Molecular Identification of Sarcocystis Halieti in Birds of Prey From Spain

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Abstract

Background: Members of the genus *Sarcocystis* are protozoan parasites characterized by a prey-predator two-host life cycle. Sarcocysts are formed in muscles or CNS of the intermediate host (IH), while sporocysts develop in the small intestine of the definitive host (DH). Various birds of prey were confirmed to be DH for *Sarcocystis* spp. By contrast, only two species, *S. wobeseri* and *S. falcatula* were identified in the muscles of birds of prey. The latter species is pathogenic and can cause encephalitis in various birds. The aim of the present study was to identify *Sarcocystis* species in the muscles of birds of prey from Spain.

Methods: In the period between 2019 and 2020, muscle tissues of 59 birds collected from Spain were examined for the presence of *Sarcocystis* spp. Sarcocysts in fresh squashed samples were morphologically characterised under a light microscope (LM). *Sarcocystis* species were identified by means of 28S rRNA and ITS1 sequence analysis.

Results: With the help of methylene blue-staining microscopic sarcocysts were detected in 3/59 (5.1%) birds of prey from Spain. Under LM, one type of sarcocysts was observed. Sarcocysts were thread-like (1050–2160 × 130–158 µm), had a thin (0.7–1.4 µm) and smooth cyst wall. Septa divided the cysts into compartments filled with banana-shaped (5.9 × 1.7 µm) bradyzoites. On the basis of DNA sequence results, *S. halieti* was identified in the western marsh harrier (*Circus aeruginosus*) and the black kite (*Milvus migrans*) for the first time. Sarcocysts of *S. halieti* detected in the black kite and the western marsh harrier were shorter and wider as compared to those observed in the great cormorant (*Phalacrocorax carbo*) and the herring gull (*Larus argentatus*). Hence, *S. halieti* might infect birds belonging to three different orders, Suliformes, Charadriiformes and Accipitriformes.

Conclusions: This is the first report of *S. halieti* in birds of prey as IH. Due to the inconsistency of research on *Sarcocystis* spp. from birds of prey, further complex morphological, histopathological, and molecular studies are required.

Background

Representatives of the genus *Sarcocystis* are protozoan parasites characterized by an obligatory two-host prey-predator life cycle. Asexual multiplication with sarcocyst formation occurs in the muscles and/or CNS of the intermediate host (IH), whereas sexual multiplication stages, oocysts/sporocysts develop in the small intestine of the definitive host (DH) [1]. Currently, 27 valid *Sarcocystis* species using birds as IH are known [2].

Raptors are indicators of biodiversity and environmental health, they are recognized in ecosystems as top predators and scavengers, and as flagship species [3]. Various birds of prey (eagles, hawks, falcons, and owls) were exhaustively examined as possible DH [1]. Also, there are numerous studies about birds of prey as IH of *Sarcocystis* spp. [4–10]. It was recorded that pathogenic *S. falcatula* may cause encephalitis in the free-ranging great horned owl (*Bubo virginianus*) [6], the golden eagle (*Aquila chrysaetos*) and the

bald eagle (*Haliaeetus leucocephalus*) [7]. Likewise, an undescribed *Sarcocystis* species causing encephalitis has been detected in an immature northern goshawk (*Accipiter gentilis atricapillus*) from Minnesota [4]. Recently, *S. wobeseri* was identified in pectoral and cardiac muscles of the white-tailed sea eagle (*Haliaeetus albicilla*) [10]. Thus, sarcocysts of two *Sarcocystis* species, *S. falcatula* and *S. wobeseri*, were recorded in the tissues of birds of prey [7, 10]. Three morphological types of sarcocysts were detected in the Eurasian buzzard (*Buteo buteo*) and the long-eared owl (*Asio otus*), and the third type of sarcocyst distinguished in the owl was recognised as *S. otus* [11]. However, this species is considered to be invalid [1].

The present paper describes a molecular identification of *S. halieti* in the muscles of birds of prey from Spain.

Methods

In the period between 2019 and 2020, leg muscles of 59 birds of prey (Accipitriformes, Falconiformes and Strigiformes) from Navarra (Spain) were examined for *Sarcocystis* (Table 1). The analysed samples come from the birds admitted to the Wildlife Recovery Centre of Ilundain (Navarra). The samples were taken by the Centre's veterinary staff, during the routine diagnostic protocol of the cause of death of the specimens that enter the Centre dead or die there. This Centre belongs to the Government of Navarra and is managed by public company GAN-NIK. Muscle samples were kept frozen (-20ºC) until a morphological detection of sarcocysts. The prevalence and infection intensity of *Sarcocystis* were evaluated in methylene-blue stained muscle samples as previously described [12].

Table 1
Birds of prey from Navarra (Spain) examined for *Sarcocystis* spp.

No.	Species	Order	Infected/ investigated
1.	Black kite (*Milvus migrans*)	Accipitriformes	2/6
2.	Western marsh harrier (*Circus aeruginosus*)	Accipitriformes	1/1
3.	Booted eagle (*Hieraaetus pennatus*)	Accipitriformes	0/2
4.	Common buzzard (*Buteo buteo*)	Accipitriformes	0/4
5.	Eurasian sparrowhawk (*Accipiter nisus*)	Accipitriformes	0/1
6.	Red kite (*Milvus milvus*)	Accipitriformes	0/9
7.	European honey buzzard (*Pernis apivorus*)	Accipitriformes	0/1
8.	Northern goshawk (*Accipiter gentilis*)	Accipitriformes	0/3
9.	Griffon vulture (*Gyps fulvus*)	Accipitriformes	0/4
10.	Common kestrel (*Falco tinnunculus*)	Falconiformes	0/7
11.	Eurasian scops owl (*Otus scops*)	Strigiformes	0/13
12.	Long-eared owl (*Asio otus*)	Strigiformes	0/2
13.	Brown owl (*Strix aluco*)	Strigiformes	0/3
14.	Little owl (*Athene noctua*)	Strigiformes	0/3
Total			**3/59**

Muscle samples of infected birds were delivered to the Laboratory of Molecular Ecology, Nature Research Centre, Vilnius, Lithuania for further detailed morphological and molecular analysis. The morphological characterization of sarcocysts and bradyzoites was performed in fresh-squashed samples. Sarcocysts with a small amount of host tissue were excised with the help of two preparation needles and were measured under a light microscope (LM) at × 40–1000 magnification.

Genomic DNA was isolated from individual sarcocysts using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania). Partial 28S rDNA was amplified with the help of the KL-P1F/KL-P2R primer pair [13] and the complete ITS1 (internal transcribed spacer 1) region was amplified using the SU1F/5.8SR2 primer pair [14]. The PCRs were conducted as described previously [15]. Visualisation, purification, and sequencing of PCR products were carried out using the previously described protocol [16]. The sequences obtained in this study were compared with those of various *Sarcocystis* spp. using the nucleotide BLAST program (megablast option) [17]. The multiple alignment was conducted using the MUSCLE algorithm loaded in MEGA7 software [18]. Selection of a nucleotide

substitution model and phylogenetic analysis under Bayesian inference were carried out using TOPALi v2.5 [19].

In leg muscles of one of the black kites (*Milvus migrans*) sarcocysts were detected in methylene-blue stained muscle samples; however, they were not observed in fresh-squashed samples. Therefore, the muscle sample of this bird was digested with pepsin according to the modified protocol of Dubey et al. [1]. Five grams of leg muscles were cut into small pieces and suspended in 15 ml of saline solution (0.9%). The entire substance was homogenized in a commercial blender at top speed for 2 min with breaks. The homogenate was transferred into a 150 ml flask and 15 ml of digestion solution was added to it (pepsin, 0.26 g; NaCl 0.5 g; water up to 15 ml and 37% HCl to pH 1.1). The entire substance was incubated at 37°C for 2 hours and the suspension was used for DNA extraction. Genomic DNA was extracted as described above. External PCR primers were SU1F/5.8SR2 [14], meanwhile internal primers GsShalF1 (5'-GATAATTGACTTTACGCGCCATTAC-3') and GsShalR1 (5' GTGCACATCCATATATGCTCATTCT-3') were designed in the present study. The first run of a nested PCR assay was conducted as described by [15]. The second run of a nested PCR was carried out in the final 12.5 µl volume consisting of 6.3 µl of DreamTaq PCR Master Mix, 0.5 µM of each primer, 1 µl from the first run of PCR, and nuclease-free water. The cycling conditions began with one cycle at 95 ℃ for 5 min followed by 35 cycles at 94 ℃ for 45 s, 65 ℃, for 45 s and 72 ℃ for 50 s, and ending with one cycle at 72°C for 7 min. Visualisation, purification, and sequencing of PCR products were performed as described above. Sequences generated in the present study were deposited in GenBank under accession numbers MW926916-MW926917, and MW929599-MW929601.

Results

Sarcocysts were detected in 3/59 (5.1%) birds of prey from Spain. From three to seven sarcocysts were observed in 1 g of methylene blue-stained muscle samples. Under LM, one type of sarcocyst

was observed. Sarcocysts seemed thread-like, 1560 × 143 µm (1050–2160 × 130–158 µm; n = 6) in size, with a thin (0.7–1.4 µm) apparently smooth cyst wall. The sarcocysts were clearly divided by septa into the compartments filled with mature banana-shaped bradyzoites, 5.9 × 1.7 µm (4.8–7.1 × 1.3–2.1 µm; n = 45) in size.

The obtained 830 bp ITS1 sequence of *S. halieti* from the western marsh harrier (*Circus aeruginosus*) (isolate: CaEs1) was 100% identical with *S. halieti* from the great cormorant (*Phalacrocorax carbo*) (JQ733513, MH130209) and from the herring gull (*Larus argentatus*) (MN450340, MN450341). The 569 bp ITS1 sequence generated from the digested muscle sample of the black kite (MmEs2) demonstrated 100% identity to *S. halieti* from the herring gull (MN450344-MN450356) and from the white-tailed eagle (MF946589-MF946590), whereas 830 bp ITS1 sequences obtained for sarcocyst isolated from the black kite (MmEs1) showed 97.2–97.7% similarity to *S. halieti* from the herring gull (MN450340-MN450356), the white-tailed eagle (MF946589-MF946596), and the great cormorant (JQ733513, MH130209), had 96.0% similarity to *Sarcocystis* sp. from the Chilean skua (*Stercorarius chilensis*) (MW160469), 93.2%

similarity to *Sarcocystis* sp. from the Cooper's hawk (*Accipiter cooperii*) (KY348755), and 92.3–92.5% similarity to *S. columbae* from the herring gull (MN450338-MN450339) and from the woodpigeon (*Columba palumbus*) (GU253885, HM125052). In ITS1 phylogenetic tree, the obtained sequences of *Sarcocystis* from the black kites and the western marsh harrier were placed in one cluster together with *S. halieti* and *Sarcocystis* sp. from the Chilean skuas (Fig. 1). It should be noted that the sequence of *Sarcocystis* from the black kite (MmEs1) formed a sister branch to other *S. halieti* sequences. The 1488 bp 28S rRNA sequence of *Sarcocystis* from the black kite (MmEs1) differed in 1–2 SNP from those of *S. halieti* (JQ733512, MF946610, MH130210) and in 7 SNP from those of *S. columbae* (HM125053), while 1508 bp sequence of *Sarcocystis* from the western marsh harrier (CaEs1) demonstrated 99.3–100% identity with *S. halieti*. Thus, on the basis of a molecular examination, *S. halieti* was identified in two black kites and a single western marsh harrier.

Discussion

The present study revealed new IH record for *S. halieti*. This *Sarcocystis* species was identified in the black kite and the western marsh harrier for the first time. Thus far, *S. halieti* have not been observed in the muscles of birds of prey. Previously, *S. halieti* was detected in the great cormorant [20] and the herring gull [21]. The results of the present study extend the knowledge of *S. halieti* specificity for the IH and indicate that this species could form sarcocysts in the birds belonging to at least three different orders, Accipitriformes (present study), Charadriiformes [21] and Suliformes [20]. More avian *Sarcocystis* species, *S. calchasi*, *S. columbae*, *S. falcatula*, *S. wobeseri* can form sarcocysts in IH belonging to different orders [1, 10, 21, 22]. The development of molecular research and expansion of the diversity of the examined host species leads to the detection of the known *Sarcocystis* species in different bird orders [22]. Such investigations are particularly important in terms of pathogenic species. It should be noted that highly pathogenic *Sarcocystis* species, such as, *S. neurona*, *S. canis*, *S. felis*, *S. calchasi*, *S. falcatula* are multi-host specific [1]. Up to date, it is not known whether *S. halieti* is pathogenic. Therefore, extensive histopathological studies of this species are recommended.

Sarcocysts of *S. halieti* detected in muscles of birds of prey seemingly differed morphologically from those previously described in other IH. For comparative purposes, sarcocysts of *S. halieti* from the great cormorant were very long, up to 6.5 × 0.1 mm [20], whereas sarcocysts from the herring gull were from 3960 µm to 7930 µm in length and from 43 µm to 128 µm in width [21]. Sarcocysts identified from the black kites and the western marsh harrier were shorter and wider (1050–2160 × 130–158 µm). Different shapes of *S. halieti* sarcocysts may be associated with a diverse type of the anatomical structure of a host. The distribution of muscle forces of accipitrids, falconids and strigiforms tend to possess greater proportions of distally inserted digital flexor musculature (53–64%, on average) [23].

Due to a lack of a comprehensive microscopical examination it is difficult to compare morphologically the sarcocysts of *S. halieti* identified in the present work with those observed in other birds of prey. Two types of sarcocysts have been reported in the bald eagles from the USA [24]. The first type of sarcocyst was microscopic, had a thin cyst wall with spines and contained bradyzoites 5 × 1 µm in size. Type II

microscopic sarcocysts were immature and had a 2 μm thick striated cyst wall [24]. These sarcocysts were not similar to those observed in our study. By contrast, type II sarcocysts detected in the Eurasian buzzard [11] measured 694–1850 × 42–235 μm, had a seemingly smooth cyst wall and resembled *S. halieti*. Also, histologically thin walled (0.5 μm) sarcocysts having a smooth surface with no visible protrusions were found in the cardiac muscle of the white-tailed sea eagle [25]. The length of the sarcocysts was not determined, however, the diameter of the largest cross-sectioned cyst was 40 μm. Subsequently, *S. wobeseri* was identified in the muscles of the white-tailed sea eagle from the UK [10]. Based on the current knowledge, sarcocsyts of *S. halieti* and *S. wobeseri* are morphologically indistinguishable [21]. Lastly, thin-walled (≤ 1 μm) and thick-walled (2–4 μm) sarcocysts were detected in the muscles of raptors from the south- eastern USA, however, no detailed microscopical examination was performed [8]. The most recent studies on *Sarcocystis* from birds of prey focused on diagnosis of this apicomplexan genus using muscle digestion and subsequent nested PCR [9] or an immunofluorescence antibody test [26]. The above-mentioned methods are relatively sensitive to the detection of *Sarcocystis* spp. However, in case of such methods, morphological characteristics of sarcocysts, which are important to phenotypic diagnosis of *Sarcocystis* species, cannot be determined. In summary, studies on *Sarcocystis* of birds of prey are fragmentary, and it is difficult to compare the results obtained by means of different morphological and molecular methods.

Conclusions

In the present study, *S. halieti* was identified in the black kite and the western marsh harrier from Navarra (Spain) by means of 28S rDNA and ITS1 sequence analysis. This is a third *Sarcocystis* species detected in the muscles of birds of prey. Studies on *Sarcocystis* spp. from birds of prey are fragmentary. Therefore, further complex morphological, histopathological and molecular methods should be employed to provide a comprehensive description of *Sarcocystis* found in birds of prey.

Abbreviations

LM
light microscope; ITS1:internal transcribed spacer; CNS:central nervous system; SNP:single nucleotide polymorphisms; IH:intermediate host; DH:definitive host.

Declarations

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Authors' contributions

Conceptualization, P.P., A.B., S.Š and D.B.; formal analysis, P.P. and S.Š.; investigation, E.J.N., A.B., P.P. and D.B.; resources D.V. and I.O.; writing–original draft preparation, P.P., A.B., E.J.N., S.Š. and D.B.; writing–review and editing, P.P., A.B., E.J.N., S.Š. and D.B.; visualization P.P. and E.J.N.; supervision, P.P. and D.B.; project administration, P.P. and S.Š.; funding acquisition, A.B. P.P. and S.Š. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article. The 28S rRNA and ITS1 sequences generated in the present study were submitted to the GenBank database under accession numbers MW926916-MW926917 and MW929599-MW929601, respectively.

Ethics approval and consent to participate

For this type of formal study consent is not required.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

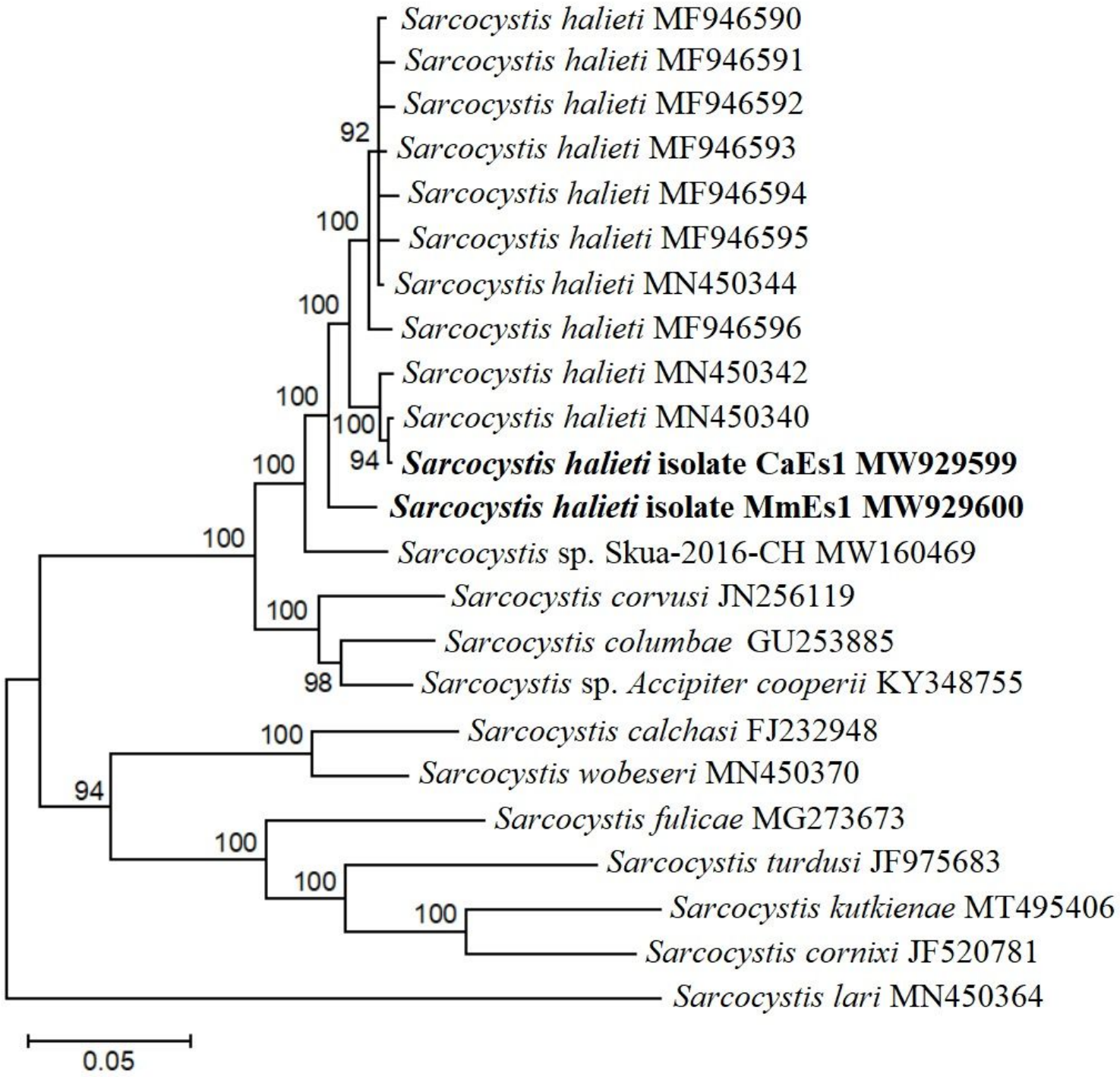

Figure 1

Phylogenetic tree of selected Sarcocystis species based on ITS1 sequences. The tree was constructed using Bayesian methods, scaled according to the branch length, and rooted on S. lari. The final alignment of ITS1 contained 23 taxa and 982 aligned nucleotide positions. Figures next to branches show the posterior probability support values. Sequences generated in the present study are indicated in boldface.

Supplementary Files

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