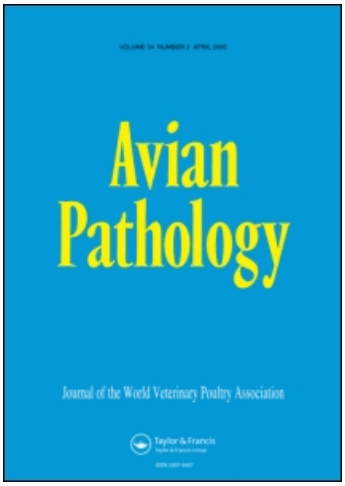

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A novel *Sarcocystis*-associated encephalitis and myositis in racing pigeons

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Sarcosporidian cysts in the skeletal muscle of domestic pigeons (*Columba livia* f. *domestica*) have previously been attributed to infection with *Sarcocystis falcatula*, which is shed in the faeces of the opossum (*Didelphis virginiana*). Here, we describe fatal spontaneous encephalitis and myositis associated with *Sarcocystis* infections in three flocks of racing pigeons with 47 of 244 animals affected. The clinical course was characterized by depression, mild diarrhoea, torticollis, opisthotonus, paralysis and trembling. Histopathological examination of 13 pigeons revealed generalized severe granulomatous and necrotizing meningoencephalitis and myositis with sarcosporidian cysts. Light and transmission electron microscopy identified cysts in heart and skeletal muscle of 1 to 2 mm in length and 20 to 50 μm in width. These were subdivided into small chambers by fine septae and filled with lancet-shaped cystozoites (7.5×1.5 μm) and dividing metrocytes, which is characteristic for *Sarcocystis.* The cysts had smooth walls and were devoid of protrusions typical of *S. falcatula*. Polymerase chain reaction amplification and sequencing of the internal transcribed spacer region (ITS-1) and the complete 28S rRNA identified a novel *Sarcocystis* species with only 51% ITS-1 nucleotide sequence similarity with *S. falcatula*. A phylogenetic comparison of the 28S rRNA revealed close sequence homologies with *Frenkelia microti*, *Frenkelia glareoli* and *Sarcocystis neurona.* The clinical, histopathological, electron microscopic and genetic data are unlike any previously described protozoan infections in pigeons, suggesting a novel, severe disease due to an as yet undescribed *Sarcocystis* species.

Introduction

Sarcocystis organisms are apicomplexan parasites with a two-host lifecycle between herbivores or omnivores as intermediate hosts and carnivores as definitive hosts. Intermediate hosts (prey) become infected through the ingestion of sporocysts released in the faeces of definitive hosts (predators). After schizogony in various organs in the intermediate host, the final stage consists of mature cysts containing cystozoites, mostly located in striated muscle. Definitive hosts become infected through ingestion of tissue cysts, whereby cystozoites enter directly into gamogony in the intestinal wall, where they develop into sporulated oocysts of the *Isospora* type (Mehlhorn & Heydorn, 1978).

Although to date more than 189 species of *Sarcocystis* have been identified, little is known of the incidence and lifecycle of *Sarcocystis* parasites in birds (Odening, 1998). Among the few species that have been characterized is *Sarcocystis horvathi*, which cycles between the chicken (*Gallus gallus*) as intermediate host and the dog (*Canis lupus*) as definitive host (Wenzel *et al.*, 1982). *Sarcocystis wenzeli* uses the same hosts, but with cats (*Felis catis*) as the alternative definitive host (Wenzel *et al.*, 1982). The *Sarcocystis rileyi* lifecycle includes ducks (Anatidae) and skunks (*Mephitis mephitis*; Riley, 1931; Cawthorn *et al.*, 1981). *Sarcocystis falcatula* uses miscellaneous avian species as intermediate hosts and the North American opossum (*Didelphis virginiana*) as the definitive host (Box & Duszynski, 1978; Box *et al.*, 1984). The brown headed cowbird (*Moluthrus ater*) has recently been identified as the intermediate host of *Sarcocystis neurona* (Mansfield *et al.,* 2008), the definitive host of which is the opossum (*D. virginiana* and *Didelphis albiventris*) (Dubey *et al.*, 2001a).

Few cases of sarcocystosis have been reported in pigeons since their first mention in the literature (Dylko, 1962). Barrows & Hayes (1977) detected sarcosporidian cysts in the striated muscle of 32 mourning doves and in the cardiac muscle of six out of 255 mourning doves (*Zenaida macroura*) (12.5% and 2.3%, respectively) with no evidence of clinical or pathological consequences. A second epidemiological study found asymptomatic *Sarcocystis* infections in the pectoral muscle of mourning doves (*Z. macroura*; prevalence of 8.9%) and white-winged doves (*Zenaida asiatica*; prevalence of 10.4%) in

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Florida (Conti & Forrester, 1981). Kaiser & Markus (1983) detected sarcosporidian cysts in three out of 70 laughing doves (*Streptopelia senegalensis*) in South Africa. In Victoria crowned pigeons (*Goura victoria*), three cases of spontaneous acute fatal pneumonia due to a *Sarcocystis falcatula*-like protozoan species have been described (Suedmeyer *et al.*, 2001). In experimental infection studies reported so far, domestic pigeons (*Columba livia* f. *domestica*) developed *S. falcatula* cysts only in the skeletal muscle without evidence of clinically relevant muscle damage or brain lesions (Box & Smith, 1982; Box *et al.*, 1984; Smith *et al.*, 1990). Clinical signs have not been reported in any of these studies.

In the present investigation, we describe the clinical, histopathological, electron microscopic and genetic findings of 13 domestic pigeons from three different flocks of racing pigeons infected with an as yet undescribed *Sarcocystis* species.

Materials and Methods

Case history. Between 2006 and 2008, 47 racing pigeons from three different flocks with a total of 244 pigeons in Berlin, Germany showed clinical signs of apathy, weakness, depression, mild diarrhoea, torticollis, opisthotonus, muscle tremor, paralysis and trembling. All 47 pigeons were humanely killed. The severity of the clinical signs varied between individuals (Table 1). The pigeons from the different flocks had no known contact with each other.

Pathological and histological examination. A complete necropsy was performed on 13 racing pigeons with neurological signs. Tissue samples from the lung, heart, liver, spleen, kidneys, intestine, brain and skeletal muscle (pectoral, gastrocnemius, neck muscles) were fixed in 4% phosphate-buffered formalin or 3% glutaraldehyde. Unfixed tissue samples were immediately snap frozen at −80°C. Formalin-fixed tissues were routinely embedded in paraffin, and sections 4 µm thick were stained with haematoxylin and eosin. In addition samples from the pectoral muscle of 15 healthy pigeons from five neighbouring, unaffected flocks were similarly processed for histological examination.

Bacterial examination. Samples of the heart blood, lung and liver were cultured on Columbia agar with 5% bovine blood and water-blue metachrome-yellow lactose agar (Gassner Agar). Plates were incubated at 37°C for 24 to 48 h under aerobic conditions. For *Salmonella* diagnosis, standard microbiological enrichment techniques were used. For pre-enrichment, samples from liver and intestine were incubated in peptone water at 37°C for 24 h. For enrichment, sample material was transferred to *Salmonella*-selective Rappaport–Vassiliadis medium and incubated for 48 h at 41°C. After enrichment, the samples were streaked on Rambach Agar plates and incubated at 37°C for 24 h.

Virus isolation. Pooled organ samples from the lung, brain, kidney, spleen and intestine were inoculated into the allantoic cavity of 11-day-old embryonating specific pathogen free chicken eggs. The eggs were incubated at 37°C for 6 days with subsequent testing of the allantoic fluid for haemagglutinating activity. Allantoic fluids with negative results were re-inoculated into another batch of eggs. Samples were considered negative if the second egg passage also revealed no haemagglutinating activity.

Table 2. *Histological findings of 13 pigeons with central nervous lesions*

Lesions	Number of pigeons affected
Lymphohistiocytic and granulomatous encephalitis with glia cell proliferation	13
Demyelination of white matter	6
Lymphocytic meningitis	2
Schizonts in neuropil	1
Sarcocystic cysts in skeletal muscle cells	13
Lymphohistiocytic myositis with degeneration and rhabdomyolysis	9
Embolism of fragmented striated muscle in large pulmonary veins	1
Heart muscle cells with sarcocysts	13
Lymphohistiocytic interstitial nephritis and eosinophilic and lymphocytic glomerulonephritis in the kidneys	7
Follicular hyperplasia in the spleen	5

Electron microscopy. The tissue samples were collected from different organs of the affected pigeons and fixed with 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C, then further processed, embedded, and prepared for light and electron microscopy using standard laboratory methods described elsewhere (Mielewczik *et al.*, 2008). For light microscopy, semi-thin sections were stained with methylene blue and studied with an Olympus photomicroscope, while the electron micrographs were taken using Zeiss electron microscopes (EM-9-S, EM 902 A).

Sequence analysis. To confirm the presence of protozoal DNA in the tissues of affected pigeons, six overlapping fragments of the 28S rRNA of *Sarcocystis* species were selected for polymerase chain reaction (PCR) amplification (Table 2; Mugridge *et al.*, 1999). Sequences from the internal transcribed spacer region (ITS) were amplified using primers ITS-5 and ITS-2 as described previously (White *et al.*, 1990). Briefly, 25 mg pectoral muscle of each animal was minced into small pieces and total DNA was extracted using overnight proteinase K digestion at 56°C and affinity column separation following the instructions of the supplier (QIamp®DNA Mini Kit; Quiagen, Hilden, Germany). PCR reactions were carried out using GoTaq® Flexi DNA Polymerase (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The PCR reactions were carried out using the following PCR protocol: initial incubation at 95°C for 5 min, followed by 40 cycles at 94°C for 1 min, 52°C for 2 min, 72°C for 2 min; and final extension at 72°C for 7 min. Amplification products were purified using the NucleoSpin® Extract II system (Macherey-Nagel) and sequenced by a commercial DNA sequencing service (Seqlab GmbH, Goettingen, Germany) using the same forward and reverse primers. Sequences were compared with all sequences listed in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul *et al.*, 1990). Multiple sequence alignments of the full-length ITS-1 region and 28S rRNA were constructed using the ClustalW program (http://www.ebi.ac.uk/clustalw/index.html; Higgins *et al.*, 1996). Proportional nucleotide distance values of the ITS-1 region were calculated based on pairwise analysis using the MEGA4

Table 1. *Case history of three different flocks of racing pigeons*

Flock	Year	Number of pigeons in the flock	Number with mild signs[a]	Number with signs of encephalitis[b]	Number necropsied
1	2006	120	16	30	4
2	2007	83	10	12	8
3	2008	41	8	5	1

[a]Reduced general health, depression, diarrhoea.
[b]Torticollis, opisthotonus, paralysis, muscle tremor, trembling.

program, which was also used to obtain the phylogenetic relationships (Tamura *et al*., 2007) with different tree building methods (neighbour-joining and minimum evolution using Kimura two-parameter and maximum parsimony with close-neighbour-interchange search).

GenBank accession numbers. The obtained ITS-1 and 28S rRNA sequences were deposited in the GenBank database with accession numbers FJ232948 and FJ232949, respectively.

Results

Necropsy results. Postmortem examination of 13 pigeons with neurological signs revealed no gross lesions in any organs examined. The nutritional status was considered normal in all animals.

Histological findings. All 13 pigeons had varying degrees of multifocal to coalescing granulomatous and necrotizing encephalitis involving all compartments of the brain, with prominent perivascular lymphocytic cuffing and glia cell proliferations (Figure 1 and Table 2). Encephalomalacia was primarily observed in the brain stem and the cerebellum. A few protozoan schizonts were observed in the neuropil of the cerebrum of one bird only. Two animals from flock 1 also had severe multifocal lymphohistiocytic meningitis. The examined skeletal muscles (pectoral, gastrocnemius and neck muscles) of all birds were severely infested with slender cysts up to 2 mm in length and 20 to 50 μm in width (Figure 2). Cysts were subdivided into small chamber-like hollows, separated by fine septae that were only visible in wet preparations (Figure 2e). The chambers were filled with lancet-shaped cystozoites 7.5×1.5 μm^2 in size and dividing metrocytes. In addition to areas of the musculature without inflammatory reactions next to cysts, severe lymphohistiocytic, granulomatous and, occasionally, eosinophilic myositis was present in all animals with marked Zenker′s degeneration and loss of affected fibres (Figure 2c). In the myocardium only a few cysts were detected (Figure 2f) with no or only mild multifocal lymphohistiocytic and granulomatous myocarditis. In the kidneys of seven birds from flocks 1 and 2, moderate multifocal lymphohistiocytic interstitial nephritis and multifocal eosinophilic and lymphohistiocytic glomerulonephritis was observed. The spleens of five birds from flocks 1 and 2 had chronic follicular hyperplasia.

Histological examination of the pectoral muscles of 15 healthy pigeons from five unrelated, unaffected neighbouring flocks without clinical signs revealed no cysts or other lesions.

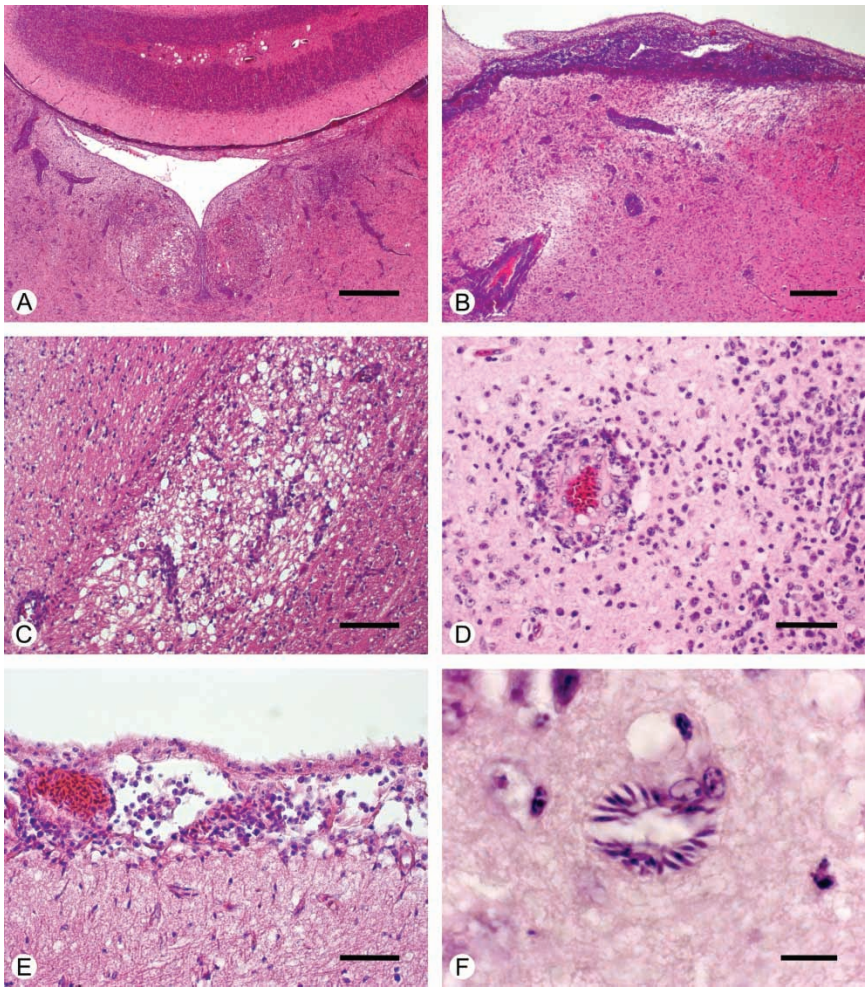

Figure 1. *Photomicrographs of the brain of pigeons with neurological signs. Severe multifocal to coalescing lymphohistiocytic and granulomatous encephalitis with glial cell proliferation was present in multiple brain compartments, including the (1a) cerebellum and brain stem, (1b) internal capsule, (1c) medulla oblongata and (1d) cerebral cortex. 1c: Demyelination in the white matter, associated with perivascular lymphocytic infiltrations. Glial cell proliferation was primarily observed in the (1b) brain stem and (1d) cortex. 1e: Lymphocytic meningitis. 1f: Schizont in the neuropil. Haematoxylin and eosin stain. Bars = 500 μm (1a), 200 μm (1b), 100 μm (1c), 50 μm (1d, 1e), and 10 μm (1f).*

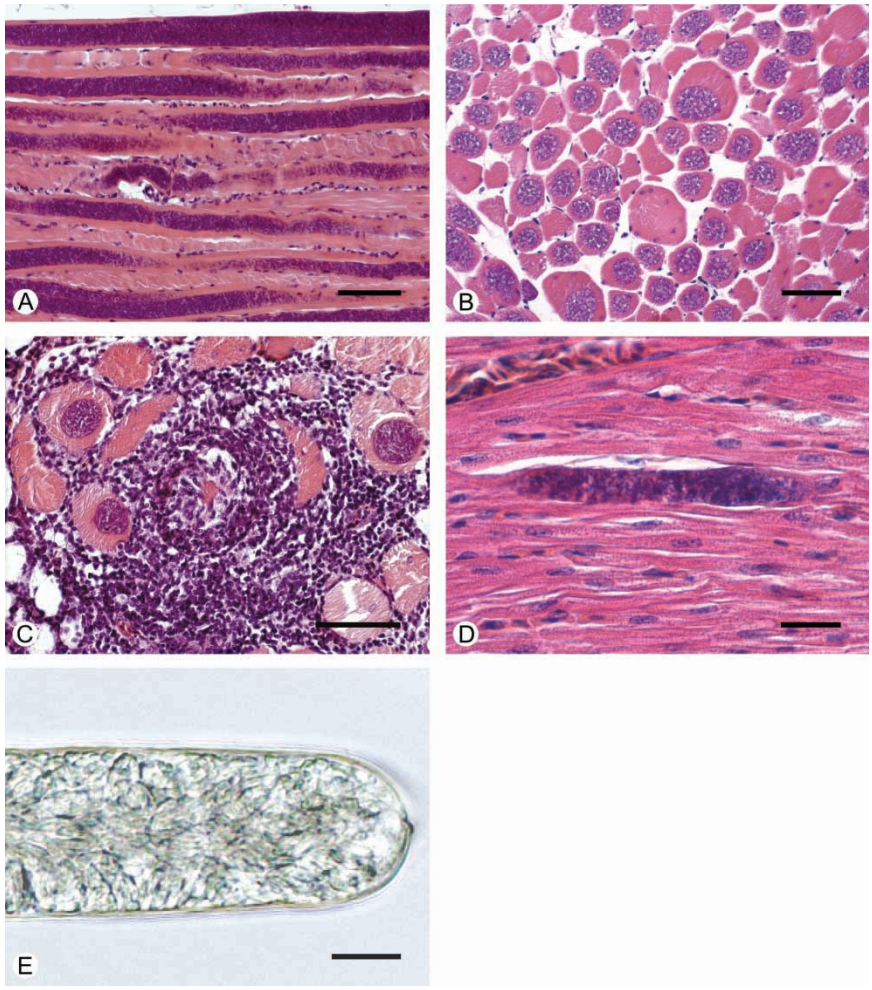

Figure 2. *Sarcocysts present in the pectoral muscles, centrally located in muscle fibres (1a: longitudinal section; 1b: cross-section). Lancet-shaped cystozoites 7.5 μm ×1.5 μm in size and dividing metrocytes. 1a, 1b: Muscle tissue without inflammatory reactions. 1c: Severe lymphohistiocytic myositis with degeneration and rhabdomyolysis. 1d: Sarcocystic cyst in the myocardium. 1e: Light microscopy of fresh preparations of sarcocysts from muscle tissue with visible subdivisions of the cysts by fine septae. 1a to 1d: Haematoxylin and eosin stain. 1e: Unstained wet preparation. Bars = 50 μm (1a to 1c), 20 μm (1d) and 10 μm (1e).*

Bacterial culturing. No bacteria were cultured from any samples examined.

Virus detection. No haemagglutinating agents were detected in any samples examined.

Transmission electron microscopy. The section through infested tissues of pigeons revealed that the tissue cysts possessed the typical fine structures of tissue cysts of *Sarcocystis* species (Figure 3). In cross-section the cysts appeared round, while in longitudinal sections they often showed spindle-like structures. They were delineated by a typical primary cyst wall, which did not form protrusions but had a smooth and wavy surface with some slight invaginations (Figure 3). An electron-dense ground substance was present subjacent to the primary cyst wall. This ground substance extended to the interior of the cyst and formed thin septae that subdivided the cysts into chambers. While in young tissue cysts all chambers were filled with ovoid metrocytes (mother cells) each produced by an endodyogeny process, the two finally infectious cyst merozoites (bradyzoites), older cysts, contained mainly such infectious stages and only a few metrocytes at the periphery (Figure 3). The cyst merozoites were about 8 μm in length and showed the typical *Sarcocystis* aspects with a conoid, numerous closely packed micronemes, dense bodies, rhoptries, one nucleus, a long tubular mitochondrion, a single golgi apparatus and a large apicoplast anterior to the nucleus. A comprehensive systematic electron microscopic investigation will be published elsewhere.

Sequence analysis. Comparison of the highly variable first internal transcribed spacer region ITS-1 with known sequences of *Sarcocystis* species and other protozoans failed to identify highly homologous sequences. Instead, varying degrees of sequence homologies were found with *S. falcatula*, *S. neurona*, *Sarcocystis dasypi*, *Sarcocystis felis* and *Sarcocystis canis* with nucleotide substitutions ranging from 0.170 to 0.529 (Table 4). Comparison of the 28S rRNA sequences with publicly accessible sequences (GenBank database) again failed to identify matching sequences. The closest sequence homologies were detected with *Frenkelia microti*, *Frenkelia glareoli* and *S. neurona* within the family *Sarcocystidae* (Figure 4).

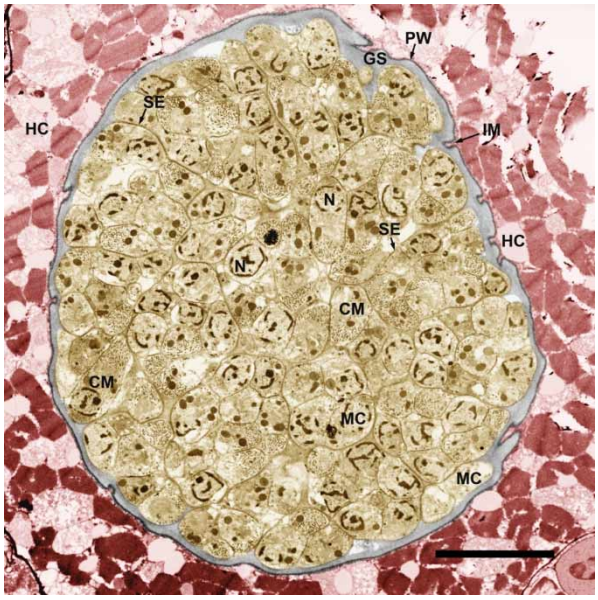

Figure 3. *Transmission electron micrograph of a cross-section through an older cyst found in the muscle tissues of pigeons. Note that the host cell (HC)-based cysts are limited by a smooth, protrusion-less primary cyst wall (PW). In the interior, mainly cyst merozoites (CM) occur in chambers formed by small septae (SE) of the ground substance (GS). At the periphery of the cyst, a few metrocytes (MC) occur. N, nucleus; IM, invagination of the primary cyst wall. Bar = 10 μm.*

Discussion

The clinical signs initially observed in the diseased domestic pigeons of the three flocks of racing pigeons suggested that *Paramyxovirus* infection or salmonellosis may have been the cause (Faddoul & Fellows, 1964; Rupiper, 1998; Marlier & Vindevogel, 2006). However, *Paramyxovirus* could not be isolated from any of the pigeons examined and bacterial culturing was negative for bacterial pathogens including *Salmonella*. Instead, the marked encephalitis and myositis were clearly associated with a massive infection with sarcocysts in muscle tissues. Complete pathological examinations failed to identify any other possible cause. Moreover, 15 healthy pigeons randomly chosen from five neighbouring unaffected flocks had no evidence of such infection in their pectoral muscles. Thus, it is assumed that the encephalitis and myositis that probably caused the clinical problems were induced by the *Sarcocystis* infection. Ultimate proof of a direct and exclusive causal role of this parasite will await fulfilment of Koch's postulates.

Neurological signs associated with sarcocystosis have previously been described in several avian species (Jacobsen *et al*. 1984; Aguilar *et al*., 1991; Dubey *et al*., 1991, 1998, 2001b; Hillyer *et al*., 1991; Mutalib *et al*., 1995; Teglas *et al*., 1998; Spalding *et al*., 2002; Olson *et al*., 2007; Villar *et al*., 2008). However, encephalitis due to *Sarcoystis* infection has not so far been reported in pigeons (Smith *et al*., 1990; Suedmeyer *et al*., 2001). Since only one pigeon showed schizonts in the brain tissue, future immunohistochemical and experimental investigations must determine whether the parasite itself or metabolism products are accountable for the severe neurological lesions. Also, the histological and electron microscopic characteristics of the parasites described here are different from previously described cysts in pigeons, especially *S. falcatula* that is thought to be the principal *Sarcocystis* species in domestic pigeons. The typical electron microscopic features of *S. falcatula* include protrusions of the cyst wall of 1 to 5 μm, microtubules originating in ground substance and running to the tip of protrusions, as well as numerous invaginations of the cyst wall into the osmophilic layer (Box *et al*., 1984). Importantly, the cyst walls were smooth and devoid of protrusions that are typically seen with *S. falcatula.* Moreover, the genetic characterization of the ITS-1 and 28S rRNA sequences of the sarcocysts discovered here identified as yet unknown sequences within the Apicomplexa. Highly variable loci of rRNA are generally necessary for identification of a new species (Elsheikha & Mansfield, 2007). Consequently, we used the complete ITS-1 of the rRNA (Marsh *et al*., 1999) and computed the proportional nucleotide distance values. Small genetic variations were found among six different isolates of *S. falcatula* (0.009 to 0.042). The same was true for *S. falcatula* and *S. neurona*, as well as *S. dasypi* (0.003 to 0.041). In contrast, the average genetic distance between *Sarcocystis* species (*C. livia f. domestica*) found in this study compared with *S. falcatula* and *S. neurona* was 0.488 and 0.499, respectively. Additionally, the full-length 28S rRNA

Table 3. *Primers used for amplification and sequencing of ITS-1 and 28S rRNA*

Primer	Sequence (5′ → 3′)	Amplicon size (base pairs)	Location within the published sequence	Source
ITS-5 forward	GGAAGTAAAAGTCGTAACAAGG	838		White *et al.* (1990)
ITS-2 reverse	GCTGCGTTCTTCATCGATGC			White *et al.* (1990)
KL1 forward	GCTGCGTTCTTCATCGATGC	714	1 to 714	Mugridge *et al.* (1999)
WE1 reverse	TTCAGCCAGCATCACAGAAC			Present study
KL1 forward	GCTGCGTTCTTCATCGATGC	1517	1 to 1517	Mugridge *et al.* (1999)
KL3 reverse	CCACCAAGATCTGCACTAG			Mugridge *et al.* (1999)
KL4 forward	AGCAGGACGGTGGTCATG	753	1334 to 2086	Mugridge *et al.* (1999)
KL6b reverse	CCCTCAGAGCCAATCC			Mugridge *et al.* (1999)
KL6a forward	GGATTGGCTCTGAGGG	514	2971 to 2584	Mugridge *et al.* (1999)
KL5b reverse	GTCAAGCTCAACAGGGTC			Mugridge *et al.* (1999)
KL5a forward	GACCCTGTTGAGCTTGAC	723	2567 to 3289	Mugridge *et al.* (1999)
KL2 reverse	ACTTAGAGGCGTTCAGTC			Mugridge *et al.* (1999)
KL6a forward	GGATTGGCTCTGAGGG	1219	2071 to 3288	Mugridge *et al.* (1999)
KL2 reverse	ACTTAGAGGCGTTCAGTC			Mugridge *et al.* (1999)

Table 4. *Proportional distances of* Sarcocystis *spp. based on the aligned internal spacer region 1*

		1	2	3	4	5	6	7	8	9	10	11	12
1	New *Sarcocystis* species (*C. livia* f. *d.*)												
2	*S. canis* DQ176645	0.170											
3	*S. felis* AY190082	0.195	0.213										
4	*S. falcatula* clone 1255 AY082638	0.490	0.520	0.508									
5	*S. falcatula* clone 1256 AY082639	0.495	0.524	0.511	0.012								
6	*S. falcatula* UCD 1 AF098245	0.485	0.514	0.504	0.042	0.047							
7	*S. falcatula* Florida 1 AF098244	0.488	0.515	0.502	0.021	0.027	0.025						
8	*S. falcatula* Cornell 2 AF098243	0.487	0.517	0.504	0.031	0.037	0.016	0.018					
9	*S. falcatula* Cornell 1 AF098242	0.485	0.514	0.502	0.009	0.014	0.031	0.013	0.021				
10	*S. neurona* UCD 1 AY082644	0.499	0.529	0.521	0.035	0.041	0.029	0.026	0.026	0.028			
11	*S. neurona* AY082648	0.499	0.529	0.521	0.033	0.039	0.027	0.026	0.024	0.026	0.004		
12	*S. dasypi* clone 217 AY082631	0.498	0.528	0.520	0.034	0.040	0.028	0.025	0.025	0.027	0.001	0.003	

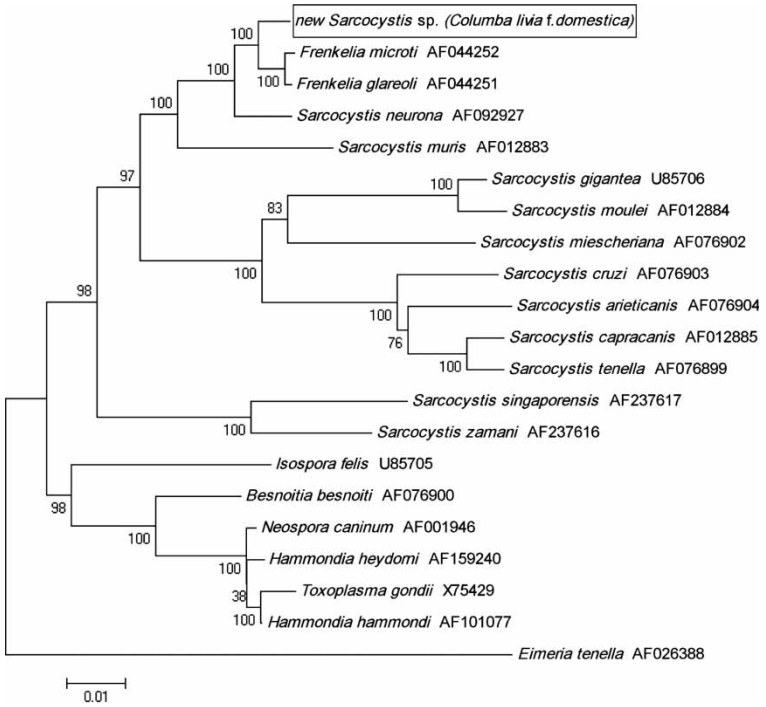

Figure 4. *Phylogram based on alignment of full-length 28S rRNA sequences of Apicomplexa with neighbour-joining analysis, rooted on* Eimeria tenella. *The branch lengths are proportional to the degree of inferred evolutionary change and the numbers indicate bootstrapping values (%). Based on the sequence comparison, the putatively new* Sarcocystis *species described here (boxed) is a member of the subfamily Sarcocystinae and is closely related to parasites also infecting avian hosts.*

was compared with 20 different apicomplexan sequences. Together with *F. microti*, *F. glareoli* and *S. neurona* the pigeon *Sarcocystis* formed a well-supported group with high bootstrap values and clearly distinct branching. Members of this group use birds as definitive or intermediate hosts (Odening, 1998; Mansfield *et al*., 2008). Some authors consider the taxons *Frenkelia* spp. to be a synonym of *Sarcocystis* spp. (Volypka *et al*., 1998; Mugridge *et al*., 1999). In summary, these data strongly suggest that the sarcocysts described here represent a novel species. The pigeon probably plays an important role in the prey spectrum of the definitive host. We plan to name this species once its lifecycle and other host species have been identified.

Remarkably, despite massive infection of skeletal muscles and cell destruction, we found only limited number of cysts in the heart. This is consistent with previous reports of *Sarcocystis* infections in doves (Barrows & Hayes, 1977). The infection of pigeons with the *Sarcoystis* sp. described in this study may best be detected by histological examination of skeletal muscle tissue. Cysts of this species measured only 20 to 50 μm in width and 1 to 2 mm in length, and therefore were macroscopically invisible. Because routine histological examination of birds does not necessarily include skeletal muscle tissue, infections with *Sarcocystis* species may have been overlooked previously, particularly when other causes (e.g. *Paramyxovirus*, *Salmonella*) were present or suspected. Although racing pigeons have been monitored continuously in Berlin and throughout Germany, no such sarcocyst parasites have been observed before. Further studies—in particular, retrospective studies of conserved pigeon material from the area—are need to determine whether the parasite was recently introduced or has been overlooked.

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