Domestic cats (*Felis catus*) are definitive hosts for *Sarcocystis sinensis* from water buffaloes (*Bubalus bubalis*)

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ABSTRACT. The definitive hosts of *Sarcocystis sinensis* in water buffaloes have hitherto been unknown, but the close similarity of this species to the cat-transmitted *Sarcocystis bovifelis* in cattle suggested they were felids. In a previous study, two domestic cats were fed macroscopic sarcocysts of *Sarcocystis fusiformis* contained within or dissected from the esophageal muscles of water buffaloes, while no microscopic sarcocysts of *S. sinensis* were noticed. Both cats started shedding small numbers of sporocysts 8–10 days post infection (dpi) and were euthanized 15 dpi. Using a PCR-based molecular assay targeting the mitochondrial *cox1* gene of *S. fusiformis*, both cats were shown to act as definitive hosts for this species. In the present study, DNA samples derived from oocysts/sporocysts in the intestinal mucosa of both cats were further examined by PCR for the presence of *S. sinensis* using 2 newly designed primers selectively targeting the *cox1* gene of this species. All 6 DNA samples examined from each cat tested positive for *S. sinensis*. A 1,038-bp-long portion of *cox1* was amplified and sequenced as 2 overlapping fragments from 5 of these DNA samples. The 5 sequences shared 99.3–100% identity with 7 previous *cox1* sequences of *S. sinensis* obtained from sarcocysts in water buffaloes. Additionally, amplification of the ITS1 region with primers targeting various *Sarcocystis* spp., yielded amplicons of 2 different lengths, corresponding to those obtained from sarcocyst isolates of *S. sinensis* and *S. fusiformis*, respectively. This is the first study to show that cats act as definitive hosts for *S. sinensis*.
KEY WORDS: *Bubalus bubalis*, cat, *cox1*, *Sarcocystis fusiformis*, *Sarcocystis sinensis*

Sarcocystis spp. are protozoan parasites with an obligatory two-host life cycle comprising sexual development and oocyst formation in the intestinal mucosa of their definitive hosts and asexual multiplication in vascular endothelial cells (schizont stage) and striated muscle cells (sarcocyst stage) of their intermediate hosts. The Indian water buffalo (*Bubalus bubalis*) is the natural intermediate host of 4 named *Sarcocystis* spp., that is, *Sarcocystis fusiformis*, *Sarcocystis buffalonis*, *Sarcocystis levinei* and *Sarcocystis sinensis* [6–9]. In addition, a *Sarcocystis hominis*-like species has been found in water buffaloes in China both by morphological and molecular methods [18]. The 2 species, *S. fusiformis* and *S. buffalonis*, form macroscopically visible sarcocysts in water buffaloes (about 3–35 × 1–5 mm and 1–8 × 0.1–0.5 mm, respectively), whereas the 3 other species form microscopic to barely visible sarcocysts (about 0.5–2 × 0.1–0.2 mm) [1, 7–9, 13, 14, 19, 20]. The sarcocysts of the 4 named species are, however, morphologically indistinguishable from those of certain *Sarcocystis* spp. in other intermediate hosts. Moreover, previous limited molecular comparisons using the 18S ribosomal (r) RNA gene as a genetic marker suggested that some of these species might be shared between water buf-

faloes and cattle, as discussed before [7–9]. Recently, a more comprehensive molecular characterization of these species has been performed based on sarcocyst isolates, and the mitochondrial cytochrome c oxidase subunit I gene (*cox1*) has been shown to be superior to previously used markers for species delimitation. These studies have established that *S. fusiformis* is indeed different from *Sarcocystis cafferi* in the African buffalo [8]; that *S. sinensis* is different from *Sarcocystis bovifelis* and *Sarcocystis bovini* in cattle [7]; and that *S. buffalonis* and *S. levinei* are different from *Sarcocystis hirsuta* and *Sarcocystis cruzi*, respectively, in cattle [9].

As regards the life cycle of the S*arcocystis* spp. in water buffaloes, dogs have been found to act as definitive hosts for *S. levinei* [1–3, 5, 14, 17], whereas cats have been shown to be definitive hosts for *S. fusiformis* [1–3, 8, 15–17] and *S. buffalonis* [13]. So far, the definitive host of *S. sinensis* has remained unknown. In 3 separate infection experiments in China in 1988, 1989/1990 and 1995, respectively, a total of 5 cats, 5 dogs, 1 sparrow hawk (*Accipiter nisus*), 1 little banded goshawk (*Accipiter badius*), 1 boreal owl (*Aegolius funereus*), 1 tawny owl (*Strix aluco*), 2 black vultures (*Aegypius monachus*), 2 rhesus monkeys (*Macaca mulatta*) and 2 human volunteers, were each given 60–2,000 isolated sarcocysts of *S. sinensis* from water buffaloes and examined for fecal excretion of oocysts/sporocysts for up to 40 days post infection (dpi). In addition, most of the experimental animals were euthanized about 40 dpi, and their intestinal mucosa was examined for developmental stages. In spite of these efforts, no oocysts/sporocysts were found in any of the fecal samples or mucosal scrapings, and the researchers therefore concluded that none of these species served as

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definitive hosts for *S. sinensis* [19, 20].

A thorough review of the literature by Gjerde [6] recently established that *S. sinensis* was morphologically indistinguishable from the species *S. bovifelis* described from cattle in Germany in the early 1970s [4, 12]. It then became clear that *S. sinensis* was probably also transmitted by cats in spite of the negative outcome of the attempts to infect 5 cats in the abovementioned experiments in China [19, 20]. At the time this similarity was first recognized, we had already conducted a small infection experiment on 2 cats with muscle tissue containing apparently only macroscopic sarcocysts of *S. fusiformis* from water buffaloes and had confirmed by using a species-specific primer targeting the *cox1* gene that cats were definitive hosts for this species [8]. Hence, it was possible to conduct a preliminary test of the hypothesis that *S. sinensis* was transmitted by cats without using additional experimental animals by simply examining the available material for the presence of DNA of the latter species also. Moreover, the partial *cox1* gene of *S. sinensis* had in the meantime been characterized from 2 sarcocyst isolates [7]. The latter achievement had made it possible to design 2 primers selectively targeting *S. sinensis* and to develop a sensitive molecular assay for identification of sarcocysts [7], which might also be suitable for identification of oocysts/sporocysts. The major aim of this study was therefore to determine whether the 2 cats of the previous infection experiment had also acted as definitive hosts for *S. sinensis* by using the newly developed *cox1*-based molecular assay.

The infection experiment was conducted in August 2014 after having been approved by the Ethical Research Committee of the Faculty of Veterinary Medicine, Cairo University, and all procedures were in compliance with the Egyptian animal welfare regulations. The details of the experimental procedures have been described previously [8]. Briefly, three 10-week-old domestic cats (*Felis catus*) were used, and sarcocysts/muscle tissue were collected from 5 naturally infected and freshly slaughtered adult water buffaloes at the El Warak slaughterhouse, Giza, Egypt. Cat #1 was fed 50 macroscopic sarcocysts consistent with those of *S. fusiformis*, which had been dissected from the esophagus of 3 water buffaloes; cat #2 was fed about 250 g of esophageal muscles containing similar macroscopic sarcocysts from 2 other water buffaloes; and cat #3 served as an uninoculated control. Feces from the cats were collected daily during the experimental period (from 2 weeks before infection until 15 dpi), and the entire 24-hr collection of feces was examined microscopically for coccidian oocysts (without enumeration) using flotation with Sheather's sugar solution. Following the detection of small numbers of sporocysts in fecal samples from the 2 inoculated cats, they were euthanized 15 dpi. The intestinal mucosa of both cats was collected by scraping with glass slides and homogenized in water, and the resulting suspension was centrifuged in order to concentrate the released sporocysts/oocysts. The presence of sporocysts/oocysts was ascertained by examining small portions of the mucosal suspension on microscope slides under a light microscope (Fig. 1).

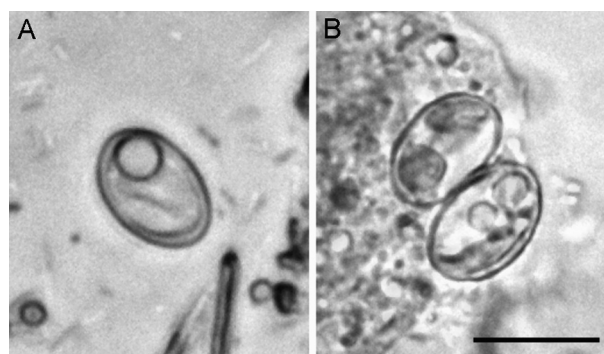Genomic DNA was extracted from 0.5 m*l* aliquots of the

Fig. 1.   Unstained sporocyst and oocyst of *S. fusiformis* and/or *S. sinensis* (based on molecular identification) in the digested intestinal mucosa (following fixation in ethanol) of two cats fed fresh sarcocysts/esophageal muscles from water buffaloes. (A) Sporocyst from cat #1. (B) Sporulated oocyst from cat #2. Bar=10 μm.

mucosal suspension from each inoculated cat as described previously [8], except that DNA was extracted from 3 additional subsamples of the mucosa from each cat, making it a total of 6 DNA samples from each cat. As already reported [8], initial attempts to amplify about 1,100 bp of *cox1* from some DNA samples using forward primer SF1 and 5 different reverse primers, including SR9, all failed, in spite of the fact that primer SR9 worked well with many sarcocyst-derived DNA samples of *S. fusiformis*. Reverse primer SffR was therefore designed to target *cox1* of *S. fusiformis* and used successfully together with primer SF1 to amplify a 972-bp-portion of *cox1* of this species from both cats (GenBank accession nos. KR186114 and KR186115) [8]. One sample from each cat was also amplified and sequenced with reverse primer SR66, which matches many *Sarcocystis* spp. [7], yielding low-quality *cox1* sequences of *S. fusiformis*, which seemed to be due to the presence of DNA of another species in the samples. Since the internal transcribed spacer 1 (ITS1) region may vary in length between different *Sarcocystis* spp. [7, 8], this marker was also examined to explore the possibility of a mixed infection and, if so, to get a clue about the identity of the second species present. However, the ITS1 region of *S. sinensis* is of about the same length as in the 2 closely related species *S. bovifelis* and *S. bovini* from cattle [7]. The ITS1 region was amplified with primer pair SU1F/5.8SR2, targeting most *Sarcocystis* spp., from all 6 DNA samples from each cat, and the size/length of the PCR amplicons was evaluated after staining with ethidium bromide and electrophoresis on agarose gel.

Following the isolation of 2 sarcocysts of *S. sinensis* and the generation of *cox1* sequences from both isolates (GenBank nos. KT900954 and KT900955) with primer pair SF1/SR9 [7], 2 primers specifically targeting *cox1* of *S. sinensis* could be designed to the exclusion of other *Sarcocystis* spp. in water buffaloes and cattle, for which *cox1* sequences were available for comparison. Forward primer SinF and reverse primer SinR targeted nucleotides 497–517 and 958–979, respectively, of the abovementioned 1,038-bp-long sequences, which start immediately downstream of forward primer SF1

[7]. All 6 DNA samples from both cats were first tested with primer pair SinF/SinR, using 2 rounds of amplification and evaluation of the amplification products on agarose gel after the second round only. Selected DNA samples (2 from cat #1 and 3 from cat #2) were then subjected to PCR amplification with primer pair SF1/SinR and SinF/SR9, followed by sequencing of the amplicons. Again, 2 rounds of amplification were used, and the products were only evaluated on agarose gel after the second round. The annealing temperature for the aforementioned PCRs was 54°C.

Selected mucosal DNA samples from both cats were also evaluated for the presence of DNA of *S. bovifelis* and *S. bovini* using forward primer SF1 and various reverse primers (Ss1R, Ss2R; Sb1R Sb2R) designed to target each of these species [7], since a co-infection with these species could not be ruled out based on the results from the ITS1 marker. Likewise, selected samples were examined for the presence of DNA of *S. buffalonis*, using the specific forward primer 18ShsF [7, 9], targeting the 18S rRNA gene, together with reverse primer 5.8SR2 in an attempt to amplify the ITS1 region of this species. All procedures concerning PCR amplification; evaluation, purification and sequencing of PCR products; and sequence assembly and comparisons, were as described previously [7, 8]. The sequences of all primers used are given in Gjerde [7].

As reported previously [8], no coccidian oocysts or sporocysts were detected in the feces of the 3 cats during the 2-week-period prior to the experimental infection or in the feces of cat #3 (uninoculated control) until the end of the experiment 15 dpi. In the feces of cats #1 and #2, *Sarcocystis* sporocysts were first detected 8 and 10 dpi, respectively, and both cats shed small numbers of sporocysts until euthanized 15 dpi. When examined post-mortem, only small numbers of *Sarcocystis* oocysts/sporocysts were seen in the scrapings of the intestinal mucosa of both cats (Fig. 1). No measurements of sporocyst size were made, since all sporocysts were initially assumed to belong to *S. fusiformis*.

Amplification of the ITS1 region with primer pair SU1F/5.8SR2 yielded PCR products of 2 different sizes, about 600 and 900 bp long, respectively, in all 6 samples from each inoculated cat. In 8 samples, the shortest band (~600 bp) was slightly stronger (thicker) than the longest band, whereas the 2 bands were of similar strength in the remaining 4 samples. The 2 amplicon sizes were consistent with those obtained with the same primer pair from sarcocysts of *S. sinensis* (~600 bp) and *S. fusiformis* (~900 bp), respectively, as determined both from bands on agarose gels and after sequencing [7, 8]. However, the *S. fusiformis*-specific forward primer SFF, targeting a variable region of the 18S rRNA gene 13–34 bp downstream of primer SU1F, was actually used in the previous study to amplify the ITS1 region of *S. fusiformis* before cloning and sequencing (of sarcocyst isolates) and to confirm that both cats acted as definitive hosts for *S. fusiformis* [8].

Amplification of the partial *cox1* gene with primer pair SinF/SinR resulted in strong bands of the expected size (573 bp) on agarose gel for all 6 DNA samples from each inoculated cat. Two DNA samples from cat #1 and 3 samples from cat #2 were thereafter amplified with primer pairs SF1/SinR and SinF/SR9 in order to obtain sequences of the same length (1,038 bp) as with primer pair SF1/SR9 alone. Both PCR assays yielded strong bands of the expected size (1,003 and 573 bp), and the resulting PCR products were sequenced using SF1, SinR and SinF as sequencing primers. Fine sequences were obtained with both primer pairs for all 5 samples. The 2 overlapping sequences were then joined into a 1,038-bp-long sequence for each isolate, and the latter 5 sequences were compared with each other and with 7 previous *cox1* sequences (GenBank nos. KT900954–KT900960) obtained from sarcocysts in water buffaloes [7]. The 2 sequences derived from cat #1 (GenBank nos. KU196747 and KU196748) differed from each other at only 1 nucleotide position, and these sequences differed at 1 or 2 positions from the 3 sequences obtained from cat #2, which were identical (GenBank no. KU196749). The 2 positions that separated the sequences from each other were also polymorphic in individual isolates (double peaks in chromatograms). Overall, the 5 new sequences differed at 0–2 nucleotides from each other (99.8–100% identity). The 2 haplotypes from cat #1 differed from the 4 previous *cox1* haplotypes of *S. sinensis* from water buffaloes at 1–7 nucleotide positions, whereas the single haplotype from cat #2 was identical with the most common haplotype derived from sarcocysts and differed at 1–5 positions from the 3 other previous haplotypes [7]. Overall, the new sequences of *S. sinensis* derived from sporocysts in cats differed at 0–7 nucleotide positions (99.3–100% identity) from the 7 previous *cox1* sequences derived from sarcocysts in water buffaloes. No amplification products were seen on agarose gels after PCRs using DNA samples from the cats as templates and primers either targeting *cox1* of *S. bovifelis* and *S. bovini* or intended to amplify the ITS1 region of *S. buffalonis*.

The present study has demonstrated that domestic cats indeed are definitive hosts for *S. sinensis* as predicted from the close morphological and molecular similarities of this species to *S. bovifelis* in cattle [6, 7]. The latter species was shown through a series of experiments in Germany in the early 1970s to be transmitted by cats, and its development in the intestinal mucosa of cats and its sporocyst morphology and mode of sporocyst shedding were studied in considerable detail [4, 10–12] as summarized by Gjerde [6]. The use of cats as definitive hosts is also in accordance with the phylogenetic position of *S. sinensis* [7], and it may partly explain the common occurrence of *S. sinensis* in water buffaloes, since cats are prevalent where water buffaloes are kept. The failure to demonstrate sporocysts in 5 cats experimentally infected with isolated sarcocysts of *S. sinensis* in China [19, 20], may have been due to a low-level infection of the cats and/or a low sensitivity of the light microscopical detection method used.

The original objective of the present small infection experiment was to demonstrate that cats were definitive hosts of *S. fusiformis* by using *cox1* as the molecular marker [8]. Hence, cat #1 was fed macroscopic sarcocysts of this species excised from the esophagus, whereas cat #2 was fed esophageal muscles containing such sarcocysts. Based on

the present findings, this material must also have contained some microscopic sarcocysts of *S. sinensis*, which were overlooked. Similarly, 2 small sarcocysts of *S. sinensis* were found in the muscle tissue adjacent to a large *S. fusiformis* sarcocyst in a previous study only after careful examination of the material under a stereo microscope [7]. It is therefore possible that microscopic sarcocysts of *S. sinensis* may have gone unnoticed also in previous infection experiments in which cats have been fed isolated macroscopic sarcocysts of *S. fusiformis*, or muscle tissue containing such sarcocysts, and that the sporocysts recovered from those cats belonged to more than one species. Such mixed infections of cats may now easily be detected by using molecular methods with the appropriate primers as demonstrated in the present study. Moreover, the molecular methods may be more sensitive than traditional microscopic methods. Thus, both cats shed only small numbers of sporocysts, despite the fact that they had been given several macroscopic sarcocysts of *S. fusiformis*. However, the central portion of such large sarcocysts frequently contains degenerate cystozoites, fluid and cell debris rather than intact and infective cystozoites as discussed previously [8], which may partly explain the low oocyst formation. Anyway, the *cox1*-based molecular assays managed to demonstrate the presence of *S. fusiformis* in the intestinal mucosa of both cats in the previous study [8] and of *S. sinensis* in the present study. Likewise, the ITS1-based assay suggested a mixed infection with both species in both cats.

The *cox1*-based assays employed to identify sporocysts of *S. sinensis* in the present study were also used successfully to identify 5 sarcocysts of *S. sinensis* from water buffaloes [7]. The 2 specific *cox1* primers (SinF and SinR) may be used together or singly in combination with a more general primer. A semi-nested PCR is also possible, that is, by using SF1/SinR or SinF/SR9 in the first round of amplification and SinF/SinR in the second round. These assays may be employed to identify sarcocysts or sporocysts of *S. sinensis* in future studies of the development and prevalence of this species in water buffaloes and cats, as well as in other potential intermediate and definitive hosts (probably various felids). Thus, the *cox1* marker unambiguously differentiates *S. sinensis* from the closely related species *S. bovifelis* and *S. bovini* in cattle, whereas these species cannot be separated on the basis of their ITS1 sequences [7]. Therefore, no attempts were made in this study to isolate, clone and sequence the shortest (~600 bp) amplicons obtained with the ITS1 primers, but the results obtained with the different *cox1* primers suggested that these amplicons indeed belonged to *S. sinensis* rather than to *S. bovifelis* and/or *S. bovini*. Available data also indicate that *S. sinensis* might be differentiated from the abovementioned species in cattle by a careful comparison of the 18S and 28S ribosomal RNA gene sequences [7]. However, an identification based on these markers is much more cumbersome than the use of the *cox1* marker, since the amplicons have to be cloned before sequencing due to intraspecific sequence variation at these markers. Moreover, as for *cox1*, it would have been necessary to design primers targeting *S. sinensis* to the exclusion of *S. fusiformis* in order to avoid a co-amplification of DNA from both species.

In summary, the present study has shown for the first time that cats act as definitive hosts for *S. sinensis* in water buffaloes. Hence, cats are involved in the transmission of 3 *Sarcocystis* spp. in this intermediate host. In order to study the development of *S. sinensis* in cats in more detail, including its prepatent and patent periods and sporocyst morphology, a more extensive infection experiment will have to be performed. In addition to using a few more cats, the experimental animals should preferably be fed muscle tissue containing only microscopic sarcocysts of *S. sinensis* over several days. The identification of cats as definitive hosts for *S. sinensis* will also make it possible to infect water buffaloes with sporocysts obtained from cats in order to study the development and pathogenicity of *S. sinensis* in its natural intermediate host.

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