing method were fixed in 10% buffered formalin to pause autolysis. After fixation, each sample was trimmed as appropriate, pre-embedded to infiltrate tissue samples with paraffin and to replace water content of tissue with wax, followed by embedding. The paraffin blocks were stored at room temperature until sectioned on a microtome to yield 5-μm-thick sections. These were stained with hematoxylin and eosin. After staining, a coverslip was placed on the tissue section, mounting medium was added, and the section was microscopically examined at × 400 magnification (Slaoui and Fiette 2011).

### DNA extraction and amplification

Genomic DNA was extracted from individual samples using a NucleoSpin® tissue kit (Macherey–Nagel GmbH & Co., Duren, Germany), according to the manufacturer's instructions. Resultant DNA samples were stored at -20 °C until use. Polymerase chain reaction (PCR) was performed using a primer pair specific for the 18S rRNA gene of *Sarcocystis* (Table 1). Genus and species of host tissue was identified using sequences of mitochondrial DNA, amplified with primers (spanning tRNA-PRO and part of the control region) listed in Table 1. For both primer pairs, a 50 μL reaction volume contained 1 × FastStart High Fidelity Reaction Buffer with 1.8 mM $MgCl_2$ (Roche Applied Science, Mannheim, Germany), 0.2 mM dNTP (Vivantis, Malaysia), 0.2 μM of each primer, 1.25 U of FastStart High Fidelity Enzyme Blend (Roche Applied Science), and 5 μL of DNA template. PCR was done using a SimpliAmp™ Thermal Cycler (Applied Biosystems (ABI), Singapore). The PCR product was loaded on 1% agarose gel, electrophoresed, and stained with ethidium bromide for visualization, and its molecular weight estimated against a 100-bp DNA ladder RTU (GeneDireX®, Taiwan). The amplified products were sequenced at First BASE Laboratories Sdn Bhd (Selangor, Malaysia) using the PCR primers as sequencing primers by the BigDye terminator v3.1 cycle sequencing kit (ABI).

### Sequence alignment and phylogenetic analysis

A nucleotide BLAST search was done using each sequence through the National Center for Biotechnology Information (NCBI). Reference sequences were downloaded from NCBI and aligned with our new sequences using the ClustalW program (Thompson et al. 1994) in Bioedit (Hall 1999: http://www.mbio.ncsu.edu/bioedit/bioedit.html).

**Table. 1** Primers for PCR and sequencing of the 18S rRNA gene of *Sarcocystis* species and a portion of the mitochondrial genome of intermediate hosts

| Primer specific organism/genes | Primers and approximate PCR products amplicon sizes (bp) | PCR programs |
|---|---|---|
| *Sarcocystis* spp. (18S rRNA region) | SarcoF<br>5′- CGC AAA TTA CCC AAT CCT GA -3′<br>SarcoR<br>5′- ATT TCT CAT AAG GTG CAG GAG -3′<br>(Moré et al. 2011) PCR product size was 676 bp | Pre-incubation at 94 °C for 5 min followed by 35 cycles of amplification step: denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 45 s, and finally extension at 72 for 10 min and cooling to 4 °C to ∞ |
| Intermediate host cattle (mitochondrial DNA) | Forward primer<br>5′- CCT CCC TAA GAC TCA GGG AA -3′<br>Reverse primer<br>5′- AGC GGG TTG CTG GTT TCA CG -3′<br>(Guan et al. 2018) PCR product size was 481 bp | Pre-incubation at 94 °C for 5 min followed by 35 cycles of amplification step: denaturation at 61 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 45 s, and finally extension at 72 for 10 min and cooling to 4 °C to ∞ |

Phylogenetic analyses of partial 18S rRNA sequences of *Sarcocystis* were conducted using MEGA7 (Kumar et al. 2016). The phylogenetic tree was constructed using the maximum likelihood method with the Tamura 3-parameter model of sequence evolution (Tamura 1992) and tested using 1000 bootstrap resamplings. *Toxoplasma gondii* was the outgroup for rooting the tree.

## Results

### Prevalence and morphology of sarcocysts

The origin of each host sample (24 from taurine cattle *Bos taurus* and 15 from water buffalo *Bubalus bubalis*) and the associated mitochondrial sequence from each host is shown

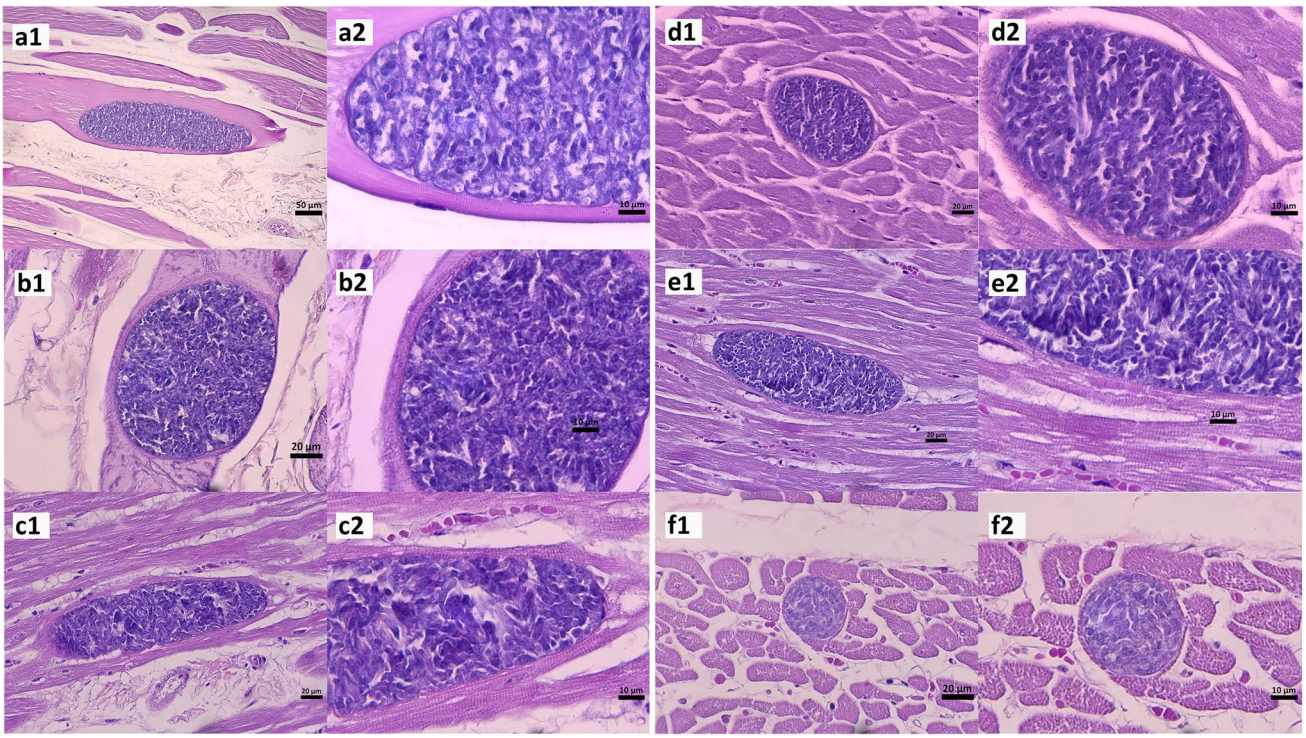


**Fig. 1** Histological sections (longitudinal and cross-sections) of sarcocysts in cattle and buffalo heart muscle from Cambodia (**a1–2** and **b1–2**), Lao PDR (**c1–c2** and **d1–2**), and Thailand (**g1–2** and **f1–2**) stained with hematoxylin and eosin (HE), (**a1** at 200 magnification; **b1**, **c1**, **d1**, **e1**, and **f1** at 600 magnification; whereas **a2**, **b2**, **c2**, **d2**, **e2**, and **f2** at 1000 magnification). Panels **a** and **e** are from taurine cattle (*Bos taurus*) specimens, while panels **b**, **c**, **d**, and **f** show sarcocysts from water buffalo (*Bubalus bubalis*)

in Table S1. Out of 39 samples, 31 (79.5%) were positive by microscopic examination which detected sarcocysts or bradyzoites. Positive samples included 8/8 (100%) from Cambodia, 9/10 (90%) from Lao PDR, and 14/21 (66.7%) from Thailand. Light microscopy showed fusiform sarcocysts varying in length:width ratios. Histological sections showed thin cyst walls (0.61 to 0.92-μm thick) (Fig. 1). Sarcocysts ranged from 183.67 to 329.21 μm in length and 64.27 to 80.83 μm in width. Bradyzoites were packed tightly into compartments of irregular shape separated by thin septa. Bradyzoites found by use of the squeezing method are not figured here.

## Molecular analyses

All 31 samples positive by microscopic examination were also positive by PCR, while the remaining eight microscopically negative samples were negative by PCR. Each unique partial 18S rRNA *Sarcocystis* sequence has been submitted to GenBank, as shown in Table 2. *Sarcocystis heydorni* was found in taurine cattle, and *S. cruzi* was found in both in taurine cattle and water buffalo. *Sarcocystis levinei* was found in water buffalo.

*Sarcocystis* 18S sequences from Cambodia (KHM 5–KHM 11) and Thailand (THA 1–THA 14) shared 97.7–100% similarity with *S. cruzi* from China, Germany, the USA, Australia, and Argentina (Fig. 2). All sequences from Lao PDR (LAO 1–LAO 9) were identical to that of *S. levinei* from Egypt (Fig. 2). In the phylogenetic tree, sequences of *S. cruzi* from taurine cattle and water buffalo in Thailand and Cambodia were intermingled in the tree with sequences of *S. levinei* from water buffalo in Lao PDR (Fig. 2). *Sarcocystis cruzi* exhibits some variation in its 18S rRNA gene. The KHM 1 sequence from Cambodia was 99.8% similar to that of *S. heydorni* from China and the Netherlands (Fig. 2), indicating the presence of *S heydorni* in Cambodia.

## Discussion

*Sarcocystis* is a globally important foodborne zoonotic pathogen (Dubey 2015). Consuming raw beef infected with *Sarcocystis* can cause sickness in humans. This study investigated the prevalence of *Sarcocystis* infection in hearts of cattle and buffaloes sold in local markets of three countries Thailand, Lao PDR, and Cambodia using microscopic observation and molecular identification. Histological examination showed that all sarcocyst walls were less than 1-μm thick, which means that all belonged within the thin-walled group (i.e. *S. cruzi*-like, *S. heydorni*-like and *S. levinei*-like) (Dubey et al. 2015; JyothiSree et al. 2017). Molecular

**Table 2** Summary of samples of *Sarcocystis* spp. collected in Cambodia, Lao PDR, and Thailand

| Countries/number of samples (N) | Accession nos | Sequence names | Genus species identification |
|---|---|---|---|
| Cambodia | MT825552 | KHM 1 | *S. heydorni* |
| (*N* = 8) | MT825553 | KHM 6, KHM 7, and KHM 9 | *S. cruzi* |
| Siem Reap | MT825554 | KHM 8 and KHM 5 | *S. cruzi* |
| | MT825555 | KHM 10 | *S. cruzi* |
| | MT825556 | KHM 11 | *S. cruzi* |
| Lao PDR | MT825557 | LAO 1, LAO 2, LAO 3, and LAO 4 | *S. levinei* |
| (*N* = 9) | | | |
| Champasak | | | |
| Vientiane | MT825558 | LAO 5, LAO 7, and LAO 9 | *S. levinei* |
| | MT825559 | LAO 6 | *S. levinei* |
| Khammouan | MT825558 | LAO 8 | *S. levinei* |
| Thailand | MT825560 | THA 1, THA 2, and THA 3 | *S. cruzi* |
| (*N* = 14) | MT825561 | THA 4 | *S. cruzi* |
| Khon Kaen | MT825562 | THA 5 | *S. cruzi* |
| Mahasarakham | MT825563 | THA 6, THA 7, and THA 8 | *S. cruzi* |
| | MT825564 | THA 9 | *S. cruzi* |
| Kalasin | MT825565 | THA 10 | *S. cruzi* |
| Phrae | MT825566 | THA 11 | *S. cruzi* |
| | MT825567 | THA 12 | *S. cruzi* |
| Phetchabun | MT825568 | THA 13 and THA 14 | *S. cruzi* |
| Chaiyaphum | | | |
| Roi Et | | ND | ND |

*ND* not done (negative by PCR)


**Fig. 2** Phylogenetic tree of *Sarcocystis* spp. based on 18S rRNA sequences obtained during this study and reference sequences from GenBank. The tree was inferred using the maximum likelihood (ML) method and the Tamura 3-parameter model of sequence evolution. Bootstrap resampling (1000) was used to assess support for individual nodes. *Toxoplasma gondii* was selected as the outgroup. Symbols designate whether samples were derived from taurine cattle (*Bos taurus*) (animal symbol with small horns) or water buffalo (*Bubalus bubalis*) (animal symbol with large horns)

identification is therefore essential for distinguishing among these species. Our molecular work identified *S. cruzi*, *S. levinei*, and *S. heydorni* (Decker Franco et al. 2018; Dubey et al. 2015; Dissanaike and Kan 1978).

Based on the 18S rRNA gene, we identified that *S. cruzi* from taurine cattle and water buffalo in Thailand and Cambodia and *S. levinei* from water buffalo in Lao PDR. *Sarcocystis levinei* and *S. cruzi* are very closely related (Gjerde et al. 2016) and our analysis also demonstrates that sequences of *S. cruzi* fall into the same clade as those of *S. levinei* (Fig. 2) (Gjerde et al. 2016). Although the 18S rRNA gene sequence is said to be a suitable marker for identification of genus and species (Xue et al. 2019), it cannot discriminate between *S. cruzi* and *S. levinei.* The relationship between these species and their taxonomic status requires further investigation, including use of full-length 18S rRNA sequences and other genetic markers (i.e. the mitochondrial cytochrome c oxidase subunit I, nuclear 28S ribosomal (r) RNA genes, and the internal transcribed spacer regions).

*Sarcocystis cruzi* is global in distribution and is transmitted by canids (Dissanaike and Kan 1978). It most generally causes subclinical infection in cattle, but can lead to fatal systemic disease with fatal myocarditis (Aráoz et al. 2019). This was the most common species found in the myocardium tissue of domestic cattle (*B. taurus*) in Cambodia and Thailand, in line with other reports that it is the most common parasite in cattle worldwide (Dubey et al. 2016). We also found this species in water buffalo (*B. bubalis*), which reflects the fact that both bovid species often live in the same area. We noted that two main groups of 18S rRNA variants were represented among *S. cruzi* isolates from our study region (Fig. 2), similar to those previously reported in infected Malaysian cattle (Ng et al. 2015). This indicates a degree of intra-specific variation within *S. cruzi*.

*Sarcocystis levinei* was described by Dissanaike and Kan (1978) from water buffaloes in Malaysia. We found it in water buffalo in Lao PDR. Ultrastructure of *S. levinei* sarcocysts was described from water buffaloes in the Philippines

(Claveria and Cruz 2000). This species is evidently typically found in water buffaloes from Southeast Asian countries.

*Sarcocystis heydorni* was described as a new species of *Sarcocystis* with thin-walled sarcocysts (< 1 μm) in cattle (Dubey et al. 2015). Its sarcocysts differ from those of other thin-walled species in having distinctive villar protrusions (Dubey et al. 2015). We found *S. heydoni* in a sample of cattle heart tissue from Cambodia. This species was experimentally shown to infect a human volunteer (Dr. Heydorn) who ate raw minced beef from Turkey (Dubey et al. 1988). There is a report of *S. heydorni* from cattle in China: Ingestion by a human volunteer did not produce a patent infection in that case (Hu et al. 2016).

One limitation of our study is that we only examined heart tissue. We may therefore have failed to find other species of *Sarcocystis*. For example, felid-transmitted species parasitize other tissues and are not found in the heart (Dubey et al. 2015). Examination of other parts of the body, such as tongue, esophagus, heart tissue and diaphragm, would increase the chances of finding additional *Sarcocystis* species (Dubey et al. 2016).

## Conclusions

We have presented the first molecular characterization of *Sarcocystis* infections within the heart samples of cattle and buffalo in three countries, Thailand, Cambodia, and Lao PDR. Phylogenetic relationships among several *Sarcocystis* species were analyzed. *Sarcocystis cruzi* appears to be the most prevalent *Sarcocystis* species in cattle in Cambodia and Thailand. *Sarcocystis levinei* was commonly found in water buffalo in Lao PDR, whereas *S. heydorni*, a known human pathogen, *S. heydorni*, was found in cattle from Cambodia. It is important to investigate the prevalence and molecular characterization of *Sarcocystis* infections in meat products more widely to fully evaluate the potential public health risk from bovine *Sarcocystis* infections in Asian countries.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00436-021-07241-5.

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## Declarations

**Conflict of interest** The authors declare no competing interests.

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