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Sarcocystis calchasi is distinct to *Sarcocystis columbae* sp. nov. from the wood pigeon (*Columba palumbus*) and *Sarcocystis* sp. from the sparrowhawk (*Accipiter nisus*)

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ABSTRACT

Sarcocystis calchasi has been identified as causative agent of a newly discovered central nervous disease in domestic pigeons (*Columba livia* f. *domestica*) observed for the first time in Germany in 2006. Initial studies have indicated that this parasite is highly pathogenic for domestic pigeons after ingestion of low doses of sporocysts shed by the Northern goshawk (*Accipiter gentilis*). Here we tested whether phylogenetically related birds might regularly harbor *Sarcocystis* species. Five wood pigeons (*Columba palumbus*) and five sparrowhawks (*Accipiter nisus*) from Northern Germany were examined. All birds were PCR negative for *S. calchasi* by universal primers. Instead, both avian species harbored two as yet undescribed *Sarcocystis* species. Light and transmission electron microscopy identified cysts in the skeletal muscle of wood pigeons of 56–156 μm in width. The cysts had a smooth surface without protrusions. Sporocysts derived from the small intestine of the sparrowhawks measured 11.88 μm × 8.34 μm. Polymerase chain reaction amplification and sequencing of the first internal transcribed spacer region (ITS-1), the 18S rRNA and the 28S rRNA gene comprising the variabel D2 and D3 domains further characterized them as two novel *Sarcocystis* species. *S. calchasi* displays a pairwise distance value of the ITS-1 region ranging between 0.165 and 0.195 with the *Sarcocystis* spp. from the wood pigeon and the sparrowhawk, respectively. A phylogenetic analysis further supported the existence of two new species.

1. Introduction

Sarcocystis species are apicomplexan parasites with a heteroxeneous life cycle between herbivores or omnivores as intermediate hosts and carnivores as definitive hosts (Dubey et al., 1989; Mehlhorn and Heydorn, 1978). In general, *Sarcocystis* spp. are regarded to be host-specific. However, some bird-infecting *Sarcocystis* spp. such as *Sarcocystis neurona*, *Sarcocystis falcatula* and *Sarcocystis dispersa* are known for their potential multi-host transmission (Box and Smith, 1982; Cerna and Kolarova, 1978; Dubey et al., 2001, 2003, 2006; Mansfield et al., 2008). In particular *S. neurona* is reported to infect a wide variety of intermediate and incidental hosts.

Although many avian host species are known for *Sarcocystis* spp., only very few are well characterized. We have recently reported a new central nervous disease in domestic pigeons (*Columba livia* f. *domestica*) induced by the newly discovered *Sarcocystis calchasi* which is transmitted by the Northern goshawk (*Accipiter gentilis*; Olias et al., 2009, 2010a,b). The sudden emergence of this parasite and its high pathogenic for domestic pigeons raised questions as to whether the disease may have been over-

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looked clinically due to its similarity with paramyxovirosis and salmonellosis (Olias et al., 2009, 2010a). It has been speculated that *S. calchasi* has been newly introduced into the area and might exhibit an increased virulence for domestic pigeons due to a low parasite–host adaptation. In host–parasite systems, amongst other processes, host switching and failure of the parasite to speciate in response to host speciation have been proposed to explain incongruence in host–parasite co-speciation (Johnson et al., 2003; Paterson and Gray, 1997). Incongruence in host–parasite evolution can express itself as increased parasitic virulence as it is known for old world psittacines infected by *S. falcatula* (Clubb and Frenkel, 1992; Ridley, 2004; Riper et al., 1986). Recent evidence, however, tends to refute this notion and indicates that parasites in novel hosts are often less virulent than in their original host (Poulin, 2007).

To gain insight into these questions, we tested closely related potential hosts, namely the wood pigeon (*Columba palumbus*) and the sparrowhawk (*Accipiter nisus*) for infection with *Sarcocystis* spp. Although a few cases of sarcocystosis have been reported in wild pigeons and doves, no report exists about sarcocystosis in wood pigeons (Olias et al., 2010b). Wood pigeons share large areas in Europe with the domestic pigeon and the Northern goshawk (Baptista et al., 1997; Ferguson-Lees et al., 2001). Notably, like the domestic pigeon, the wood pigeon serves as principal prey for European Northern goshawks (Altenkamp, 2001; Penteriani, 1997). In contrast, the diet of sparrowhawks as generalist predator comprises of primarily small sized birds and only infrequently pigeons and doves (Frimer, 1989; Solonen, 1997). Sparrowhawks have already been reported to harbor *Sarcocystis* spp., however, morphological or genetic characteristics have not been provided in these studies (Cerna and Kolarova, 1978; Svobodova, 1997).

2. Materials and methods

2.1. Sources of wood pigeons and sparrowhawks

Five wood pigeons hunted in Northern Germany, during the hunting season in 2008 were obtained. Three female and two male sparrowhawks found dead in Northern Germany were necropsied in 2008 and 2009.

2.2. Histopathology and electron microscopy

Tissue samples from the gastrocnemius muscle of the wood pigeons and the intestine of the sparrowhawks were fixed in 4% phosphate-buffered formalin, routinely embedded in paraffin and sections of 4 μm thickness were stained with haematoxylin and eosin. For transmission electron microscopy, isolated muscle cysts were fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4 °C, further processed, embedded, and prepared using standard laboratory methods (Mielewczik et al., 2008).

2.3. Sequence analysis

The complete first internal transcribed spacer region (ITS-1), the 18S rRNA gene and a partial region of the 28S rRNA gene comprising the variable D2 and D3 domains were selected for PCR amplification with primer combinations shown in Table 2 and Fig. 4. Briefly, single sarcocysts were excised with a fine needle from leg muscles of five wood pigeons. Sporocysts were derived from the small intestine of five sparrowhawks as described elsewhere (Rommel et al., 1995). DNA from sarcocysts and sporocysts, respectively, was extracted, amplified and sequenced as described (Olias et al., 2009). Nucleotide sequences of the ITS-1 region and of a fused sequence comprising 1630 base pairs of the 18S rRNA and 1278 base pairs of the 28S rRNA were aligned using ClustalW2 (Larkin et al., 2007). Nucleotide distance values of the maximum likelihood phylogenetic tree were calculated with PhyML (Guindon and Gascuel, 2003) under the HKY substitution model (Hasegawa et al., 1985) with base frequency estimates obtained by ML. The program was set to estimate the proportion of invariable sites and the gamma distribution parameter, while the number of substitution rate categories was set to four. The input tree was built using the BIONJ algorithm implemented in PhyML. The maximum parsimony phylogenetic tree was calculated with the PHYLIP package (Felsenstein, 2005). Bootstrapping (1000 replicates) was done using seqboot and the resulting set of alignments was analyzed with DNApars using ordinary parsimony. *Besnoitia besnoiti* was set as outgroup. A consensus tree was computed with consense using the extended majority-rule. Trees in newick format were visualized and processed using MEGA4 (Tamura et al., 2007; Kumar et al., 2008). The same program was used to calculate the percent identity between the ITS-1 regions of *S. calchasi*, *Sarcocystis columbae* sp. nov. and *Sarcocystis* sp. ex *A. nisus* derived from uncorrected pairwise distance (*p*-distance) values.

2.4. GenBank accession numbers

The obtained 18S rRNA, ITS-1 and partial 28 rRNA sequences were deposited into the GenBank database, accession numbers GU253883–GU253888.

3. Results

3.1. Histopathology and electron microscopy of *Sarcocystis* spp. in wood pigeon muscles

Histologically, all examined leg muscles of the five woodpigeons were mildly infested with sarcocysts of varying diameter. The cysts were 106 μm (±50 μm; $n = 20$) in diameter and appeared without visible protrusions (Table 1 and Fig. 1A and B). Some larger cysts exhibited thin-walled chambers free of cystozoites in the center of the cyst (Fig. 1C). Only in one case an area of the muscle next to a cyst with severe lymphohistocytic myositis was present (Fig. 1D).

Utrastructurally, all sarcocysts exhibited an identical cyst wall morphology. The primary cyst wall had a smooth and wavy surface with only few rather short invaginations (Fig. 2). Subjacent an electron-dense ground substance extended to the interior of the cyst and formed septae that subdivided the cysts into chambers filled with cyst merozoites (cystozoites; Fig. 3).