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INFECTIOUS DISEASE

Sarcocystis calchasi in a captive Patagonian conure (*Cyanoliseus patagonus*) in Finland

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Summary

Sarcosystis calchasi is an emerging pathogen causing encephalitis in many avian species and has been documented in North America, Germany and Japan. In November 2019, a captive Patagonian conure (*Cyanoliseus patagonus*), kept in a zoological aviary in Finland, was euthanized due to acute respiratory distress. At necropsy, histopathological examination revealed numerous parasitic tissue cysts in the skeletal muscles and myocardium, chronic moderate multifocal lymphoplasmacytic and histiocytic meningoencephalitis and acute moderate multifocal purulent pneumonia caused by aspiration of foreign material. By light and transmission electron microscopy, tissue cysts had structures typical of *Sarcocystis* organisms. The ultrastructure of the cyst wall was compatible with *S. calchasi* and *Sarcocystis columbae*. *S. calchasi*-specific semi-nested polymerase chain reaction testing resulted in amplification of the internal transcribed spacer (*ITS*) gene, which had 100% identity with *S. calchasi ITS* sequences. This is the first report of *S. calchasi* in Fennoscandia and of a naturally-occurring *S. calchasi* infection in a captive psittacine bird in Europe. Our finding suggests that captive psittacine birds kept in outdoor facilities may be at risk of *S. calchasi* infection throughout the Holarctic.

Keywords: Apicomplexa; protozoal encephalitis; psittacinebirds; *Sarcocystiscalchasi*

Sarcocystis calchasi is a novel emerging apicomplexan parasite that causes encephalitis and myositis in birds (Olias *et al*, 2009, 2010a). Like other *Sarcocystis* species, it has an obligatory two-host prey–predator life cycle (Dubey *et al*, 2016a). *S. calchasi* is non-pathogenic for its definitive hosts, the European *Accipiter* hawks (genus *Accipiter*, order Accipitriformes) (Olias *et al* 2010c, 2011), but highly pathogenic for its intermediate hosts, including avian species from the order Columbiformes (Olias *et al*, 2010b; Ushio *et al*, 2015; Hodo *et al*, 2016; Mete *et al*, 2019), order Psittaciformes (Rimoldi *et al*, 2013; Olias *et al*, 2014) and order Suliformes (Bamac *et al*, 2020). *S. calchasi* DNA has also been detected in woodpeckers (family Picidae, order Piciformes) (Ziegler *et al*, 2018). *S. calchasi* was first reported in domestic pigeons (*Columba livia* f. *domestica*) in Germany in 2009 (Olias *et al*, 2009; Olias *et al*, 2010a). The fatal central nervous system disease caused by the parasite, pigeon protozoal encephalitis (PPE), is a significant disease in racing pigeon flocks in Germany (Parmentier *et al*, 2019). In columbids, *S. calchasi* infections have been reported in North America (Wünschmann *et al*, 2011; Hodo *et al*, 2016; Mete *et al*, 2019), Central Europe (Olias *et al*, 2010a; Parmentier *et al*, 2018, 2019) and Japan (Ushio *et al*, 2015), suggesting a wide global distribution of the parasite. In 2013, an outbreak of severe neurological disease in five psittacine birds of three different species in a zoological exhibit was reported in California, USA. The causative agent was confirmed to be *S. calchasi* (Rimoldi *et al*, 2013).

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Experimental studies have shown cockatiels (*Nymphicus hollandicus*) to serve as intermediate hosts of *S. calchasi* and to develop a severe neurological disease resembling PPE (Olias *et al*, 2014). In this paper we report *S. calchasi* infection in a captive Patagonian conure (*Cyanoliseus patagonus*) in a zoological aviary in Finland. To our knowledge, this is the first report of naturally-occurring *S. calchasi* infection in a captive psittacine bird in Europe and the first documentation of the presence of *S. calchasi* in Fennoscandia.

In November 2019, a captive adult male Patagonian conure, kept in an enclosed zoological aviary in Helsinki (Helsinki Zoo), developed acute respiratory distress. The bird was approximately 15 years old, had been born in the same aviary and had not shown any abnormalities prior to the acute clinical signs. A group of 14 psittacine birds had been kept in the outdoor aviary, which had a mesh roof, from April to September. During winter, the birds were kept inside the tropical house. The mesh size allowed house sparrows (*Passer domesticus*) to enter the outdoor aviary. The affected bird received supportive medication with a non-steroidal anti-inflammatory drug (meloxicam, 0.5 mg/kg; Metacam 20 mg/ml; Boehringer Ingelheim Vetmedica, Lyon, France) and systemic antibiotics (oxytetracycline, 50 mg/kg; Engemycin LA Vet 100 mg/ml; Intervet International, Boxmeer, the Netherlands). Despite treatment, the bird deteriorated and was euthanized due to severe breathing difficulties and was sent for necropsy to the Department of Veterinary Biosciences, Faculty of Veterinary Medicine, University of Helsinki, Finland. A week after this bird had started showing clinical signs, another Patagonian conure in the same group developed similar but milder respiratory signs. The second bird, however, responded to the treatment described above and fully recovered.

The euthanized bird underwent a complete necropsy. Tissue samples were collected from the internal organs, including trachea, sinuses, gonads, gastrocnemius muscle and brain, and were fixed in neutral buffered 10% formalin and routinely processed. Paraffin-embedded tissues were cut (5 µm) and stained with haematoxylin and eosin (HE) for light microscopic examination. Selected sections of lung and brain tissue were also stained with Gram stain and by periodic acid–Schiff (PAS). Unfixed tissue samples from the heart, lungs, liver, kidney and spleen were stored at −18°C.

Formalin-fixed skeletal muscle samples were prepared for transmission electron microscopy (TEM) as described (Hagner *et al*, 2018). Briefly, tissue samples were cut into 1 mm^3 cubes and re-fixed with 2% glutaraldehyde in 100 mM phosphate buffer overnight at 10°C. The samples were then fixed in 1% buffered osmium tetroxide for 1 h. After rehydration, the samples were embedded in embedding resin (TAAB, Aldermaston, UK). Semithin sections were cut and stained with toluidine blue (TB) to select areas with tissue cysts for preparation of ultrathin sections, which were set on pioloform-coated single-slot copper grids and stained with 1% aqueous uranyl acetate and lead citrate (Leica Ultrostain ll; Leica, Wetzlar, Germany). Ultrathin sections were examined with the JEM-1400 transmission electron microscope (Jeol, Tokyo, Japan) and images were taken with an Orius SC1000B bottom-mounted CCD camera (Gatan, Pleasanton, California, USA).

The total DNA was extracted from frozen muscle tissue sample with the Tissue and Hair Extraction Kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The incubation time was 3 h at 56°C with 100 µl of incubation buffer. After extraction, the DNA concentration was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the extracted DNA was stored at −20°C until polymerase chain reaction (PCR) assay. DNA was extracted in triplicate. A negative control (nuclease-free water sample) was included in DNA extraction.

To confirm the presence of *Sarcocystis* spp, a nested PCR amplifying the 18S rRNA locus was performed according to Li *et al* (2002), slightly modified by Hagner *et al* (2018). For a specific amplification, we used a *S. calchasi*-specific semi-nested PCR targeting the internal transcribed spacer (*ITS*) gene, as described by Olias *et al* (2011). The initial amplification was performed using Sca1/Sca2 primers. Amplicons were diluted 1:500 for the second amplification with primers Sca1/SNCa3. The predicted amplicon size was 136 bp. Amplification reactions were performed in 50 µl reaction mixtures containing 200 pM of each primer, 25 µl of PCR Master Mix (Promega) and 17 µl of nuclease-free water. A sample of nuclease-free water served as a negative control. Amplicons were visualized on a 2% agarose gel stained with ethidium bromide and photographed under ultraviolet light.

PCR products were purified using ExoSAP-IT PCR Product Cleanup (Thermo Fisher Scientific) following the manufacturer's instructions. Sequencing was performed by the Sequencing Unit of the Institute for Molecular Medicine Finland, FIMM Technology Centre, University of Helsinki. PCR products were sequenced in duplicate. The results of the sequencing reactions were analysed and edited using Snap Gene Viewer (https://www.snapgene.com/snapgene-viewer/). Sequences were compared with data in GenBank using BLAST searches.

At necropsy, the major gross pathological findings were moderate hydrocephalus with symmetrical dilation of the lateral ventricles. Skeletal muscles of the legs were multifocally pale and unevenly coloured. On histological examination, numerous parasitic intrasarcoplasmic tissue cysts were seen in the gastrocnemius muscle without any associated inflammatory reaction (Fig. 1). The cysts contained multiple bradyzoites and paler-staining metrocytes and the maximal size of the tissue cysts was 600 µm in length and 40 µm in width. Many similar intrasarcoplasmic tissue cysts were also scattered in the myocardium without any associated inflammatory reaction. In the central nervous system, chronic moderate multifocal lymphoplasmacytic and histiocytic meningoencephalitis was seen. Multifocal perivascular infiltration (perivascular cuffing) of histiocytes, lymphocytes and plasma cells was detected in the cerebrum, cerebellum and brainstem (Fig. 2). Lesions were most evident in the brainstem. A single small ovoid parasitic schizont was found in the neuropil of the brainstem, with no associated inflammatory reaction (Fig. 2). This structure measured approximately 14 × 12 µm and was negative for *Toxoplasma* immunohistochemical labelling. No schizonts were detected in any other organ. Histological examination of the lungs revealed acute, moderate, multifocal, purulent pneumonia with numerous foreign plant-like particles in the parabronchi, suggesting acute aspiration pneumonia. Moderate infiltration of heterophils in the parenchyma and parabronchi was seen with moderate parenchymal congestion and haemorrhage. Gram and PAS staining of brain and lung sections did not reveal any specific pathogens.

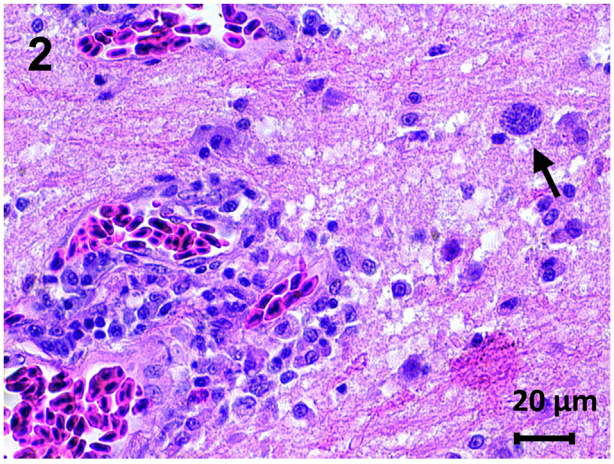

Fig. 2. Sarcocystosis, brainstem, Patagonian conure. Chronic lymphoplasmacytic and histiocytic encephalitis with perivascular cuffing of mononuclear inflammatory cells and a single small schizont in neuropil (arrow). HE.

By TEM examination, all tissue cysts had a similar structural appearance, which was typical of *Sarcocystis* spp (Figs. 3 and 4). The tissue cysts were filled with variable proportions of bradyzoites and pale-staining ovoid metrocytes. Immature cysts contained mostly dividing metrocytes and mature cysts, mainly infectious, peripherally arranged bradyzoites with only a few metrocytes. The bradyzoites had structures typical of an apicomplexan organism including a conoid, multiple micronemes, rhoptries, one mitochondrion and one nucleus. The primary tissue wall was thin, (approximately 60 nm thick) with no protrusions. The surface of the wall was slightly wavy and had occasional small invaginations (Fig. 4).

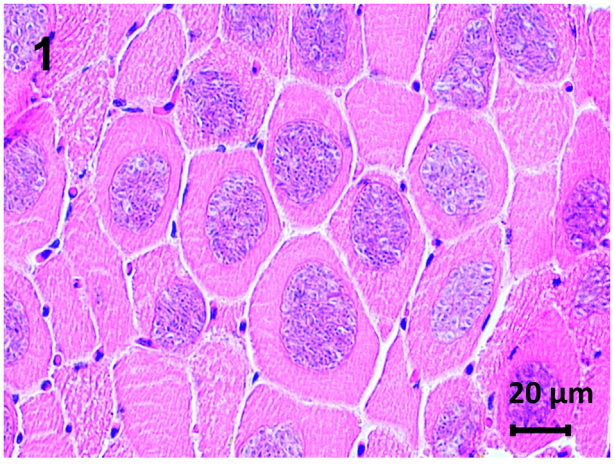

Fig. 1. Sarcocystosis, skeletal muscle, Patagonian conure. Numerous intrasarcoplasmic tissue cysts without any associated inflammatory reaction. HE.

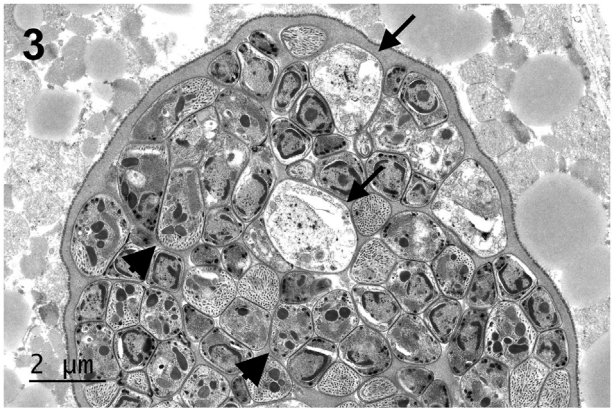

Fig. 3. Sarcocystosis, skeletal muscle, Patagonian conure. Intrasarcoplasmic tissue cyst contains pale-staining ovoid metrocytes (arrows) and smaller mature bradyzoites (arrowheads) with smooth, slightly wavy primary cyst wall but no protrusions. TEM.

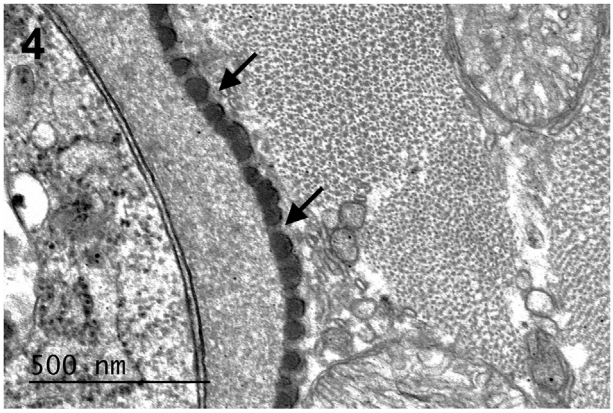

Fig. 4. Sarcocystosis, skeletal muscle, Patagonian conure. Intrasarcoplasmic tissue cyst wall has small knob-like blebs (arrows), typical of cyst wall type 1a. TEM.

Different *Sarcocystis* cyst walls have been categorized into 42 types, with multiple subtypes, based on their ultrastructural differences (Dubey *et al*, 2016a). With this classification system, the cyst wall analysed was compatible with type 1a. The ultrastructure was identical to the previously described cyst wall structure of *S. calchasi* (Olias *et al*, 2010a) and *Sarcocystis columbae*, a species closely related to *S. calchasi* and known to infect multiple avian species (Olias *et al*, 2010d; Dubey *et al*, 2016b; Prakas *et al*, 2020).

Molecular methods were used to specify the species of the detected *Sarcocystis* organism. *S. calchasi*-specific semi-nested PCR testing resulted in amplification of the *ITS* gene, which had 100% identity with *S. calchasi ITS* sequences (GenBank accession numbers FJ232948, MG283135, KT945022, KT945021, KC733715 and MT565292). All negative controls and processing controls were negative.

In the affected bird, multiple mature intramuscular tissue cysts and chronic inflammatory lesions in the central nervous system were suggestive of a chronic *Sarcocystis* infection. These histopathological findings resembled those of naturally-occurring *S. calchasi* infection in psittacine birds (Rimoldi *et al*, 2013). The bird had developed severe acute respiratory distress, which was explained by the suppurative inflammation seen in the lungs. It was considered likely that chronic inflammatory lesions in the central nervous system had predisposed the bird to the development of acute pneumonia as a result of aspiration of foreign material. The hydrocephalus found in this bird at necropsy was considered to be a congenital lesion, rather than due to sarcocystosis. Experimental infections with *S. calchasi* have resulted in a biphasic clinical disease in domestic pigeons (Olias *et al*, 2010b) and cockatiels (Olias *et al*, 2014). Infected cockatiels had severe lethargy and polyuria at 7–13 days post infection (dpi) and some birds died during the acute phase. The histopathological lesions included multifocal hepatic and splenic necrosis and mononuclear inflammation, inflammation and necrosis of the lungs, kidneys and bone marrow and the presence of multifocal schizonts in the liver, spleen and endothelial cells of other visceral organs. In cockatiels surviving the acute phase, a chronic phase followed 57–63 dpi after an asymptomatic period and manifested clinically as moderate to severe neurological signs. Histopathological findings during the chronic phase included severe lymphohistiocytic meningoencephalitis and the presence of tissue cysts in skeletal muscles (Olias *et al*, 2014), which were compatible with the lesions in our case. Therefore, it was likely that the infection in our case had occurred several weeks previously.

Intermediate hosts become infected by ingesting infectious *Sarcocystis* sporocysts in water or food. Sporocysts are infective immediately after faecal shedding by the definitive host and may remain viable for several months in the environment (Dubey *et al*, 2016a). In this case, psittacine birds in the aviary often foraged on the ground while being outside and contamination of soil with the faeces of free-ranging birds flying over the cages was considered a possibility. Southern Finland is within the natural habitat range of both European *Accipiter* hawks, namely the Eurasian sparrowhawk (*Accipiter nisus*) and the Northern goshawk (*Accipiter gentilis*) (Ferguson-Lees and Christie, 2001), both of which are known to act as definitive hosts of *S. calchasi* (Olias *et al*, 2010c; Olias *et al*, 2011). Therefore, we considered the most probable source of infectious sporocysts to be the soil of the outdoor cages contaminated by faeces of *Accipiter* hawks. In racing pigeon flocks in Germany, defaecation by *Accipiter* hawks into the aviaries has been considered an important route of transmission of *S. calchasi* to pigeon flocks (Parmentier *et al*, 2019). Rimoldi *et al* (2013) also suspected faeces-contaminated soil as a possible route of transmission of *S. calchasi* in an outbreak in captive psittacines.

Previous studies have indicated a high prevalence of *S. calchasi* infection in *Accipiter* hawks (Olias *et al*, 2011; Parmentier *et al*, 2018), domestic pigeons (Parmentier *et al*, 2019) and common woodpigeons (*Columba palumbus*) (Parmentier *et al*, 2018) in Germany. Northern goshawks have a wide distribution across the Holarctic and Eurasian sparrowhawks across the Palearctic (Ferguson-Lees and Christie 2001) and the distribution of *S. calchasi* has been suspected to reflect that of its free-ranging definitive and intermediate hosts (Olias *et al*, 2011; Parmentier *et al*, 2018). Migratory hawks have also been speculated as having a role in dispersing the parasite to

novel areas (Olias *et al*, 2011). Our current report of the presence of *S. calchasi* in Fennoscandia supports the earlier hypothesis that the distribution of *S. calchasi* is wider than previously documented (Olias *et al*, 2010c; Olias *et al*, 2011).

Combined with previous reports, our current findings indicate that captive psittacine birds kept in open or semi-open outdoor facilities, with potential exposure to the faeces of free-ranging birds, are at risk of *S. calchasi* infection. Therefore, *S. calchasi* should be included in the list of differential diagnoses for neurological or unspecific clinical signs in psittacine birds having access to outdoor facilities in the Holarctic. Many avian species of the order Psittaciformes are threatened and *S. calchasi* seems to be highly pathogenic for multiple psittacine species (Rimoldi *et al*, 2013; Olias *et al*, 2014). Further epidemiological and experimental studies are required to enable a more detailed risk assessment of this emerging pathogen.

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Conflict of Interest Statement

The authors declared no potential conflicts of interest with respect to the research, authorship or publication of this manuscript.

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