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NOTE

Pathology

Meningoencephalitis with malacia caused by *Sarcocystis calchasi* in a rock pigeon in Japan

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ABSTRACT. *Sarcocystis* spp. cause pigeon protozoan encephalitis, a neuronal disease. A female pigeon exhibiting torticollis had a necrotic area in the cerebral hemisphere surrounded by lesions with perivascular cuffing, gliosis, granulomatous foci, and meningitis. Non-necrotic lesions were also observed in the brainstem. Intact and degenerative schizonts were observed within the neuropils and neurons in the lesions. Deoxyribonucleic acid (DNA) was extracted from paraffin-embedded brain tissues and genetically analyzed after gel electrophoresis to determine *Sarcocystis* spp. using specific primer sets for 28S ribosomal ribonucleic acid and internal transcribed spacer region-1. DNA sequencing confirmed a significant homology with *S. calchasi*. This is the first report of meningoencephalitis with malacia caused by *S. calchasi* in a rock pigeon in Japan.

KEYWORDS: malacia, pigeon protozoan encephalitis, rock pigeon, *Sarcocystis calchasi*

Apicomplexan parasites of the genus *Sarcocystis* cause diseases in various species, though they have a highly pathogenic predilection for the central nervous system of wild birds [1, 10, 13]. *Sarcosystis falcatula* infection induces neuronal signs in free-ranging horned owls [16] and free-ranging eagles [17] in the USA. Olias *et al.* reported *Sarcocystis* spp., which exhibited only 51% nucleotide sequence similarity in the internal transcribed spacer region (ITS-1) with *S. falcatula* in Germany [7]. *Sarcocystis* spp. was identified as *Sarcocystis calchasi*, which causes pigeon protozoan encephalitis (PPE), a novel neuronal disease in domestic pigeons [8]. Spontaneous PPE associated with *S. calchasi* has also been reported in white-winged doves and domestic pigeons in the USA [3, 18] and in a rock pigeon in Japan [15]. Protozoa, schizonts, and merozoites have been observed in brain lesions associated with lymphohistiocytic, lymphoplasmacytic, or granulomatous encephalitis and meningoencephalitis [3, 7, 15, 18]. Herein, we encountered the first case of PPE in a rock pigeon with granulomatous meningoencephalitis and malacia in Japan, best to our knowledge, and confirmed *S. calchasi* caused the lesion.

A female wild rock pigeon (*Columba livia*) was picked from Western Tokyo because it exhibited torticollis. The pigeon was diagnosed with a poor prognosis and euthanized using pentobarbital anesthesia by a veterinarian. Macroscopically, the left cerebral hemisphere had collapsed and softened (Fig. 1). Tissue specimens including brain, lung, heart, spleen, and liver were fixed in 10% neutral buffered formalin, embedded in paraffin, and sliced into 3 µm-thick sections, which were then stained with hematoxylin-eosin (HE), and Luxol Fast Blue (LFB)-Periodic acid-Schiff (PAS). Immunohistochemical staining using anti-CD3 antibody, anti-paired box 5 (PAX5) antibody, anti-glial fibrillary acidic protein (GFAP) antibody, and anti-ionized calcium-binding adapter molecule 1 (Iba1)

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antibody was conducted on brain lesions to identify T and B lymphocytes, microglia, and astrocytes, respectively (Supplementary Table 1).

Histopathologically, we observed an extensive necrotic area in the left cerebral hemisphere in the HE- and LFB-PAS-stained sections, which were notably coarsened, with aggregation of fat granule cells and neovascularization in the central parts (Fig. 2). The necrotic area contained Iba-1-positive microglia/macrophages and GFAP-positive reactive astrocytes (Fig. 3). It was surrounded by relatively intact neuropils with perivascular cuffing, gliosis, and granulomatous lesions (Fig. 4). Perivascular cuffing and gliosis with heterophils and mononuclear cells were observed in the right cerebral hemisphere and brainstem. Mononuclear cells had infiltrated the meninges of the cerebrum and brainstem (Fig. 5). Iba1-positive microglia/macrophages and CD3-positive T lymphocytes were observed in the non-necrotic areas and meninges, and a smaller population of PAX5-positive B lymphocytes was also observed in these lesions (Supplementary Figs. 1–4).

Subsequently, we identified pathogens associated with neuropathological changes in HE- and LFB-PAS-stained sections. In the non-necrotic lesions, immature to mature schizonts (Fig. 6) were scattered within the neuropil and neurons. Intact schizonts were PAS-negative; however, degenerative schizonts contained PAS-positive granules observed in the neuropils and microglia. Protozoa were not identified in either malacia or severe granulomatous lesions.

Some sarcocysts and mononuclear cell infiltrates were observed in the heart. Mononuclear cell infiltrates and necrotic foci were observed in the liver, and diffuse mononuclear cell infiltration was observed in the kidney; however, protozoa were not identified in these organs.

We extracted deoxyribonucleic acid (DNA) from formalin-fixed paraffin-embedded (FFPE) sections of the cerebrum, cerebellum, midbrain, medulla oblongata, heart, kidneys, lungs, liver, and spleen using NucleoSpin® DNA FFPE XS (MACHEREY-NAGEL GmbH & Co., KG, Duren, Germany). The extracted DNA samples were amplified using specific primer sets for the ITS-1 region and complete D2 in the conserved regions of the 28S ribosomal ribonucleic acid (rRNA) of *Sarcocystis* spp. (Supplementary Table 2). For ITS1, polymerase chain reaction (PCR) was conducted using the primer pairs *SCa1* and *SCa2* for initial amplification, and the amplicons were amplified using the primer pair *SCa1/SNca3* [11, 14]. For the 28S rRNA, PCR was conducted using the primer pairs *SAD2F* and *SAD2R* [18]. According to the manufacturer's instructions, real-time PCR was performed using the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Specific bands of 350 and 220 bp were detected in the midbrain and cerebrum for the 28S rRNA and in the heart, medulla oblongata, midbrain, and cerebrum for ITS-1, respectively (Figs. 7 and 8, Supplementary Figs. 5 and 6). Mixtures of the midbrain and cerebrum samples for 28S rRNA and mixtures of the heart and cerebrum

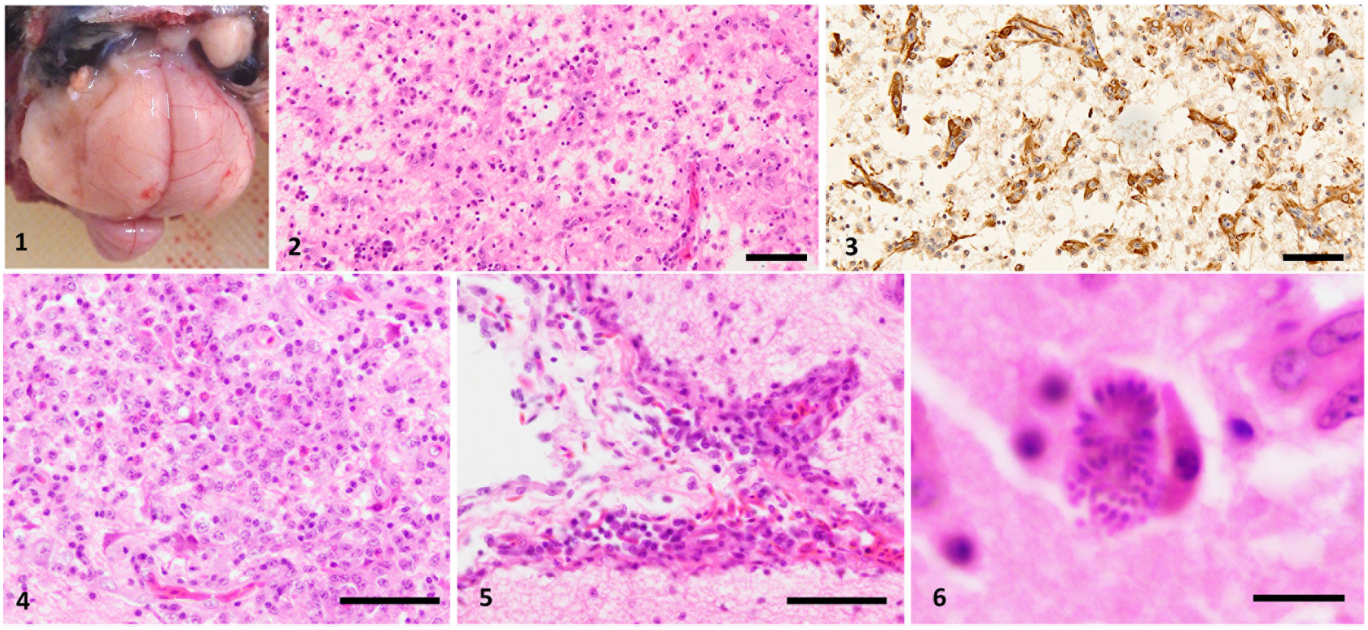

Fig. 1. Macroscopic image of the brain. The left cerebral hemisphere is collapsed, suggesting cerebral malacia.

Fig. 2. Necrotic area with fat granule cells in the left cerebral hemisphere. Hematoxylin and eosin stain, bar=50 µm.

Fig. 3. Necrotic area in the left cerebral hemisphere. Reactive astrocytes are observed in the necrotic neuropils. Immunohistochemistry for glial fibrillary acidic protein, bar=50 µm.

Fig. 4. Granulomatous lesions in the right cerebral hemisphere. Microglia, lymphocytes, and heterophils are observed. Hematoxylin and eosin stain, bar=50 µm.

Fig. 5. Mononuclear cell infiltration in the meninges of the right cerebral hemisphere. Hematoxylin and eosin stain, bar=50 µm.

Fig. 6. A schizont with merozoites. Hematoxylin and eosin stain, bar=20 µm.

samples for ITS-1 were isolated and purified for sequencing. Sequences were aligned using the MUSCLE algorithm implemented in the MEGA X software (Megasoftware. net). Evolutionary history was deduced using the maximum-likelihood method. The Tamura 3 parameter model with gamma distribution and the Hasegawa-Kishino-Yano model with gamma distribution were selected as the nucleotide substitution models that best fit the aligned sequence dataset. These models were used to construct phylogenetic trees for the 28S rRNA and ITS-1. A bootstrap test with 1,000 replicates was carried out to evaluate the robustness of the implied phylogeny [2, 4]. For each *Toxoplasma gondii* was selected as an outgroup for recombinant DNA sequencing. ITS-1 and 28S rRNA nucleotide sequences were deposited in the DNA Data Bank of Japan under accession numbers LC796271 and LC796270, respectively.

Genetic analysis of the FFPE sections of the brain and heart, but not of other organs, detected two sets of specific bands by gel electrophoresis, and *S. calchasi* was identified by BLAST analysis of the nucleotide sequences of the PCR products (Figs. 7 and 8, Supplementary Figs. 5 and 6). Using the 350 bp sequences from 28S rRNA, BLAST analysis showed 99.68 and 99.66% similarity with *S. calchasi* from roller pigeons (KU220951) as well as white-winged doves and Eurasian collared doves (KT945019), respectively, and 99.68% similarity with *Sarcocystis* spp. from racing pigeons (FJ232949) (Fig. 8). Using the 220 bp sequences from ITS1, BLAST analysis showed 96.32% similarity with *S. calchasi* from *Accipiter gentilis*, *Accipiter nisus* (OQ848675), and racing pigeons (FJ232948) (Supplementary Fig. 6). Based on phylogenetic analysis, 350 bp sequences from 28S rRNA and 220 bp sequences from ITS1 formed one cluster with those of *S. calchasi* identified in Columbiformes as intermediate hosts. The 350 bp sequences from 28S rRNA clustered with 95% bootstrap support in the tree branch corresponding to *S. calchasi*, whereas the 220 bp sequences from ITS1 showed 52% bootstrap support within the same branch. Other *Sarcocystis* spp. grouped outside this branch. Hence, DNA sequencing of the rock pigeon examined here confirmed significant homology to *S. calchasi*. The sequences were classified into a group distinctively identified as *S. calchasi*, and distinct from *S. falcatula* and *S. halieti*. However, all three species are known to induce encephalitis and meningitis in the central nervous system, and myositis in the skeletal muscles [3, 6, 15–17]. Newcastle disease virus, avian herpesvirus, and avian influenza virus genes were not detected in the FFPE sections of the brain samples.

S. calchasi and *S. falcatula* are highly pathogenic to the brains of intermediate hosts, including pigeons, owls, and eagles [3, 7, 12, 15–17] (Supplementary Table 3). *S. halieti* can also migrate to the brain and induce cerebral damage in a juvenile owl [6]. These *Sarcocystis* spp. induced similar pathological changes, that is, lymphohistiocytic or granulomatous encephalitis and meningoencephalitis in the cerebrum, cerebellum, or brain stem [3, 6, 7, 12, 15–17]. However, some variations in pathological changes were identified, depending on the pathogen and the intermediate host. T lymphocyte-mediated inflammation was mainly observed in the brains of pigeons infected with *S. calchasi* [12, 15], whereas plasma cells and Mott cells were highly observed in eagles [3]. More severe damage, that is necrotizing encephalitis or malacia, was found in the brains of pigeons infected with *Sarcocystis* spp., which could potentially be *S. calchasi* [7], and in the great horned owl infected with *S. falcatula* [16]. Necrotic changes were also experimentally induced by oral inoculation with 10^{2-4} sporocysts of *S. calchasi* derived from the northern goshawk [12]. In Japan, multifocal inflammatory changes, including mononuclear cell infiltration, perivascular cuffing, and meningitis, have been reported in the brain of a rock pigeon [15]; however, necrotic changes have not been reported. Although the location of the brain lesion has not been reported in detail, it has been reported to occur in the cerebellum and brainstem in spontaneous cases of *S. calchasi* infection [7]. It is considered rare for a lesion confined to the left cerebral hemisphere, as in the present case.

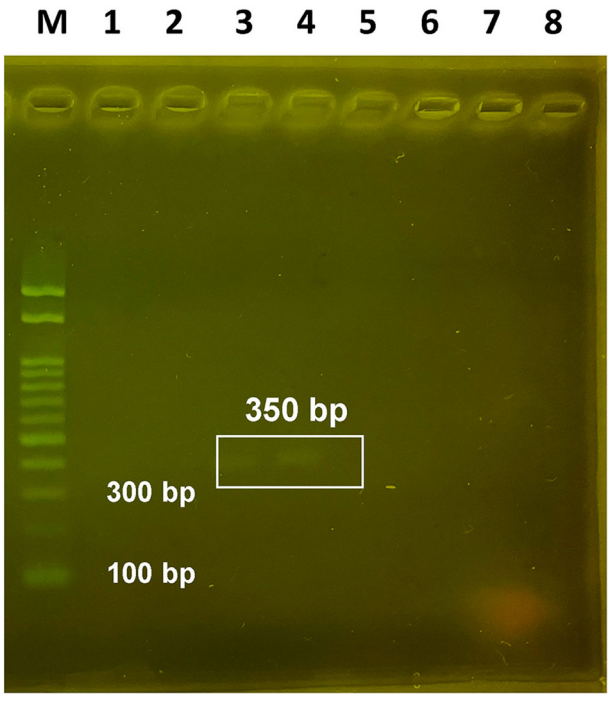

Fig. 7. Agarose gel electrophoresis (2%) shows the expected sizes (350 bp) of PCR products from DNA using the primer pairs SAD2F/SAD2R for 28S rRNA. Lane M, DNA ladder; Lane 1, heart; Lane 2, medulla oblongata; Lane 3, midbrain; Lane 4, cerebrum; Lane 5, kidney; Lane 6, lung; Lane 7, liver; Lane 8, spleen. PCR, polymerase chain reaction; DNA, deoxyribonucleic acid; rRNA, ribosomal ribonucleic acid.

Fig. 8. Phylogenetic analysis for 28S rRNA using the maximum likelihood method with 1,000 bootstrap replications. Values <50% are not shown. The nucleotide sequences of 28S rRNA detected in this study were contained within *Sarcocystis calchasi* and formed a separate branch within a group encompassing *Sarcocystis* spp. obtained from birds as intermediate hosts or definitive hosts. *Toxoplasma gondii* was included as the outer group. Bootstrap values are shown on the interior branch nodes, and scale bars indicate the number of substitutions per site. Individual *Sarcocystis* spp. are named as follows: accession number, definitive or intermediate host, and species of *Sarcocystis* (country). A red circle represents the *Sarcocystis* spp. identified as LC796270 in this study. rRNA, ribosomal ribonucleic acid.

Previous studies reported neurological signs and brain lesions in detail in pigeons infected with *S. calchasi* in the USA, Germany, and Japan [3, 8, 15, 18]. The *S. calchasi*-infected pigeons exhibited neurological signs such as polyuria, diarrhea, torticollis, tremors, and paralysis, which appeared more severe than that of the pigeon with torticollis examined in this study. Previous studies also reported the biphasic pathogenesis of brain lesions in PPE, classified into early acute and late chronic stages [9, 10]. One group of infected pigeons died of multi-organ failure caused by many protozoan infections in the acute phase. In contrast, other groups of infected pigeons died of neuronal and cardiac dysfunction caused by a low number of protozoan infections in the chronic phase. Encephalitis develops slowly through a delayed-type hypersensitivity against schizonts and immature sarcocysts [5, 9, 10, 12]. Thus, the chronic stage is characterized by a marked upregulation of interferon-γ expression, accompanied by massive mononuclear cell infiltration, consistent with the T cell-mediated granulomatous lesions in the present case and other reports [12, 15]. The pathogenesis of meningoencephalitis with malacia caused by *Sarcocystis* spp. is not fully understood; therefore, the relationship between antigens derived from degraded protozoa and the progress of granulomatous encephalitis should be explored further in future studies.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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