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Short Communication

No evidence of *Sarcocystis calchasi* involvement in mammalian meningoencephalitis of unknown origin

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ABSTRACT

Sarcocystis calchasi has recently been identified as the cause of pigeon protozoal encephalitis, PPE, a lethal brain disease in pigeons and parrots. While only avian species have been identified so far to be susceptible to this pathogen as definitive or intermediate hosts, we speculated whether mammals may be susceptible as well, as in *Sarcocystis neurona* and other related apicomplexan parasites. Specifically, we hypothesized its involvement in mammalian meningoencephalitis of unknown origin, MUO. A total of 143 archived formalin fixed, paraffin embedded brain samples with MUO from dogs, cats, pigs, cattle, sheep, guinea pigs, horses, goats, mice, raccoon, ferret, hamster, mink and maned wolf were examined pathohistologically and by PCR for parasitic stages or DNA, respectively, of *Sarcocystis calchasi* or other apicomplexan parasites. All samples had non-suppurative, lymphoplasmacytic and/or granulomatous encephalitis or meningoencephalitis typical of MUO with many similarities to PPE in pigeons. However, neither parasitic structures nor DNA of *Sarcocystis calchasi* or other apicomplexan parasites were detected. It thus appears that, despite histological similarities between mammalian MUO and pigeon PPE and despite seemingly high prevalence of PPE and a persistent threat by *Sarcocystis calchasi* in pigeons, based on histopathology and PCR there is no evidence for a role of this parasite in MUO in mammals as intermediate or aberrant hosts.

1. Introduction

Sarcocystis calchasi was recently discovered as the cause of pigeon protozoal encephalitis (PPE), a highly fatal neurologic disease in domestic pigeons, *Columba livia f. domestica* (Olias et al., 2013). It has been demonstrated in the Berlin area, Germany, in 2006 where the infection continues to be highly prevalent (Olias et al., 2009). Subsequent cases have been reported from the USA also in parrots (Rimoldi et al., 2013; Wunschmann et al., 2011) and in Japan (Ushio et al., 2015) and recent epidemiologic surveys have suggested a wide parasite distribution throughout Germany (Mayr et al., 2015, personal communication).

Sarcocystis calchasi has a close phylogenetic relationship to other well-known pathogenic species of its family, such as *Sarcocystis neurona*, *Sarcocystis tenella*, *Neospora caninum* and *Toxoplasma gondii* (Olias et al., 2009). It has an obligatory two-host life cycle, similar to other members of the family Sarcocystidae. Hawks of the genus *Accipiter* are the definitive hosts, and Columbiformes (Olias et al., 2010a, 2014) and Psittaciformes (Olias et al., 2014; Rimoldi et al., 2013) have been identified as intermediate hosts. The sporocysts excreted by the hawk are ingested by the pigeon where the infection results in severe brain lesions that make the infected pigeon an easily accessible pray for its predators which serve as definitive hosts (Maier et al., 2015; Olias et al., 2013). PPE is clinically a biphasic disease in the intermediate host. The first phase is caused by schizogony of the parasite, mostly in the liver, and results in polyuria, diarrhea and apathy. In the second phase pigeons develop non-suppurative lymphocytic and granulomatous meningoencephalitis with severe clinical neurological deficits including torticollis, paralysis and trembling. This phase is also accompanied by cyst formation in the striated muscles and, in case of cyst rupture, myositis (Maier et al., 2015; Olias et al., 2009). This depends on the infectious dose as high doses cause mortality during the first phase and low doses might skip the first phase, at least from the clinically visible point of view. The hawks acquire the infection by consumption of pigeon tissue cysts. These are then digested and cystozoites liberated from the cysts undergo gamogony in the mucosa of the small intestine, in some cases causing mild diarrhea in the definitive host (Olias et al., 2010b).

Several representatives of the Sarcocystidae family, including *Sarcocystis neurona*, *Neospora caninum* and *Toxoplasma gondii*, are infectious for more than one intermediate or aberrant hosts and are capable of evoking meningoencephalitis in virtually all of them (Dubey et al., 2001; Dubey and Schares, 2011; Elmore et al., 2010). *Toxoplasma gondii* which is the best studied apicomplexan so far may infect virtually all warm-blooded animals as intermediate hosts (Elmore et al., 2010).

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Sarcocystic calchasi has already been schown to cause encephalitis in more than one avian intermediate host species including cockatiels (*Nymphicus hollandicus*), North American rock pigeons (*Columbia livia f. dom.*), Alexandra parrots (*Polytelis alexandrae*), long-billed corellas (*Cacatua tenuirostris*) and rose-breasted cockatoos (*Eolophus roseicapilla*) (Olias et al., 2014; Rimoldi et al., 2013). However, dogs, ferrets, rats and mice failed to serve as definitive host (Olias et al., 2010a). The close phylogenetic relationship between *Sarcocystis calchasi* and *Sarcocystis neurona*, the latter capable of infecting both birds and mammals, led to our hypothesis that *Sarcocystis calchasi* may also be infectious and may cause disease in mammals as intermediate or aberrant hosts.

Non-suppurative inflammation of the brain is common in many mammalian species and may be induced by a wide range of pathogens, autoimmune diseases and toxins. Non-infectious causes include entities such as granulomatous meningoencephalitis (GME), necrotizing meningoencephalitis (NME), necrotizing leukoencephalitis (NLE; Talarico and Schatzberg, 2010) and non-suppurative meningoencephalitis (Schwab et al., 2007). However, such lesions with unknown causes are commonly referred to as meningoencephalitis of unknown origin, MUO. Typical histological patterns include infiltration of mononuclear leukocytes i.e. lymphocytes, plasma cells, and histiocytes, into the Virchow-Robin space and neuropil, often accompanied by a glial reaction (Sanchez et al., 2013). Macrophages as hallmark of granulomatous inflammation are involved to varying degrees. By definition, the etiology and pathogenesis of MUO remain unclear until a cause is discovered in any specific case. For example, cases that historically had been included in the MUO group have eventually been identified as *Sarcocystis neurona* induced equine protozoal myeloencephalitis, EPM (Schwab et al., 2007). Moreover, PPE in pigeons shares many pathological similarities with typical cases pf mammalian MUO. Therefore, we speculated that *Sarcocystis calchasi* may also be involved in case of mammalian MUO. To test our hypothesis, we tried to detect stages of *Sarcocystis calchasi* histologically and their DNA using PCR in 143 archived cases of MUO in a wide range of mammalian species from the Berlin region where the parasite is highly prevalent.

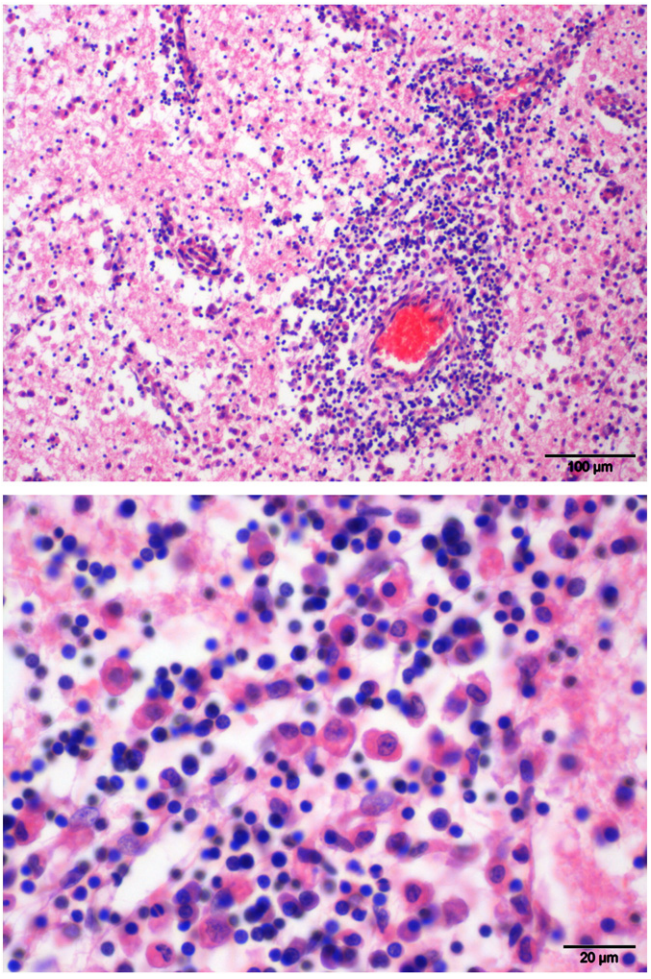

Fig. 1. A. Cerebral cortex, severe chronic multifocal lymphohistiocytic encephalitis with multilayered perivascular cuffs in a dog with meningoencephalitis of unknown origin. B. Lymphocytes, macrophages and fewer plasma cells present in the inflammatory infiltrate (H&E stain).

2. Materials and methods

2.1. Biological samples

Formalin-fixed, paraffin embedded (FFPE) brain tissue samples from 143 animals of 15 mammalian species from the Berlin area, necropsied in the period between 1989 and 2012, were selected from the archive of the Institute of Veterinary Pathology of the Freie Universitaet Berlin. The sample pool consisted of 60 dogs (41.9%), 41 cats (28.7%), eight pigs (5.6%), seven cows (4.9%), six sheep (4.2%), six guinea pigs (4.2%), four horses (2.8%), three goats (2.1%), two mice (1.4%), one ferret (0.7%), one hamster (0.7%), one mink (0.7%), one maned wolf (*Chrysocyon brachyurus*; 0.7%), one raccoon (0.7%) and one common squirrel monkey (*Saimiri sciureus*; 0.7%). The samples included cerebral cortex, cerebellum and brain stem. The selection criterion for the paraffin blocks and corresponding hematoxylin and eosin (H&E) stained histological slides of brain tissue was the diagnosis of non-suppurative meningitis, meningoencephalitis and/or encephalitis of unknown origin. The slides were reexamined by light microscopy (Olympus BH2 BHS, Olympus, Tokyo, Japan) by a veterinary pathologist. All samples with predominantly neutrophilic inflammatory components were sorted out for not being consistent with the sought histopathological pattern. Only the lesions with lymphocytic, plasmacytic and/or histiocytic/granulomatous inflammation resembling the histomorphology of PPE in pigeons and *Sarcocystis neurona* infection in horses, for example, were included.

2.2. DNA extraction and PCR reaction

Total DNA was extracted from FFPE tissue blocks (40 slices at 1 µm) using the DNA isolation kit NucleoSpin®FFPE DNA (Macherey-Nagel, Germany) according to the manufacturers' protocol. The obtained DNA concentration varied between 2.78 ng/µl and 847.1 µg/µl. The average template amount used in one reaction equaled approximately 300 ng.

For the specific amplification of *Sarcocystis calchasi* DNA a primer pair (SCa1: 5′-CTCCTTGCTCGAGAATGAACATGAG-3′; SCa2: 5′-GATCATCTTTTCGACGACAATATCG-3′) corresponding to the ITS1 region (GeneBank Accession Number: FJ232948) of the 18S ribosomal RNA (18S rRNA) gene (GeneBank Accession Number: GQ245670), with a predicted amplicon size of 222 base pairs (bp) was used as described. This primer pair was specific only for *Sarcocystis calchasi* and did not amplify closely related species (Olias et al., 2011).

For the amplification of other species from the phylum Apicomplexa including other *Sarcocystis* spp. a second primer pair (SAD 2F: 5′-GGAA GCGATTGGAACC-3′; SAD 2R: 5′-CCTTGGTCCGTGTTTCA-3′) located in the D2 domain of the conserved 28S ribosomal RNA gene (GeneBank Accession Number: FJ232949), with predicted amplicon size of 349 bp, was used as described (Wunschmann et al., 2011). Because the target domain is a region present in a number of apicomplexan species, the use of this primer pair allowed for a consensus PCR able to detect more species.

Length and integrity of DNA extracted from the FFPE samples were tested using PCR amplification of EF1α as reference gene and primer pairs for two amplification products of two separate lengths, 198 bp