ORIGINAL PAPER

Corvid birds (Corvidae) act as definitive hosts for *Sarcocystis ovalis* in moose (*Alces alces*)

Bjørn Gjerde · Stina S. Dahlgren

Abstract Epidemiological data and a unique phylogenetic position had suggested that *Sarcocystis ovalis* in moose and red deer might use a definitive host other than canids, felids, or humans. Corvid birds and rats were therefore evaluated as potential definitive hosts for this species in a small pilot study. Four laboratory rats were each inoculated with 10 or 25 sarcocysts of *S. ovalis* isolated from moose, but no *Sarcocystis* oocysts were detected in their intestinal mucosa upon euthanasia 2 to 3 weeks later. At a site where large flocks of corvid birds (hooded crows, ravens and European magpies) fed on remnants of moose carcasses during the hunting period in October, fresh bird droppings were collected on the ground and examined microscopically and by molecular methods. By microscopy, a small number of typical *Sarcocystis* sporocysts, measuring 12.8×8.4 μm, were found in the faecal samples. These sporocysts were identified as belonging to *S. ovalis* by a polymerase chain reaction assay using specific primer pairs targeting the ssu rRNA gene, followed by sequence analysis. The intestinal contents of a crow and two magpies shot near the dumping site were also examined. *Sarcocystis* oocysts (16.1×12.4 μm) and free sporocysts (12.5×7.9 μm) were found in the intestinal mucosa/contents of one magpie (*Pica pica*). These oocysts/sporocysts were also found to belong to *S. ovalis* by the same molecular assay. This is the first report of corvid birds acting as definitive hosts for a species of *Sarcocystis*.

B. Gjerde (✉) · S. S. Dahlgren
Department of Food Safety and Infection Biology,
Section of Microbiology, Immunology and Parasitology,
Norwegian School of Veterinary Science,
P.O. Box 8146 Dep., 0033 Oslo, Norway
e-mail: Bjorn.Gjerde@nvh.no

Introduction

Among the 13 species of *Sarcocystis* hitherto identified in reindeer, moose, roe deer, and red deer in Norway (Gjerde 1985b; Dahlgren and Gjerde 2007, 2008, 2009, 2010a), three species, i.e., *Sarcocystis hardangeri*, *Sarcocystis ovalis*, and *Sarcocystis oviformis*, share the common feature of having a very distinctive sarcocyst morphology, their cysts being fairly large and oval or egg-shaped, as suggested from two of the species names, whereas the other species in these cervid hosts generally have spindle-shaped or thread-like cysts. Moreover, the surface of these oval cysts has sparsely distributed, slanting, tongue-like protrusions, and their host cells are encapsulated by a host-derived fibrous layer (Gjerde 1985a, 1986; Dahlgren and Gjerde 2007, 2008, 2009, 2010a).

Such oval sarcocysts were first recognized in wild reindeer (*Rangifer tarandus*) from Hardangervidda in Southern Norway, and the species was therefore named *S. hardangeri* (Gjerde 1984a). Shortly afterwards, similar cysts, believed to belong to the same species, were found in semidomesticated reindeer in Northern Norway (Gjerde 1984b) and, more recently, also in reindeer in Iceland (Dahlgren et al. 2007), the Icelandic reindeer population being descendants of a small number of semidomesticated reindeer imported from Northern Norway in the late 18th century. Sequence analysis of the small subunit ribosomal RNA (ssu rRNA) gene of isolates from both Norway and Iceland proved that these sarcocysts were conspecific (Dahlgren et al. 2007).

Gjerde (1984a, b, 1985a, 1986) described in detail the sarcocyst morphology of *S. hardangeri*, as revealed by light microscopy (LM), transmission electron microscopy (TEM), and scanning electron microscopy (SEM), and

noted that, by TEM, the cyst surface and host cell encapsulation of *S. hardangeri* looked very similar to that of type B cysts reported from moose (*Alces alces*) in Canada by Colwell and Mahrt (1981). However, it was not until fresh muscle tissues from moose in Eastern Norway were examined that oval cysts, indistinguishable from those of *S. hardangeri*, were clearly identified in moose and described by both LM and SEM (Dahlgren and Gjerde 2008). Molecular characterization revealed that the cysts in moose were closely related genetically to *S. hardangeri* but still sufficiently different at the ssu rRNA gene to be considered a separate species, which was named *S. ovalis*. Moreover, this study confirmed that moose in Canada also harbored *S. ovalis* (Dahlgren and Gjerde 2008).

Next, similar cysts were identified in roe deer (*Capreolus capreolus*) in Eastern Norway (Dahlgren and Gjerde 2009). These cysts were indistinguishable from those of *S. hardangeri* and *S. ovalis* by LM and SEM, and also quite similar to these species in their ssu rRNA gene sequence, but sufficiently different genetically to be regarded as belonging to a separate species, which was named *S. oviformis*. Finally, the same type of oval cysts were identified in red deer (*Cervus elaphus*) in Western Norway, but molecular examination at the ssu rRNA gene revealed that they belonged to *S. hardangeri* and *S. ovalis*, which previously had been found in reindeer and moose, respectively (Dahlgren and Gjerde 2010a).

In all four cervid hosts, sarcocysts of *S. hardangeri*, *S. ovalis*, and *S. oviformis* have generally been less prevalent and much fewer in number in infected animals than cysts of species transmitted by canids, and therefore, canids have *not* seemed to act as definitive hosts. This notion has also been supported by two unsuccessful attempts to infect foxes. Thus, Gjerde (1985b) fed 30 microisolated sarcocysts of *S. hardangeri* to a fox and a domestic cat, but no subsequent sporocyst shedding was observed. Similarly, 12 foxes were fed muscle tissues of moose containing cysts of *S. ovalis*, but none of the foxes became infected with this species, as determined by the use of specific primer pairs (Dahlgren and Gjerde 2010b).

The common occurrence, but low infection intensity, of these *Sarcocystis* spp. in different cervids all across Norway, as well as in Iceland and Canada, has further suggested that they were transmitted by widely dispersed definitive hosts, but perhaps by animals with a limited ability to contaminate the environment with large numbers of sporocysts. However, a low infection intensity might also be due to a limited ability of these species to proliferate within the intermediate host. Phylogenetic analyses subsequent to the molecular characterization of each species, until all three species were included, have consistently placed *S. hardangeri* (Dahlgren et al. 2008), *S. hardangeri*, and *S. ovalis* (Dahlgren and Gjerde 2008) and *S. hardan-*

geri, *S. ovalis*, and *S. oviformis* (Dahlgren and Gjerde 2009, 2010a) in a position clearly outside the two groups of *Sarcocystis* spp. in even-toed ungulates (Artiodactyla) using either canids or felids/humans as definitive hosts, as well as separate from all other *Sarcocystis* spp. included in the analyses. The sequence similarity at the ssu rRNA gene and their phylogenetic position have thus suggested that *S. hardangeri*, *S. ovalis*, and *S. oviformis* are sibling species using the same definitive hosts, which are not canids or felids but perhaps carnivores that have not been recognized as such previously.

Early on, it was suspected that widely distributed carrion feeders like corvid birds (Corvidae), particularly the common raven (*Corvus corax*) and the hooded crow (*Corvus cornix*), might act as definitive hosts for *S. hardangeri* in Norwegian reindeer (Gjerde 1985b). The finding of *S. hardangeri* in reindeer in Iceland supported this hypothesis, since the raven was the only commonly occurring carnivore species, beside the arctic fox, in the areas inhabited by those reindeer (Dahlgren et al. 2007). Other potential definitive hosts were common scavengers like rodents, since rats and mice were known to act as such for *Sarcocystis rodentifelis* and *Sarcocystis muris*, respectively (Grikieniene and Kutkienė 1998; Koudela and Modrý 2000). The phylogenetic analyses did, however, place the latter species far from *S. hardangeri*, *S. ovalis*, and *S. oviformis* (Dahlgren and Gjerde 2010a), but this could possibly be due to the fact that *S. rodentifelis* and *S. muris* possess both a diheteroxenous (rodent/felid) and a dihomoxenous (rodent/rodent) life cycle, and in both instances, use rodents (rats or mice) rather than ruminants as intermediate hosts. Based on the abovementioned considerations, both corvid birds and rats were evaluated as possible definitive hosts for *S. ovalis* of moose, which was the species most readily available for such studies. Moreover, specific primer pairs, targeting the ssu rRNA gene, had already been developed to facilitate the molecular identification of oocysts/sporocysts of this species (Dahlgren and Gjerde 2010b).

Materials and methods

Collection of faeces from corvid birds

The forest area known as Nordmarka immediately to the north of Oslo City has a dense moose population. The major part of this area is owned by a privately held company. Each year during the legal hunting period for moose in October, this company processes the carcasses of about 60 moose at a small abattoir-like plant located in the forest. The inedible portions of the carcasses, including the esophagus, portions of the diaphragm attached to the liver,

and any muscle tissues that have become soiled or damaged by the shooting, are dumped into a large hole in the ground, which is excavated for this purpose in the vicinity of the plant. The dumping place is kept open while the hunt is ongoing, and then finally covered with earth. While in use, this site attracts large crowds of hooded crows, common ravens, and European magpies, which feed on the remnants of the moose carcasses.

Since this practice provided a good opportunity to study the possible role of corvid birds as definitive hosts for *S. ovalis*, faecal matter deposited by these birds (no other large birds were present at the site) in the immediate vicinity of the dump was initially collected in late October 2007. The dumping place had then been in use for about 2 1/2 weeks, and there were numerous faecal deposits in the area, particularly at the resting places for the birds (on and near larger stones, small elevations, and trunks of fallen trees). About 50 fairly fresh deposits were scraped by a metal spoon from the surface on which they had been dropped and were collected in a jar. In addition, a hooded crow that had been shot about 2 weeks later while feeding at the dump was examined. (In Norway, hunting of crows, magpies and ravens is allowed from early August till the end of February.) Late in October 2009, faecal matter from corvid birds was again collected in the immediate vicinity of a new dumping place close to the processing plant. At the time of sampling, the dump had been in use for a little more than 3 weeks. However, there were only a moderate number of faecal deposits in the immediate vicinity of the carcasses, since there were few suitable resting places for the birds, as the site was closely surrounded by tall trees. Fresh faecal material was scraped with a metal spoon from the ground and the surface of a few moose carcasses, and also retrieved by picking up fallen leaves on the ground upon which faecal matter had been deposited. In total, about 15 individual droppings were retrieved. In addition, two magpies that had been shot by a hunter at the dumping site the day after the collection of faecal samples were examined.

Examination of faeces by microscopy

All the faecal material collected in 2007, as well as most of the material collected in 2009, were processed as follows: individual faecal deposits were pooled and homogenized in tap water in a blender; the resulting suspension was sieved to remove coarse particles and then centrifuged; the sediment was resuspended in saturated $NaCl/ZnCl_2$; and the floated material was examined under a microscope at 200× magnification. In 2009, a few fresh individual faecal deposits retrieved on leaves were examined by mixing small amounts of faeces in a drop of saturated sucrose on a microscope slide, followed by examination under the microscope.

Examination of the intestines of three corvid birds

The single crow shot in 2007 had been kept frozen for about 5 days before examination immediately upon thawing. The two magpies shot in 2009 had been kept frozen for about 10 days before being shipped by mail to the laboratory, which for unknown reasons took a whole week across town. The entire intestine was removed from each bird and cut open lengthwise, and mucosal scrapings were taken with a scalpel blade throughout the length of the small intestine at about 5-cm intervals, smeared onto microscope slides in a small volume of distilled water, and examined under the microscope at 200× magnification.

Molecular examination of intestinal mucosa and faeces from magpies/corvids

The following material collected in 2009, and found by microscopy to contain oocysts/sporocysts of *Sarcocystis*, was selected for examination by molecular methods: From an infected magpie, small portions of the intestinal mucosa, about 4 to 5 cm posterior to the stomach, were removed with a scalpel blade and transferred to a small volume of distilled water in each of two 1.5-ml centrifuge tubes (samples A1, A2); portions of a single faecal deposit containing *Sarcocystis* sporocysts were transferred to two 1.5-ml centrifuge tubes (samples B1, B2); a small portion of this sample was also floated in $NaCl/ZnCl_2$, and the top layer was transferred to a 1.5-ml centrifuge tube and diluted with an equal volume of distilled water (sample B3); following flotation in $NaCl/ZnCl_2$ of the pooled faecal sample comprising several individual droppings, the top layer was diluted about fivefold in tap water and centrifuged, and the sediment was transferred to two 1.5-ml centrifuge tubes (samples C1, C2). All samples selected for molecular examination were kept frozen at −20°C for 4 to 8 weeks before DNA extraction.

DNA was extracted from the two mucosal samples (A1, A2) from the magpie, from floated faecal material from the individual sample (B3), and from a subsample of the pooled faecal sample (C1) using QIAamp® DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's tissue protocol. DNA was also extracted from the unprocessed faecal sample (samples B1, B2) and from a subsample of the pooled sample (C2) using QIAamp® DNA Stool Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's pathogen detection protocol, except that proteinase K was used at a higher concentration (20 μl) and for a longer incubation period (50 min at 56°C followed by 10 min at 70°C).

All seven DNA samples were initially tested in a standard polymerase chain reaction (PCR) assay using ssu rRNA gene primer pairs that had been designed, evaluated, and found to be specific for *Sarcocystis alces* (primers

SD1f/SD1r) and *S. ovalis/S. hardangeri* (primers SD3f/ SD3r), respectively, as described previously (Dahlgren and Gjerde 2010b). In addition, a second primer pair that had been similarly evaluated and found to be specific for *S. ovalis/S. hardangeri* was used. These primers were SD4f (5′-TGGGGGCATTCGTATTTAAC-3′) and SD4r (5′-CTGTTCACATGCCGAAAAGA-3′). Based on the initial results, all seven DNA samples were further examined in a nested PCR; i.e., aliquots of genomic DNA were used as templates for primer pair 1Lf/3Hr in the first reaction, while the amplicons thus generated were used as templates for either primer pair SD1f/SD1r or SD3f/SD3r in the second reaction. In addition, DNA from three samples (A1, A2, C2) was similarly examined in a nested PCR using primer pair S5f/Primer B in the first round and primer pair SD4f/ SD4r in the second round. The nucleotide sequences of primers 1Lf, 3Hr, S5f, and Primer B are listed in Dahlgren and Gjerde (2007). Negative and positive controls (DNA extracted from sarcocysts of *S. alces* and/or *S. ovalis*) were included in each reaction. The PCR protocol for the primer pairs used in the first round of the nested PCR has been described previously (Dahlgren and Gjerde 2007), as has the protocol for the specific primer pairs that were either used separately or in the second reaction of the nested PCR (Dahlgren and Gjerde 2010b).

PCR products were separated on 1% agarose gels and visualized under UV light after staining with ethidium bromide to check for products of the appropriate size. To verify the results and to determine whether the visible PCR products represented *S. ovalis* or *S. hardangeri*, purified amplicons were sent for sequencing to Eurofins MWG Operon, Germany, using the same specific primers as in the PCR. The DNA sequences were checked for sequence similarities with published sequences in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Experimental inoculation of rats with isolated sarcocysts of *S. ovalis*

A small pilot experiment on rats was conducted at the Animal Facility of the Norwegian Institute of Public Health, Oslo, following approval by the Norwegian Animal Research Authority. Four male Wistar rats (HANTac:WH), weighing about 250 g, were used. The rats were kept in pairs in two standard cages and fed a standard diet throughout the experimental period. Fresh muscle samples from moose, mainly portions of the diaphragm, were collected on two occasions in October 2009 at the abattoir-like plant in Nordmarka described above. The muscle tissue was examined under a dissecting microscope, and a total of 70 large oval cysts (Fig. 1) consistent with those of *S. ovalis* (Dahlgren and Gjerde 2008) were excised with a needle and kept in phosphate-buffered saline overnight or for a few days until
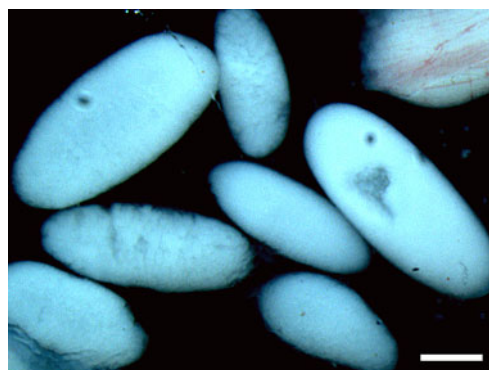

Fig. 1 Sarcocysts of *S. ovalis* isolated from the diaphragm of moose. *Scale bar*=400 μm

inoculation into the stomach of the rats by a gavage needle. Two of the rats were inoculated once with ten cysts each and were killed 14 days after inoculation. The other two rats were each inoculated twice 12 days apart with 10 and 15 cysts, respectively, and were killed 8 days after the last inoculation (20 days after the first). Immediately after euthanasia, the entire intestine was removed from each rat and cut open lengthwise, and mucosal scrapings were taken and examined as described for the birds.

Results

Faecal samples from corvids

The pooled faecal sample examined in 2007 contained a few nematode eggs and a moderate number of unsporulated, subspherical *Isospora*-like oocysts of two types, measuring about 18 and 24 μm in diameter. No *Sarcocystis* sporocysts were detected. In the samples collected in 2009, a few typical *Sarcocystis* sporocysts (Fig. 2) were found both in an individual faecal deposit and in the pooled sample comprising several deposits. The sporocysts measured, on average, 12.8×8.4 μm ($12.3–13.5 \times 7.7–9.0$ μm) ($n=20$) and contained a granular residual body and four elongate sporozoites, measuring about 8×2 μm. In addition to the sporocysts, a moderate number of unsporulated, subspherical *Isospora*-like oocysts of two types, measuring 18 to 19 μm and 24 to 25 μm in diameter, respectively, as well as a few nematode eggs, were detected in the faecal samples.

Intestinal mucosa of rats and corvids

No coccidian oocysts/sporocysts or other parasites were found in the intestinal mucosa or contents of the four rats inoculated with *S. ovalis* cysts or in the crow examined in 2007. The intestinal mucosa of both magpies obtained in 2009 was decomposed upon examination, making it
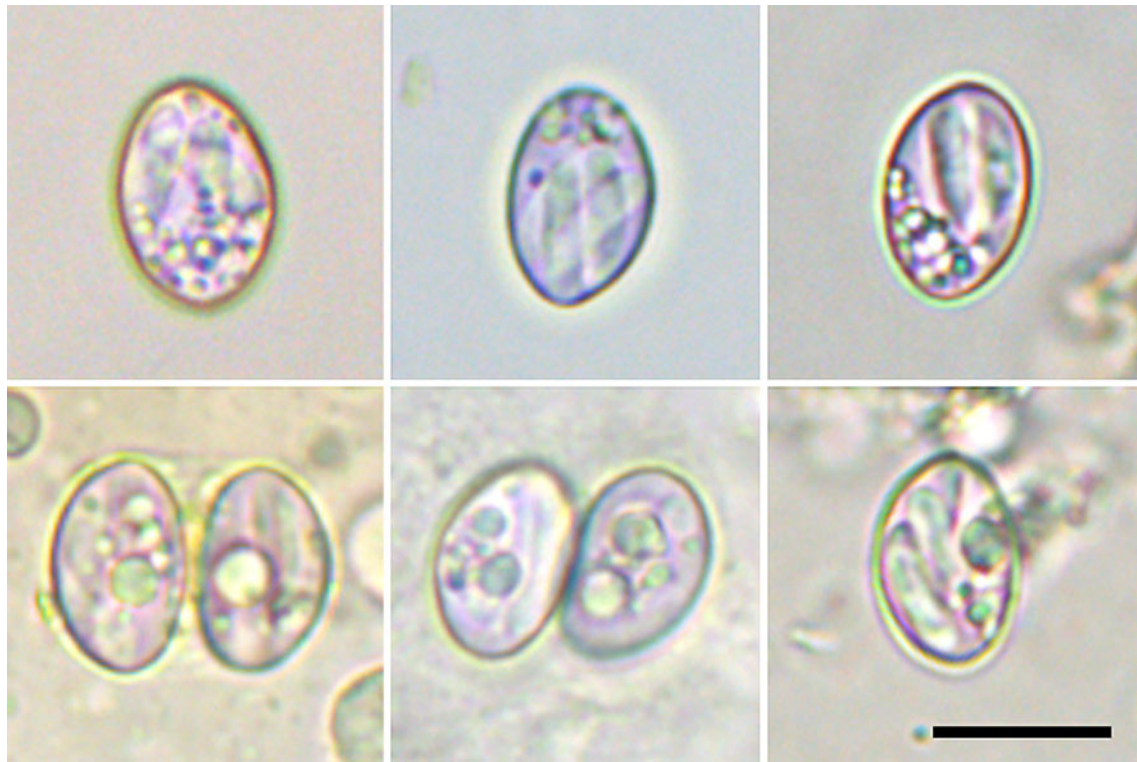
Fig. 2 Sporocysts and oocysts of *S. ovalis* from naturally infected corvid birds feeding on moose carcasses. *Upper row:* three sporocysts recovered from faecal samples of corvid birds. *Lower row:* two oocysts (*left*) and a free sporocyst (*right*) from the intestinal mucosa of a European magpie. *Scale bar*=10 μm

impossible to determine in which mucosal layer coccidian stages were located. In one of the magpies, a moderate number of unsporulated, nearly spherical *Isospora*-like oocysts, about 24 μm in diameter, and some nematode eggs were found. In the other magpie, a low number of sporulated, thin-walled *Sarcocystis* oocysts and a few free sporocysts were found in the scrapings (Fig. 2). The oocysts measured 16.1×12.4 μm (15.6–16.8×12.0–12.9 μm) (*n*=7), whereas the sporocysts measured 12.5×7.9 μm (12.0–12.9×7.3–8.3 μm) (*n*=17). *Sarcocystis* oocysts/sporocysts were mainly found in the anterior third of the small intestine, whereas a moderate number of unsporulated, subspherical *Isospora*-type oocysts, about 24 μm in diameter, were found in the posterior two thirds. The *Isospora*-type oocysts found in the intestines of both magpies were morphologically indistinguishable from the larger type of oocysts found in the pooled faecal samples from corvid birds both in 2007 and 2009.

Molecular identification

Standard PCR

By standard PCR, amplicons of the appropriate size were obtained on agarose gel with both primer pairs (SD3f/SD3r, SD4f/SD4r) specific for *S. ovalis*/*S. hardangeri* with DNA derived from the two mucosal samples from the magpie, whereas no visible PCR products were obtained with these primer pairs for any of the DNA samples derived from faecal material from the corvid birds. No PCR products were obtained with the *S. alces*-specific primer pair SD1f/SD1r for any sample from the corvids. The bands on the gel derived from the mucosal samples from the magpie were weak, and by sequencing, only short nucleotide sequences were obtained from these PCR products. However, a 338-nucleotide (nt) long sequence generated by primers SD3f/SD3r, which spanned a region of the ssu rRNA gene in which *S. ovalis* differs from *S. hardangeri*, showed the highest identity (99.7%) with an isolate of *S. ovalis* obtained from Norwegian moose (GenBank accession number EU282019).

Nested PCR

By nested PCR with outer primers 1Lf/3Hr followed by the *S. alces*-specific inner primers SD1f/SD1r, all samples, except the *S. alces* DNA control sample, were negative. However, when using primer pair SD3f/SD3r in the second reaction, both mucosal samples from the magpie (three replicates of A1 and A2), as well as one subsample of the pooled faecal sample (three replicates of C2), yielded strong bands on the gel consistent with that of the *S. ovalis*

DNA control sample, whereas the individual faecal sample (B2), yielded a weak band in the same position. Amplicons from all ten positive reactions were sequenced in both directions. For nine of these samples (six from the intestinal mucosa of the magpie, three from a pooled faecal sample from corvids), the resulting 643 nt long consensus sequences (forward/reverse), spanning the entire region between inner primers SD3f and SD3r, were identical. These sequences were also identical with the corresponding region (nucleotides 83–725) of a complete sequence of *S. ovalis* (GenBank accession number EU282019) derived from sarcocysts in moose inhabiting the same forest area as the moose and corvid birds in the present study (Dahlgren and Gjerde 2008). Moreover, the new sequences had 99.2% identity (638/643 nt) with an isolate of *S. ovalis* from Canadian moose (GenBank accession number EU282034), and 99.4% (639/643 nt) and 98.9% (636/643 nt) identities with two sequences of *S. ovalis* obtained from sarcocysts in red deer in Western Norway (GenBank accession numbers GQ250988 and GQ250989) (Dahlgren and Gjerde 2010a). In contrast, these sequences had only about 97% identity with four sequences of *S. hardangeri* from reindeer (GenBank accession numbers EF467654, EF056013, EF056014) or red deer (GenBank accession number GQ250989), and with a sequence of *S. oviformis* from roe deer (GenBank accession number FJ196262). Thus, the new sequences from corvid birds were considered to belong to *S. ovalis*, and a sequence derived from oocysts/sporocysts in the intestinal mucosa of the magpie (isolate SoCorvidPp1N), as well as a sequence derived from the pooled faecal sample from various corvid birds (isolate SoCorvidF1N), were submitted to GenBank and have been issued accession numbers HM474411 and HM474412, respectively.

The sequence obtained from the individual faecal sample was shorter (by 13 nt) and had several gaps and substitutions compared with the other sequences. This sequence showed only 92% identity with the abovementioned sequences of *S. ovalis*, *S. hardangeri*, and *S. oviformis* in GenBank. By nested PCR using primers S5f/Primer B followed by SD4f/SD4r, the two mucosal samples from the magpie (A1, A2) and the pooled faecal sample (C2) yielded strong bands on the gel consistent with that of the *S. ovalis* DNA control sample. Amplicons from the three positive reactions were sequenced in both directions. The three 764 nt long consensus sequences (forward/reverse) were identical and they were also identical with the corresponding region (nucleotides 1026–1789) of a complete sequence of *S. ovalis* (GenBank accession number EU282019) derived from moose in this area, and differed at only one or two specific positions (99.7–99.9% identity) from the three other sequences of *S. ovalis* in GenBank mentioned above. In contrast, these sequences differed at 7 or 8 specific positions (98.9–99.1% identity) from the abovementioned sequences of *S. hardangeri* and *S. oviformis* available in GenBank. Hence, the three new sequences from this region of the ssu rRNA gene, were also considered to belong to *S. ovalis*.

Discussion

The present pilot study showed that corvid birds, and specifically a European magpie, may act as definitive hosts for *S. ovalis*, whereas rats were unsuitable as definitive hosts for this species. Using nested PCR and two specific primer pairs, which together amplified a considerable portion of the ssu rRNA gene, both the oocysts/sporocysts in the intestinal mucosa of the magpie and the similarly sized sporocysts in droppings from various corvid birds could be proven to belong to *S. ovalis*. Thus, the partial ssu rRNA gene sequences of *S. ovalis* obtained from the corvid birds showed complete identity with a previous consensus sequence of *S. ovalis* obtained from four sarcocysts from four moose inhabiting the same forest area (Dahlgren and Gjerde 2008), and differed only slightly from isolates of *S. ovalis* from moose in Canada and from red deer in Western Norway, suggesting that these birds indeed had become infected by the local strain of *S. ovalis*. The corvid birds feeding at the dumping site clearly had the opportunity to become infected with *S. ovalis*, since this species was found to be present in several of the moose carcasses processed at the plant and used as a source of *S. ovalis*-cysts for the inoculation of rats. Other studies have also shown *S. ovalis* to be a fairly common species in moose in this area (Dahlgren and Gjerde 2008, 2010b).

The failure to detect *S. ovalis* DNA in the faecal samples by means of the specific primer pairs alone using standard PCR might have been due to the very small number of sporocysts present in these samples and/or to PCR inhibitors in the faeces. However, the specific primer pairs proved to be very useful in the nested PCR in order to selectively amplify DNA from *S. ovalis*, since the corvid birds harbored concurrent infections with related coccidians, i.e., with one or two species of *Isospora*. The aberrant sequence (only 92% identity with *S. ovalis*-like species) obtained from amplicons generated with primers SD3f/SD3r from the individual faecal sample was probably a defective *S. ovalis* sequence resulting from a low level of *S. ovalis* DNA in the template.

Due to the decomposed nature of the intestinal mucosa of the magpie harboring *Sarcocystis* oocysts, it could not be conclusively determined that the oocysts had actually been formed within the lamina propria. However, since several intact oocysts were found in the mucosal scrapings, and only in the anterior portion of the intestine, it is inconceiv-

able that they had been ingested with the feed and represented spurious parasites. The *Isospora*-type oocysts had most likely also been produced in the intestine of this magpie, since they were morphologically indistinguishable from oocysts found in the droppings from corvid birds both in 2007 and 2009. The fact that the PCR-based assay using *S. alces*-specific primers yielded negative results for the mucosal samples from the magpie provided further evidence that the positive outcome with the *S. ovalis*-specific primers was due to the presence of the oocysts/sporocysts rather than to a recent ingestion of *Sarcocystis* infected muscle tissues from moose, since cysts of *S. alces* are more common and more numerous in moose than those of *S. ovalis* (Dahlgren and Gjerde 2008, 2010b).

The identification of a magpie rather than other corvid birds as the first confirmed definitive host species for *S. ovalis* will probably prove to be coincidental. Thus, the faecal deposits from the feeding site, which also contained sporocysts of *S. ovalis*, might just as well have originated from crows or ravens, which seemed to be more numerous at the feeding site than magpies. Moreover, other *Sarcocystis* spp. have been shown to possess a low specificity for their definitive hosts, usually infecting several closely related species within the same family of carnivores. Thus, we expect that other corvid birds may also transmit *S. ovalis* to moose and red deer, the two known intermediate hosts for this species (Dahlgren and Gjerde 2008, 2010a). In Norway, the hooded crow and the common raven may actually play a more important role than magpies in this transmission, since they are the principal corvid species feeding on carcasses of large animals (Røskaft 1992). Nevertheless, the present findings should be confirmed by examining the intestines or faeces from more corvid birds by microscopy for oocysts/sporocysts, followed by molecular species identification, and perhaps also by experimental infections of captured corvid birds with microisolated sarcocysts of *S. ovalis*, like it was done in the experiment on rats.

The close similarity in sarcocysts morphology, ssu rRNA gene sequence, and phylogenetic position between *S. ovalis*, *S. oviformis*, and *S. hardangeri* has suggested that they are sibling species using the same definitive hosts. The present finding of corvid birds acting as definitive hosts for *S. ovalis* may therefore explain how *S. hardangeri* managed to become established in the descendants of the reindeer imported from Norway to Iceland more than 200 years ago and still be present there today (Dahlgren et al. 2007). Thus, the common raven is the only corvid bird species in Iceland, these birds are endemic in the areas of Iceland inhabited by reindeer, and they commonly feed on reindeer carcasses (Petersen 1998). It is therefore very likely that ravens act as definitive hosts for *S. hardangeri* in Iceland.

The use of different corvid birds as definitive hosts may also explain the widespread occurrence and fairly high prevalence of *S. ovalis*, *S. oviformis*, and *S. hardangeri* in cervids in Norway; since such birds are present in all areas inhabited by these cervids, they are eager scavengers on dead animals, and they are able to disseminate their droppings over large areas. Each bird may, however, ingest only one or a few cysts, resulting in the shedding of a small number of oocysts/sporocysts, and this low-level infection, coupled with the wide dissemination of their faeces, will also lead to a low-level contamination of the environment with sporocysts. This might explain previous observations of a generally low to moderate number of sarcocysts of these species in infected hosts, in contrast to the more numerous sarcocysts of species being transmitted by canids.

In their intermediate hosts, cysts of *S. ovalis*, *S. oviformis*, and *S. hardangeri* have generally been located close to the aponeuroses or tendons of the muscles, although they have also been found in the esophagus of all host species (Gjerde 1984a, b; Dahlgren and Gjerde 2008, 2009, 2010a). The apparent preference of the sarcocysts for the least fleshy portions of the muscles might be an adaptation to being transmitted by scavenging birds, since these are the portions that would tend to remain after large predators or carrion feeders have taken their share of the carcasses. The proportion of birds that ingest infective sarcocysts is probably small, as indicated in this study, in which only one of three birds examined were infected. However, the absence of *Sarcocystis* sporocysts in the faecal samples collected in 2007 was probably mainly due to sampling too early after the first carcasses had been dumped at the site, i.e., before any infected birds had started to shed sporocysts.

To our knowledge, this is the first report of corvid birds acting as definitive hosts for a species of *Sarcocystis*. Previously, such birds have only been known as intermediate hosts for members of this genus (Černá 1984; Kutkienė et al. 2009). Erber et al. (1978) fed two rooks (*Corvus frugilegus*) muscle tissues of roe deer containing sarcocysts but observed no sporocyst shedding. However, other scavenging birds, namely, vultures (*Gyps* spp.), have previously been reported to be definitive hosts for *Sarcocystis*. Thus, Markus and Mundy (1979) reported finding *Sarcocystis* sporocysts in the faeces of different vultures in southern Africa and provided measurements of sporocysts recovered from the faeces of two white-backed vultures (*Gyps africanus*). Markus et al. (1985) later proved that a Cape vulture (*Gyps coprotheres*) and a white-backed vulture (*G. africanus*) were definitive hosts for *Sarcocystis* of the impala (*Aepyceros melampus*), since both birds started to shed *Sarcocystis* sporocysts 15 to 16 days after being fed muscle tissues from this intermediate host. Two

species of *Sarcocystis*, or rather cyst types, were found in the impala meat used to infect the vultures, but the authors could not determine which one infected the vultures, since molecular species identification was not used at that time (Markus et al. 1985).

Several birds of prey (owls, falcons, hawks, and buzzards) are known as definitive hosts for four named *Sarcocystis* spp. (*Sarcocystis cernae*, *Sarcocystis dispersa*, *Sarcocystis scotti*, *Sarcocystis sebeki*) and two *Frenkelia* spp. (*Frenkelia glareoli* and *Frenkelia microti*), all of which use small rodents as intermediate hosts (Černá 1984). Some researchers are of the opinion that these *Frenkelia* spp. should be transferred to the genus *Sarcocystis* (Mugridge et al. 1999; Šlapeta et al. 2001). Ssu rRNA gene sequences have been generated for *S. dispersa* (Doležel et al. 1999) and for both *Frenkelia* spp. (Votýpka et al. 1998) in order to study their phylogenetic relationships with other members of the Sarcocystidae. When these sequences were included in the phylogenetic analyses comprising *S. ovalis*, *S. oviformis*, and *S. hardangeri*, they were found to be only distantly related to these three species from cervids (Dahlgren and Gjerde 2009, 2010a). This finding might seem at odds with the concept of coevolution of *Sarcocystis* spp. with their definitive hosts (Doležel et al. 1999; Šlapeta et al. 2001), from which one might expect all bird-transmitted species to group together. A possible explanation could be that birds are a diverse group, and while corvid birds belong to the order Passeriformes, the birds transmitting *S. dispersa* and the two *Frenkelia* spp. belong to the orders Strigiformes (owls) and Falconiformes, respectively. Nevertheless, *S. dispersa* and the two *Frenkelia* spp. seem to be fairly closely related, but they also have in common rodents as intermediate hosts, whereas *S. ovalis*, *S. oviformis*, and *S. hardangeri* form an aberrant group within the major clade comprising *Sarcocystis* spp. using even-toed ungulates as intermediate hosts. This might suggest that the phylogenetic position of *Sarcocystis* spp. is also influenced by which type of intermediate host they use.

In conclusion, the present pilot study has shown by microscopy and molecular methods that corvid birds, and specifically a European magpie, may act as definitive hosts for *S. ovalis*. The use of corvid birds, rather than mammals as definitive hosts, may explain the widespread distribution and the peculiar phylogenetic position of *S. ovalis* and the closely related species *S. oviformis* and *S. hardangeri*, which probably also use corvids as definitive hosts.

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