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First report of *Sarcocystis pilosa* sporocysts in feces from red fox, *Vulpes vulpes schrencki*, in Hokkaido, Japan

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ABSTRACT

Sarcocysts of various *Sarcocystis* spp. are highly prevalent in wild sika deer, *Cervus nippon yesoensis*, in Hokkaido, Japan, and four species have been identified based on morphological and molecular characteristics: *S. ovalis*, *S. pilosa*, *S. tarandi*-like, and *S. truncata*-like. The definitive hosts of *S. ovalis* are corvids, but the hosts of the other species have not yet been identified. Aiming to determine the definitive hosts of these species, we collected 65 red fox (*Vulpes vulpes schrencki*) fecal samples in eastern Hokkaido and examined them for fecal sporocysts using a modified sucrose flotation method. One fecal sample contained typical *Sarcocystis* sporocysts, which were identified as *S. pilosa* based on 18S ribosomal RNA and cytochrome *c* oxidase subunit I gene sequences. This is the first identification of *S. pilosa* sporocysts in the wild. These findings indicate that red foxes serve as a definitive host of *S. pilosa*, and that red foxes constitute a source of *S. pilosa* infection for deer in Hokkaido.

1. Introduction

In wild sika deer, *Cervus nippon yesoensis*, in Hokkaido, Japan, sarcocysts of protozoan parasites of the genus *Sarcocystis* are highly prevalent, and four *Sarcocystis* species have been identified in the region to date: *S. ovalis*, *S. pilosa*, *S. tarandi*-like, and *S. truncata*-like (Takano et al., 2006; Narisawa et al., 2008; Irie et al., 2019). Among these species, *S. ovalis* has been reported to have a corvid as its definitive host in the region (Irie et al., 2017). Although the definitive hosts of the remaining species remain unknown, phylogenetic and epidemiological evidence seems to indicate that members of the Felidae (or unknown animals) are the likely definitive hosts of *S. tarandi*-like and *S. truncata*-like (Dahlgren and Gjerde, 2010a; Gjerde, 2014). The remaining species, *S. pilosa*, was originally isolated from *C. nippon* in Lithuania (Prakas et al., 2016), where seven *Sarcocystis* species were characterized in farmed sika deer by means of morphological and molecular methods (Rudaitytė-Lukošienė et al., 2018). Seven partially different *Sarcocystis* species, including *S. pilosa*, were also characterized in wild sika deer (*C. nippon centralis*) in mainland Japan (Abe et al., 2019). The definitive host of *S. pilosa* is suspected to be a member of the Canidae, because *S. pilosa* falls phylogenetically within a clade that includ *Sarcocystis* spp. using Canidae as their definitive host. Further, sarcocysts that are morphologically similar to *S. pilosa* have been described in *C. nippon centralis* and *C. nippon yesoensis* in Japan, and this type of sarcocyst is experimentally able to infest and reproduce in dogs (Saito et al., 1995, 1998; Arai et al., 2010). The red fox, *Vulpes vulpes schrencki*, is a very common canid in Hokkaido, and has been observed feeding on deer carrion (Tsukada and Nonaka, 1996). It therefore seems likely that red foxes serve to maintain *S. pilosa* in sika deer in Hokkaido. To clarify the *Sarcocystis* life cycle and the cause of the high prevalence of these parasites in deer, a survey of fecal sporocysts in red fox fecal samples was conducted.

2. Materials and methods

2.1. Red fox fecal sample collection

As described by Morishima et al. (1999), red fox fecal samples were collected along road verges, agricultural fields, and paths that were likely to be utilized by red foxes in eastern Hokkaido. Fecal collection was conducted in May 2018 (n = 44) and in December 2018 (n = 21). To aim to collect feces from different foxes, the sites where feces were picked up were separated by at least 2 km. To confirm that fecal samples belonged to red foxes, fecal DNA was extracted as described by Nonaka et al. (2009), and the 12S ribosomal RNA (rRNA) gene was analyzed as described by Yagi et al. (2002). To inactivate *Echinococcus multilocularis* eggs, which are highly prevalent in red foxes in the study area, fecal samples were incubated at 70 °C for 12 h before being stored

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at −30 °C until use.

2.2. Fecal sporocyst examination

Fecal sporocysts were examined in 1 g of feces using a modified sucrose flotation method (specific gravity, 1.27) (Ito, 1980). For species identification, sporocysts were collected from the supernatant of the centrifuge tube by simple sedimentation in saline.

2.3. DNA extraction and PCR sequencing of collected sarcocysts

Genomic DNA from collected sporocysts was extracted using a PowerSoil DNA Isolation Kit (Mobio Laboratories, Solana Beach, CA) according to the manufacturer's instructions. The 18S rRNA and cytochrome *c* oxidase subunit I (COI) genes were amplified and sequencing was performed using previously described primers (Irie et al., 2019). Direct sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA), and the obtained sequences were compared with those deposited in GenBank/EMBL/DDBJ.

3. Results and discussion

Of the 65 fecal samples analyzed, one (collected in December 2018) contained *Sarcocystis* sporocysts at approximately 500 sporocysts per gram of feces. The fecal sample was confirmed to belong to *V. vulpes* based on the 12S rRNA gene sequence. To confirm the presence of sporocysts in the feces, fecal examination was repeated again, and revealed reproducibility of the examination. The fecal sample was also positive for eggs of *E. multilocularis* and Capillariidae, and negative for *Cystoisospora* oocysts. The Capillariidae species might be *Calodium hepaticum* (syn. *Capillaria hepatica*), which reproduces in the liver of vole, and the eggs pass thorough the intestines of foxes that prey on vole. In other feces, eggs of hookworm, *Toxocara canis*, *Dibothriocephalus nihonkaiensis*, and oocysts of unidentified *Cystoisospora* were detected by the flotation.

The detected *Sarcocystis* sporocysts contained four sporozoites and were 15.0 μm (SD = 0.2) long and 9.3 μm (SD = 0.4) wide (Fig. 1) (mean values for 100 sporocysts). 18S rRNA (1669bp) and COI (1029bp) gene sequences of the sporocysts were deposited in GenBank/EMBL/DDBJ with accession numbers LC496069 and LC496070, respectively. Both sequences were 99.95%–100% identical to those of *S. pilosa* obtained from sarcocysts from sika deer in Hokkaido and Lithuania, and 99.23%–99.87% identical to such samples from mainland Japan (Supplemental Table S1).

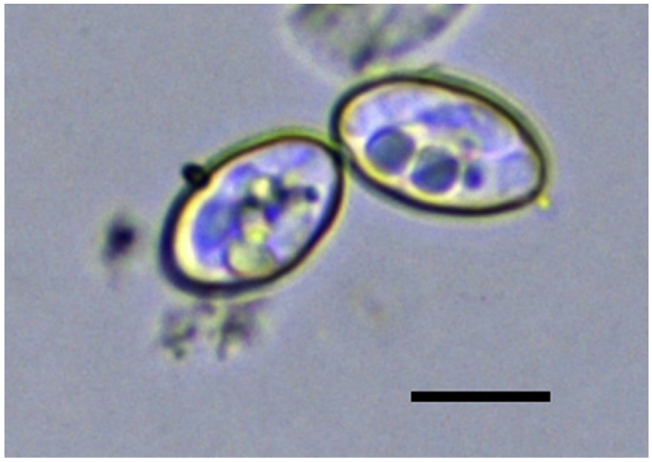

Fig. 1. Morphology of detected sporocysts under light microscopy. Scale bar: 10 μm.

Although *Sarcocystis* is considered highly endemic in sika deer from Hokkaido (Saito et al., 1998; Irie et al., 2019), very little is known about the life cycles of these *Sarcocystis* spp. Of the four prevalent species in the region, we focused on *S. pilosa*, the definitive hosts of which are suspected to belong to the Canidae. We therefore collected and analyzed red fox fecal samples and we detected *S. pilosa* sporocysts in one sample. Although it was previously experimentally demonstrated that red foxes can act as a definitive host for *S. hjorti*, which is closely related to *S. pilosa* (Dahlgren and Gjerde, 2010b), this is the first record of *S. pilosa* sporocysts in feces excreted by red foxes in the wild. The finding indicates that the red fox serves as a definitive host of *S. pilosa*, and also that red foxes could be an infection source for deer in the region.

In this study, only one fecal sample from red foxes contained sporocysts, representing 1.5% of the analyzed fecal samples and 4.8% of fecal samples collected in the winter. Prevalence of *Sarcocystis* sporocysts in feces of red foxes has been evaluated in many studies: 1.9% in Bulgaria (Kirkova et al., 2011), 3.8% in Ireland (Wolfe et al., 2001), 10.1% and 17.9% in the USA (Dubey, 1982; Davidson et al., 1992), and 84.4% in Newfoundland (Khan and Evans, 2006). Species were unfortunately not identified in those studies, and thus intermediate host animals were also not determined. Although simple comparison might not be appropriate because of biological and geographical differences, the rate of sporocyst-positive feces in the present study might relatively be low. However, the number of fecal samples examined in our study was statistically insufficient, and further investigation is necessary to evaluate the true prevalence. In addition, it is indispensable in future work to confirm that *S. pilosa* can reproduce in fox intestine by histological and/or intestinal scraping examinations, to eliminate the possibility that we have observed pseudoparasitism caused by ingestion by red foxes of carcasses of other animals that do serve as a definitive host of *S. pilosa*.

Red fox feeding habits in Hokkaido were inferred by analyzing residues in collected feces, which revealed that the annual percentage occurrence of material derived from deer was 16.7%, with the highest utilization of deer occurring in May (Tsukada and Nonaka, 1996). Considering the feeding habits of red foxes, the prevalence of *Sarcocystis* infection in red foxes may be higher than that observed in our study. Moreover, given that *S. pilosa* endemicity among deer in the region is > 90% (Irie et al., 2019), difference in the prevalence of these parasites between the definitive and intermediate hosts is considered unlikely. Excretion of sporocysts peaks during the early patent period, before dramatically decreasing in just a few weeks in the late patent period (Saito and Itagaki, 1994). In addition, in fecal samples of arctic foxes (*V. lagopus*), lower detectability of fecal sporocysts using a flotation technique was reported than that by qPCR analysis of fecal suspension (3% and 16%, respectively) (Elmore et al., 2013). Consequently, fecal sporocyst detectability might be markedly lower than the actual prevalence in the wild. Among the *Sarcocystis* species that use deer as an intermediate host in Germany (e.g. *S. tenella/S. capracanis*, and *S. gracilis*), the prevalence among red foxes was relatively high (6%–10% of surveyed animals) (Moré et al., 2016). Comparison of *Sarcocystis* detectability based on fecal examination and mucosal scraping examination revealed that the latter exhibited higher sensitivity (Scioscia et al., 2017). Thus, to more accurately evaluate *Sarcocystis* prevalence, such mucosal examination of small intestines from the definitive host animal is necessary. Further, sporocysts are known to be resilient in field conditions (especially in cold temperatures), and can remain infective for extended periods of time (Saito, 1989).

In the same way that the red fox serves as the definitive host for some *Sarcocystis* spp. (Dubey, 1983; Dahlgren and Gjerde, 2010b; Moré et al., 2016), so too does the raccoon dog (Gjerde, 1984; Moré et al., 2016). Indeed, raccoon dogs have been experimentally infested with *Sarcocystis* by feeding with sarcocysts from sika deer in Japan, and they excreted sporocysts in their feces (Saito et al., 1998). The *Sarcocystis* species used in that experiment was not clear due to the lack of molecular information at the time. Sarcocysts of *S. pilosa* were also detected

in *C. nippon centralis*, which exists on the main island of Japan (Abe et al., 2019), and slight molecular diversity was observed compared with *S. pilosa* isolates from Hokkaido and Lithuania (Supplemental Table S1). This might reflect a difference in the definitive host animals; the red fox population is suspected to be smaller on the main island of Japan, and, therefore, red foxes may not play an equally important role in the transmission of *S. pilosa* in other regions of Japan as they presumably do in Hokkaido. Moreover, the raccoon dog could also be a definitive host of *Sarcocystis* species, which could be determined by a survey of raccoon dogs in Hokkaido and the main island of Japan.

In conclusion, this is the first study to find *S. pilosa* sporocysts in red fox feces in Hokkaido, which indicates that red foxes could be a definitive host of this *Sarcocystis* species on this island. Because of the low detectability of sporocysts by fecal examination, examination of intestinal mucosal scrapings from red foxes is considered necessary to more accurately reveal the prevalence of this parasite in the region. In addition, a survey of other canids (e.g. raccoon dogs) should be undertaken to identify the primary definitive host(s) for *S. pilosa* in this region.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.12.001.

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