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Non-invasive methods for identifying oocysts of *Sarcocystis* spp. from definitive hosts

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ABSTRACT

Because the excreted sporocysts and/or oocysts of various species of *Sarcocystis* may not be discriminated morphologically, we sought to validate a diagnostic technique based on variation in the 18S rDNA sequence. Oocysts and/or sporocysts from three taxa of *Sarcocystis* were collected from human, feline, and canine definitive hosts that had fed upon meats infected with the muscle cysts of *Sarcocystis hominis*, *Sarcocystis fusiformis* and a species of *Sarcocystis* from water buffalo that could not be distinguished from *Sarcocystis cruzi*. Using a new collection method employing filter paper, these excreted oocysts and sporocysts were subjected to DNA extraction, as were the corresponding muscle cysts. Methods employing PCR–RFLP and DNA sequencing of a partial 18S rDNA gene (*ssrRNA*) sequence were then used to successfully distinguish among the three taxa. The same, unique restriction digestion pattern characterizes the tissue cysts and oocysts and/or sporocysts of each parasite taxon. The technique makes possible amplification and identification of species specific gene sequences based on DNA extracted from as few as 7 excreted sporocysts (the equivalent of 3 and 1/2 oocysts) from freshly prepared material, or as few as 50 sporocysts from feces samples that had been stored in potassium dichromate ($K_2Cr_2O_7$) for as long as 6 years. This represents the first report using molecular diagnostic procedures to diagnose oocysts of *Sarcocystis* in faecal samples, describing a valuable new tool for studying the epidemiology of various *Sarcocystis* species.

1. Introduction

The Sarcocystidae (phylum Apicomplexa) includes a wide variety of parasitic protozoa, some of which are important pathogens of livestock and humans. *Sarcocystis* spp. have obligatory two-host life cycles, alternating between hosts that serve as each other's predator and prey. Intermediate hosts (typical herbivores) become infected by ingesting water or food contaminated with oocysts excreted by the definitive host. Such oocysts are comprised of two sporocysts, each of which contains four sporozoites. Once released from their sporocysts upon ingestion, the motile sporozoites can initiate infections in intermediate hosts which culminate, after several rounds of asexual replication and development, in the establishment of tissue cysts (sarcocysts) in host musculature. Definitive hosts (carnivores or omnivores) acquire infection upon consuming prey whose tissues are infested with such sarcocysts upon the consumption of infected tissues. Replication in the intestine of the definitive host, including the parasite's sole opportunity for sexual reproduction, results in excretion of oocysts to begin the cycle anew.

Sarcocystis hominis was initially described by Railliet and Lucet in 1891, who identified cattle (*Bos taurus*) as its intermediate host. Humans, rhesus monkeys (*Macaca mulatta*), baboons (*Papio cynocephalus*), and possibly chimpanzees (*Pan troglodytes*) may serve as definitive hosts for *S. hominis* [1]. *S. hominis* causes substantial economic losses in the cattle industry [1,2] and is a serious human intestinal pathogen in South China [2]. *S. hominis* infection in humans can result in abdominal discomfort or pain, diarrhoea, vomiting, dizziness, fatigue, anaemia, and even inflammation or haemorrhage and necrosis of the lower intestine [3,4]. *Sarcocystis fusiformis* uses the water buffalo (*Bubalus bubalis*) as its intermediate host, and the cat (*Felis domesticus*) as is its definitive host. Cattle have been described as intermediate hosts for *Sarcocystis cruzi*, a parasite excreted by dogs, whereas recent work is exploring the possibility that

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water buffalo serve as intermediate hosts for the same parasite species [5–11]. Among species of *Sarcocystis* infecting cattle and water buffalo, these three parasite taxa induce the most pathology and economic loss, and all three are highly prevalent in China and elsewhere [1,2].

Light microscopy may be employed to demonstrate the occurrence of sarcocysts in mammalian tissues, and may be used to discriminate among some parasite species based on the size of the sarcocysts. However, transmission electron microscopy has traditionally been required in order to establish, more definitively, the identity of such sarcocysts based on diagnostic differences in the ultrastructure of the sarcocyst wall; neither form of microscopy affords the means to diagnose species from the oocyst stage [1,2], Molecular techniques have been applied to help differentiate among tissue cysts in the intermediate host, including PCR-based randomly amplified polymorphic DNA (RAPD-PCR) [12], sequencing and PCR–RFLP analysis of 18S rDNA [5,13]. To our knowledge, no study has yet demonstrated the utility of such methods to differentiate DNA extracted from sporozoites residing within the sporulated oocysts excreted from definitive hosts. Doing so would enable non-invasive sampling from animals and people, and would facilitate medical and veterinary diagnosis in definitive hosts. In principle, variation in DNA sequences should overcome the lack of morphological differentiation among the oocysts of various species [14]. However, the scarcity and small size of excreted oocysts in natural infections render this approach technically challenging, especially owing to the abundance of inhibitors to PCR in feces. PCR–RFLP and sequencing methods have been applied to DNA extracted from abundant sporocysts of *Sarcocystis neurona*, obtained by scraping the small intestinal mucosa of the opossum [15,16]. But non-invasive diagnostic methods would be invaluable, especially for human patients. Furthermore, it remains uncertain whether traditional methods to store fecal samples (in potassium dichromate ($K_2Cr_2O_7$) and to harvest and microscopically examine fecal oocysts and sporocysts (employing $ZuSO_4$ and Sheather's solution) render sporocysts of *Sarcocystis* spp. suitable for subsequent genetic characterization. For epidemiological research, there is an urgent need to distinguish the oocysts and sporocysts of *Sarcocystis* spp. from the definitive host. Therefore, we evaluated a genetic approach to identifying oocysts and sporocysts of *Sarcocystis* spp. excreted from experimental infected definitive hosts, employing a new method to efficiently harvest such parasites from faecal preparations.

2. Materials and methods

2.1. S. hominis cysts and oocysts

Tissue cysts were collected from fresh muscle samples of cattle using a modification of previously described methods [13]. Briefly, each fresh tissue cyst was dissected from the muscle fibers by two, new preparation needles and released by agitation in 20–30 μl distilled water or ice in a 1.5 ml Eppendorf tube. Each sample was comprised of a single tissue cyst, stored at −20 °C until subjected to molecular analysis. Six years ago [17], oocysts and sporocysts of *S. hominis* were obtained from the excreta of a human volunteer who had been previously established as free of *Sarcocystis* infection, and who was then fed 1567 fresh *S. hominis* tissue cysts. The human subject protocol for this study as part of the parasites infection epidemiological surveys of the Yunnan University was approved by the University Review Board. Since then, this faecal sample had been stored in 2% aqueous (w/v) potassium dichromate ($K_2Cr_2O_7$), at 4 °C for about 6 years.

2.2. S. fusiformis tissue cysts and oocysts

Tissue cysts of *S. fusiformis* were collected from the oesophagus of 20 water buffalo slaughtered in Kunming, Yunnan, P.R. China using the dissection and storage methods described above (but without prolonged storage in $K_2Cr_2O_7$).

Prior to feeding them each with mature tissue cysts, two cats were subjected to continuous fecal examination for a period of 2 weeks prior to experimental infection. Exposure of the cats followed previously reported methods [17], such that 50 separate tissue cysts of *S. fusiformis* were used in a single exposure to each cat. Each infected cat was caged and fed only cooked food in order to preclude exposure to other infectious agents. The faeces were collected and stored following the procedure described in section 2.1. Each cat's faeces were examined for sporocysts and oocysts, 7 days post infection (DPI), daily. The faeces from the cats were stored for approximately 4 months prior to their use in this study.

2.3. Tissue cyst and oocysts of a species of Sarcocystis resembling S. cruzi

Muscle samples without macroscopically visible sarcocysts were collected from the fresh cervix, scapular region, abdomen, psoas, and thigh of water buffalo slaughtered in Kunming, Yunnan, P.R. China. The muscle samples were examined for tissue cysts, using the methods described above [13]. Each tissue cyst was sampled individually. Some were stored at –20 °C for use in molecular studies and others were used to infect either of two dogs, confirmed as free of prior infection by checked in the same way as the cats. The muscle samples were checked carefully to ensure that the only sarcocysts present conformed in size to that expected for *S. cruzi*. Exposure of the dogs followed previously reported methods [17]. Thus, 300 g of muscle infected with cysts, were used in a single challenge for each dog. The dogs were fed and treated as above 2. 2. The faeces were collected and stored following the procedure described in section 2.1. The faeces from the dogs were stored for 1 week prior to their use in this study. For the present purposes, we designate this taxon as *Sarcocystis cruzi*-like pending ongoing investigations intended to confirm or refute the hypothesis that these, indeed, correspond to the same species (*Sarcocystis cruzi*) for which *Bos taurus* has been described as a natural intermediate host.

2.4. Harvesting of sporocysts and oocysts

Morphologically indistinguishable oocysts began to be excreted from an infected human volunteer, the cats, and the dogs 14, 11, and 12 DPI. 1–2 g of stored faecal sample was used for obtaining sporocysts and oocysts by faecal flotation in 15 ml of a $ZnSO_4$ solution, as previously described [2,17].

The meniscus of such flotations was transferred to a microscope slide by an inoculating loop for sporocysts and oocysts exam, after which two methods were compared for their ability to collect the sporocysts and oocysts for subsequent processing 1) transfer using an inoculating ring using the surface tension of the solution or 2) adsorption to a piece of filter paper measuring 2–8 mm × 1–5 mm and storage in a 1.5 ml Eppendorf tube with 500 μl distilled water at −20 °C until use. Preliminary findings guided our subsequent decision to transfer the equivalent of 4, 5, 6, 7, 40 and 50 sporocysts to separate 1.5 ml Eppendorf tubes (assuming two sporocysts in each unsporulated oocyst). Each inoculating loop and filter paper was used only once to avoid risking cross contamination.

2.5. DNA extraction, PCR, RFLP, and DNA sequencing

DNA was extracted from the tissue cysts and oocysts of *S. hominis*, *S. fusiformis* and *S. cruzi*-like using the phenol/chloroform method as previously described [13]. Each sample was eluted in 20–25 μl of buffer.

Part of the 18S rDNA gene was amplified using a semi-nested PCR procedure, as previously reported [13]. Briefly, the primary PCR used 1 μl of DNA extraction (about 100 ng/μl) as template in a 25 μl PCR