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Is there Evidence of *Sarcocystis calchasi* Involvement in Meningoencephalitis of Unknown Origin in Mammals? A Retrospective Study

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List of Abbreviations

bp	base pairs
BVDV	Bovine Viral Diarrhea Virus
DNA	Deoxyribonucleic Acid
dpi	days post infection
EDTA	Ethylenediaminetetraacetic Acid
EF1α	Elongation Factor 1 alpha
EPM	Equine Protozoal Meningoencephalomyelitis
f.	*forma*
FeLV	Feline Leukemia Virus
FFPE	Formalin Fixed Paraffin Embedded
GI	Gastrointestinal
GME	Granulomatous Meningoencephalitis
H&E	Hematoxylin and Eosin
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
ITS	Internal Transcribed Spacer
LSU	Large Ribosomal Subunit
MUO	Meningoencephalitis of Unknown Origin
MTOC	Microtubule Organizing Centers
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NLE	Necrotizing Leukoencephalitis
NME	Necrotizing Meningoencephalitis
NTC	No Template Control
PCR	Polymerase Chain Reaction
PPE	Pigeon Protozoal Encephalitis

RNA	Ribonucleic Acid
sp.	Species (singular)
spp.	Species (plural)
SSU	Small Ribosomal Subunit
TBE	Tris-Borate-Ethylenediaminetetraacetic Acid
TNF	Tumor Necrosis Factor

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1 Introduction

Sarcocystis calchasi is a new pathogenic protozoon belonging to the phylum Apicomplexa. It was discovered as the causative agent of a severe neurological disease in domestic pigeons (*Columba liva* f. *domestica*) called Pigeon Protozoal Encephalitis (PPE; Olias et al. 2013). This species and the disease caused by it have been observed for the first time in Berlin, Germany, in 2006 (Olias et al. 2009). In the meantime, cases in the USA (Wunschmann et al. 2011; Rimoldi et al. 2013) and Japan (Ushio et al. 2015) have also been reported. Further national and global prevalences are unknown. *Sarcocystis calchasi* is genetically closely related to other well-known pathogenic species of its family, such as *Sarcocystis neurona*, *Sarcocystis tenella*, *Neospora caninum* and *Toxoplasma gondii* (Olias et al. 2009). Like most organisms from the Sarcocystidae family, it has an obligatory two-host life cycle. Hawks of the genus *Accipiter* are the definitive hosts and pigeons (Olias et al. 2010a), doves (Olias et al. 2014), cockatiels (Olias et al. 2014), princes parrots and cockatoos (Rimoldi et al. 2013) have been identified as intermediate hosts so far. The sporocysts shed by the hawk are subsequently ingested by the pigeon and cause clinical symptoms and pathological lesions which make the pigeon easily accessible for its predators (Olias et al. 2013).

PPE caused by *Sarcocystis calchasi* in the pigeon, its intermediate host, is a biphasic disease. In the first phase, schizogony of the parasite in various organs results in such clinical signs as polyuria, diarrhea and apathy. The second phase consists of non-suppurative meningoencephalitis clinically apparent in form of severe neurological signs. During this phase, cyst formation takes place in the striated muscles, too (Olias et al. 2010c). Definitive hosts become infected via ingestion of tissue cysts, whereby cystozoites proceed to gamogony in the wall of the small intestine. They shed large number of sporocysts in feces, but do not develop clinical signs (Olias et al. 2010b). PPE was first observed in 2006 in Berlin, and in the period from 2006 to 2008 an increased incidence of the disease was noted which led to its extensive investigation, detailed description of the parasite and identification of the definitive host and other intermediate hosts (Olias et al. 2009). Since 2008 a steady incidence of the disease was noted in the biopsies

sent for diagnostics to the Institute of Veterinary Pathology in Berlin, which indicates an ongoing threat to the domestic pigeon population in this region.

Many organisms from the Sarcocystidae family have more than one intermediate or aberrant host and may cause meningoencephalitis in various hosts (Dubey et al. 2001b; Elmore et al. 2010; Dubey and Schares 2011). *Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* are a only few examples. *Toxoplasma gondii* which is the best investigated apicomplexan species has most species of mammals and birds as intermediate hosts (Elmore et al. 2010). In case of *Sarcocystis calchasi* more than one avian intermediate host species has been identified already (Rimoldi et al. 2013; Olias et al. 2014). Because many different Sarcocystidae species that are closely related to *Sarcocystic calchasi* cause diseases in avian as well as in mammalian species, the question arose whether this parasite may also be pathogenic and contribute to diseases in mammals. As there are still many cases of meningoencephalitis of unknown origin being constantly encountered in mammals which histopathologically resemble the lesions seen in PPE, the aim of this work was to test if *Sarcocystis calchasi* may play a role in these cases in the setting of a retrospective study using previous cases of meningoencephalitis of unknown origin in mammals.

2 Literature Survey

2.1 Apicomplexa

Protozoa of the phylum Apicomplexa include sporocyst-forming parasites which produce infectious sporozoites (Fig. 1; Dubey et al. 1988). Many of them are etiologic agents in a number of diseases of animals and people around the world. Of particular medical concern are members of the genera *Eimeria*, *Neospora*, *Plasmodium, Sarcocystis* and *Toxoplasma* (Selzer and Becker 2011). They are responsible for a group of diseases of global relevance such as malaria caused by several members of *Plasmodium* spp. and toxoplasmosis caused by *Toxoplasma gondii* affecting both humans and animals (Dubey 2004; Kim and Weiss 2004). Sarcocystosis, caused by different *Sarcocystis* spp., is included in this group as well. This disease is accounted for severe losses in animal production and also exhibits zoonotic potential (Dubey et al. 1988; Fayer 2004; Upadhayay and Evum 2013).

Organisms from the genus *Sarcocystis* are highly prevalent parasites affecting animals and men (Dubey et al. 1988). So far over 180 different species have been recognized (Tenter and Johnson 1997) and ongoing research in this area is constantly providing new species descriptions.

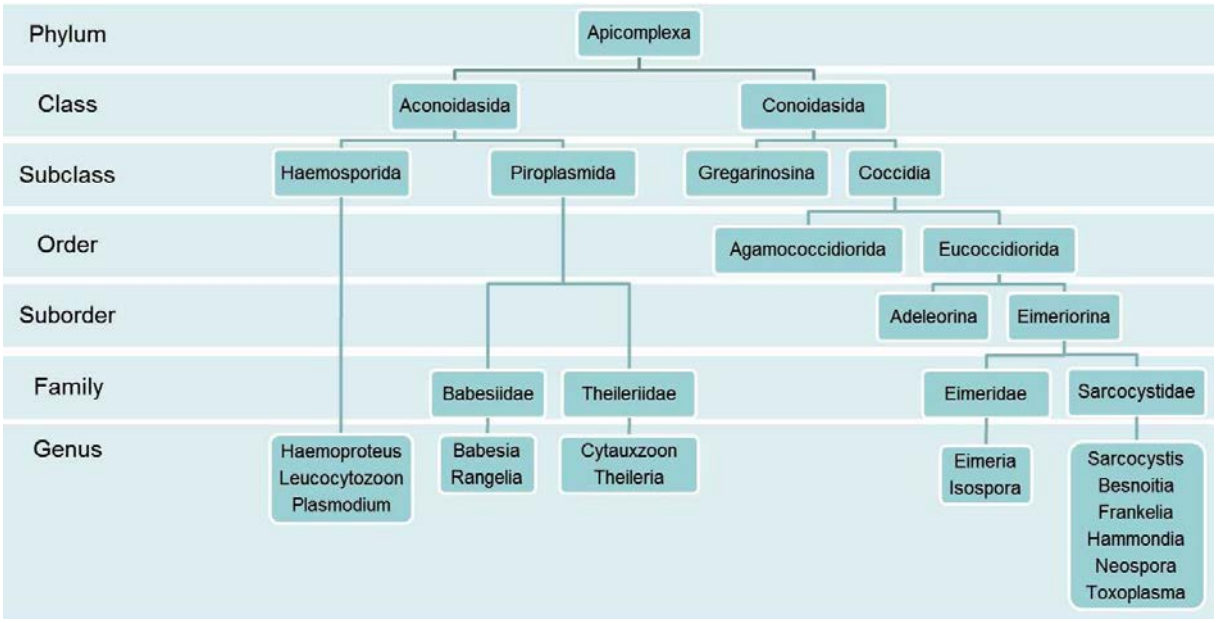

Figure 1: Taxonomy overview of the phylum Apicomplexa drawn after the information from Taxonomy Browser (National Center for Biotechnology Information 2015)

2.2 Structure and Morphology of Apicomplexan Parasites

Most members of the phylum Apicomplexa are obligate intracellular parasites. Usually they grow and replicate within the parasitophorous vacuole, a non-phagosomal, membrane-bound compartment that is separated from most cellular metabolic pathways and is formed while entering the host cell. After invading the cell, parasites proliferate, grow and divide until the host cell is destroyed by the replicating organisms (Morrissette and Sibley 2002).

Apicomplexa share many morphological traits that are considered diagnostic for this phylum (Fig. 2). The zoites of these protozoa have an elongated shape and a characteristically specialized apical region that consists of a group of unique organelles called the apical complex. They include the rhoptries, the micronemes, the apical polar ring, and the conoid. Rhoptries and micronemes are specialized secretory organelles that contain products required for motility, adhesion and invasion of host cells, and establishment of the parasitophorous vacuole (Morrissette and Sibley 2002). The conoid is a small cone-shaped structure composed of a spiral of microtubular filaments. It is thought to play a mechanical role in invasion of host cells and is present in only some members of the phyllum. The apical polar ring is an organelle characteristic for all Apicomplexa. It serves as one of the three microtubule-organizing centers (MTOC) in these parasites and constitutes an anchoring point for the subpellicular microtubules; spindle pole plaques and centrioles / basal bodies are the other MTOC (Dubey et al. 1988; Morrissette and Sibley 2002). In addition to the apical complex, most of the apicomplexan parasites possess a unique, crucial organelle, the apicoplast, which is a plastid without photosynthetic ability (Seeber and Soldati-Favre 2010). Together with the mitochondrion it is suspected to be responsible for such plastid-related metabolic pathways like fatty acid synthesis, isoprenoid synthesis and heme synthesis (Arisue and Hashimoto 2014). The parasites are bounded by the pellicle, a composite structure consisting of the plasma membrane and the closely apposed inner membrane complex (Dubey et al. 1988; Morrissette and Sibley 2002). Other organelles, such as the Golgi complex, endoplasmatic reticulum, ribosomes and mitochondria do not differ from these organelles in other

eukaryotic cells, although their activity may be reduced in bradyzoites. The nucleus in the zoites is haploid (Dubey et al. 1988).

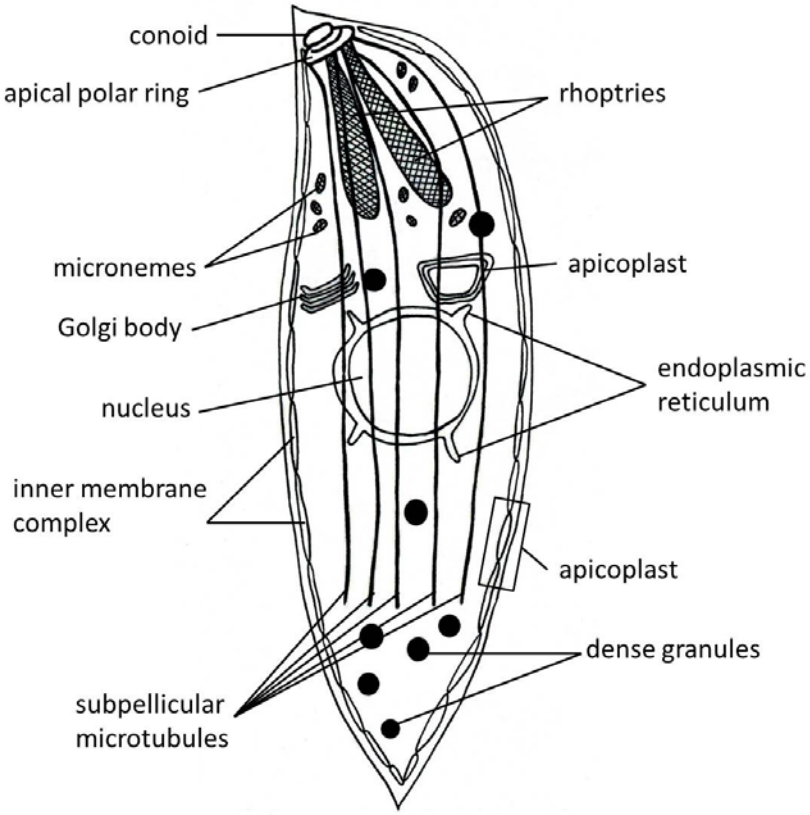

Figure 2: Characteristic morphological traits of apicomplexan zoite (modified from Morisette and Sibley, 2002)

2.3 Life Cycle of Apicomplexan Parasites

Sarcocystis spp. have an obligatory prey-predator, heteroxenous (two-host) life cycle (Fig. 3). Asexual stages develop only in the intermediate host, which usually is the prey animal. Sexual stages develop only in the definitive host, which is a carnivore. Intermediate and definitive hosts vary for each *Sarcocystis* species (Box and Smith 1982; Lindsay et al. 1995a; Fayer 2004).

The definitive host becomes infected by ingesting muscular or neural tissue containing mature sarcocysts. Bradyzoites are liberated from the sarcocysts by digestion in the stomach and intestine. Bradyzoites actively move, penetrate the mucosa of the small intestine, and transform into male (micro) and female (macro) gamonts. Microgamets liberated

from the microgamont actively move and penetrate the periphery of the macrogamont. After fertilization, a wall develops around the zygote and the oocyst is formed. Oocysts sporulate in the *lamina propria* of the intestine. During sporulation as a result of three nuclear divisions two sporocysts with four sporozoits each are formed within the oocyst (oocyst of *Isospora* type). The oocyst wall is thin and often ruptures releasing free sporocysts into the intestinal lumen. Subsequently they are passed in feces and excreted. The intermediate host becomes infected by ingesting sporocysts in food or water. Sporozoites excyst from sporocysts in the small intestine (Dubey et al. 1988). The sporozoites, after ingestion by the intermediate host, undergo asexual reproduction called schizogony or merogony forming schizonts or meronts (Dubey et al. 1988; Fayer 2004). These first divisions are a result of endopolygeny – a form of merogony consisting of multiple rounds of mitosis without nuclear division, leading to a polyploid nucleus. The later division of the nucleus coincides with budding forming merozoites (Francia and Striepen 2014). Both first and second generation schizonts are located within the host cytoplasm and are not surrounded by the parasitic vacuole.

The number of generations of schizogony and the type of host cell vary for each *Sarcocystis* species. The *Sarcocystis* species of large domestic animals (sheep, goat, cattle, pigs, horses) form first and second-generation schizonts in the vascular endothelium, whereas in *Sarcocystis* species of mice and pigeons this process takes place in hepatocytes. This is the phase in which the intermediate host exhibits disease symptoms or even dies, if infected with highly pathogenic *Sarcocystis* species. Merozoites liberated from the terminal generation of schizogony initiate the sarcocyst formation (Dubey et al. 1988). At this stage, division by endodyogeny (binary fission) takes place and bradyzoits within a parasitic vacuole are formed (Lindsay et al. 1995a).

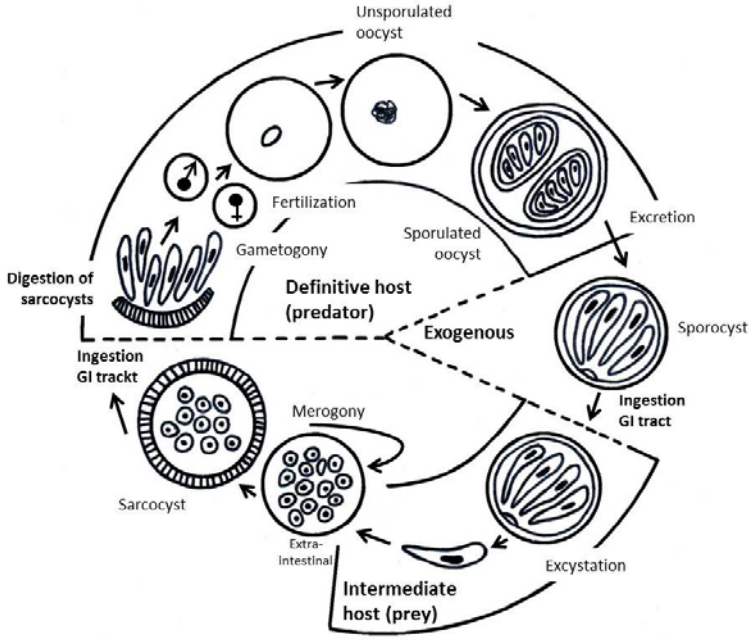

Figure 3: Life cycle of the genus *Sarcocystis,* modified from Kenyon College (Kenyon College 2010); GI tract – gastrointestinal tract

2.4 *Sarcocystis calchasi*

2.4.1 Discovery and Life Cycle

Between 2006 and 2008, 47 racing pigeons from three different flocks in Berlin, Germany showed clinical signs of apathy, diarrhea, torticollis, opisthotonus, paralysis and trembling (Olias et al. 2009). Histopathological examination revealed generalized severe granulomatous and necrotizing meningoencephalitis and cysts of a previously unknown *Sarcocystis* species in heart and skeletal musculature (Olias et al. 2009). The morphological findings in combination with experimental infection data (Olias et al. 2010c) and genetic analysis (Olias et al. 2010d), clearly indicated that the recently discovered *Sarcocystis* parasite represented a new species, which subsequently was named *Sarcocystis calchasi* (Olias et al. 2010a).

This novel, highly pathogenic parasite uses the northern goshawk (*Accipiter gentilis*) as definitive host and the domestic pigeon (*Columba livia* f. *domestica*) as intermediate host (Olias et al. 2010a).

Definitive hosts are infected through ingestion of tissue cysts and cystozoites undergo gamogony in the intestinal wall, where they develop into sporulated oocysts of the *Isospora* type (Mehlhorn and Heydorn 1978; Olias et al. 2009). Subsequently the oocysts are ingested by the pigeon and cause clinical symptoms and pathological lesions which make the pigeon an easy prey for its predator (Olias et al. 2010c).

The definitive host identification was performed by feeding pigeon tissues infected with *Sarcocystis calchasi* to different predators which could have had natural contact with the birds, in order to investigate in which of the tested carnivores the parasite's life cycle continued, i. e. sporocyst formation in the lining of intestinal wall occured. The potential definitive hosts tested in the infection trial included dogs, ferrets, rats, mice, northern goshawks and gyr-saker hybrid falcons. Sporocysts were shed only in the feces of the northern goshawk, which allowed for the assumption that this species is the natural definite host of *Sarcocystis calchasi* (Olias et al. 2010b).

The prepatent period of the parasite is five days, as the definitive hosts started shedding sporocysts six days post infection (dpi). The sporogony takes place in the small intestine of the definitive host. Unsporulated, partially sporulated and sporulated oocysts measuring 11.9 x 14.6 µm can be found in the *lamina propria* of the mucosa. Sporocysts measure 7.9 x 11.9 µm in average and contain four sporozoites and residual bodies (Olias et al. 2010a).

The schizonts found in different organs including liver, spleen, lung and bone marrow of infected pigeons are in different developmental stages. Ovoid single schizonts, which are situated in a parasitophorous vacuole inside its host cells, develop to 6 µm long merozoites formed in the process of endopolygeny. The development of merozoites leads to the rupture of the parasitophorous vacuole and their release (Olias et al. 2010a).

In the skeletal muscle microscopically visible cysts developed from 51 to 65 dpi. Slender mature and immature cysts with the size of 2 mm x 20 to 50 µm are filled with dividing metrocytes and cystozoites. Sarcocysts are divided in chambers separated by fine septae. The cystozoites are lancet-shaped and measured approximately 7,5 x 1,5 µm.

In young tissue cysts all chambers are filled with metrocytes. Each metrocyte in the endodyogeny process produce two merozoites (cystozoites). They are found in largest number in older cysts which contained metrozoites only in the periphery.

Transmission electron microscopy revealed that the cyst wall lacks protrusions, has a smooth and wavy surface with slight invaginations and does not contain any fibrils, tubules or microtubules (Olias et al. 2010a).

2.4.2 Pigeon Protozoal Encephalitis

The disease caused by *Sarcocystis calchasi* is called Pigeon Protozoal Encephalitis (PPE). This highly fatal neurologic disease of domestic pigeons (Olias et al. 2009; Olias et al. 2010b) is biphasic and its clinical course includes encephalitis and myositis (Olias et al. 2010c).

In the first phase, 7 to 12 dpi, schizogony of the parasite in liver, spleen, lung, bone marrow, connective tissue next to esophagus and crop (Olias et al. 2010a) results in clinical signs such as polyuria, diarrhea and apathy. The second phase, approximately 65 dpi, is clinically apparent as severe central neurological signs such as torticollis, opisthotonus, paralysis and trembling. These clinical signs are due to a severe, multifocal to coalescing, granulomatous and necrotizing meningoencephalitis with perivascular lymphocytic infiltrations.

The inflammation affects gray and white matter of all brain compartments, and glia cell proliferation and demyelination of the white matter as well as areas of encephalomalacia in brain stem and cerebellum are present. In this phase of the disease cysts in the striated muscles are also formed, and in case of their rupture granulomatous myositis may develop (Olias et al. 2009).

Experimental infection of pigeons with *Sarcocystis calchasi* revealed the influence of the infectious dose on the course of disease. Pigeons infected with high doses (8×10^4) of sporocysts died within 7-12 dpi with severe, parasite-induced liver necrosis. In contrast, the

individuals infected with a lower number ($10^2 – 10^4$) of sporocysts developed central nervous symptoms, but no sooner than 8 weeks post infection (Olias et al. 2010c).

Even though the parasites cause severe encephalitis, they are only rarely present in the brain tissue (Olias et al. 2009; Olias et al. 2013). The late occurrence of brain lesions and the absence of intralesional parasitic stages in the brain suggest an indirect, currently unknown immunopathological mechanism. It has been suggested that the parasite might interfere with the cell-mediated immune reaction. In the acute phase of disease *Sarcocystis calchasi* induces a down-regulated mRNA expression of important T-helper 1 cytokines, such as interleukin (IL)-12, IL-18 and interferon (IFN)-γ (Olias et al. 2013). During this phase, no inflammatory reaction is detectable in the brain, while in a second chronic phase of infection, severe encephalitis is present and IFN-γ is up-regulated (Olias et al. 2010d).

To elucidate the host specificity of the parasite, experimental infections have been conducted. After experimentally excluding chickens (*Gallus gallus domesticus*) as its intermediate host, *Sarcocystis calchasi* was previously considered to be host-specific (Olias et al. 2010c). Nevertheless, recently, after performing experimental infections in cockatiels, it turned out to be polyxenous. In this way, psittacine birds, even though only distantly related to pigeons, have been identified as further intermediate host of the parasite. They exhibit a similar pathology to pigeons in the course of the disease (Olias et al. 2014). Moreover, natural infections have recently been described in princess parrots (*Polytelis alexandrae*) and long-billed corella (*Cacatua tenuirostris*; Rimoldi et al. 2013). This cross-species infectious potential of *Sarcocystis calchasi* suggests an unmonitored prevalence of this parasite in the avian population (Olias et al. 2014).

Shortly after the first detected outbreak in Berlin, additional cases have been identified in Minnesota (United States of America), which indicates that the disease is also present outside Germany (Wunschmann et al. 2011; Rimoldi et al. 2013). It is conceivable that the disease might be present, but unidentified in other regions of the world as well, or it can be easily transmitted, which makes pigeon keeping and falconry potential risk factors (Olias et al. 2010c).

2.5 Sarcocystis-Induced Encephalitis in Avian and Mammalian Species

Sarcocystis calchasi is genetically closely related not only to other well-known pathogenic species from its family, such as *Sarcocystis neurona* and *Sarcocystis falcatula* but also to other cyst-forming coccidian genera including *Toxoplasma* and *Neospora* (Tenter 1995), all of whom are known to cause meningoencephalitis in mammalian and / or avian species (Dubey 2002; Hill et al. 2005; Siegal-Willott et al. 2005; Wunschmann et al. 2009; Elmore et al. 2010). Many species from the Sarcocystidae family are polyxenous. They are able to infect more than one species and thus end up having more than one intermediate or aberrant host. *Sarcocystis neurona*, a parasite of avian origin (Mansfield et al. 2008), can infect a wide variety of warm-blooded animals and can cause neurological fatalities affecting especially the horse industry (Dubey et al. 2001b). It is the cause of Equine Protozoal Encephalomyelitis (EPM) a severe neurological disease of horses in America (Dubey et al. 2001b). In most cases of EPM, similar to PPE, very few parasites are seen intralesionally. In most acute cases multifocal, random hemorrhages in the spinal cord are present. In subacute and chronic cases apart from the hemorrhages, small foci of necrosis and non-suppurative inflammation are observed with perivascular cuffing by mononuclear cells, mainly in the meninges. The spinal cord is the most affected organ, followed by brain stem, cerebellum and rarely other locations of the brain. The cellular infiltrates are variable and may contain lymphocytes, neutrophils and eosinophils, multinucleated giant cells and gitter cells. Horses are considered to be aberrant hosts of this parasite because of the presence of schizonts and merozoites only, with absence of muscular tissue cysts in the organism (Dubey et al. 2001b).

Another member of the Sarcocystidae family, *Toxoplasma gondii*, which is one of the best studied apicomplexan, has most species of mammals and birds as intermediate hosts (Elmore et al. 2010). *Toxoplasma* usually infects the intermediate and definitive host without causing a clinical disease. The susceptibility to the disease depends very much on the host species and on its immunological status. It has been shown that for example adult rats and dogs are relatively resistant to the infection, but young rats and puppies are fully susceptible.

Cattle and horses belong to the most resistant species whereas goats and sheep suffering from the infection show reproductive disorders ranging from embryonic death and resorption to stillbirth and neonatal death (Dubey 2004; Dubey 2009).

The clinical manifestation of toxoplasmosis is mostly seen in immunosuppressed individuals (Tenter et al. 2000; Dubey 2004). In humans immunosuppressive agents given before transplantation (Elmore et al. 2010) or acquired immunodeficiency syndrome (AIDS; Dubey 2004; Hill et al. 2005) can contribute to the clinical manifestation. The most important and most frequently reported manifestation of toxoplasmosis in immunosuppressed patients is encephalitis. It has been reported in patients receiving immunosuppressive agents and it has been estimated to contribute to 10-30 % of deaths of patients with AIDS (Tenter et al. 2000). In animals canine distemper virus (Dubey 2004; Hill et al. 2005) and feline immunodeficiency virus (Foster et al. 1998) play an immunosuppressive role in *Toxoplasma* infection.

Even though the immunocompromised state predisposes to the development of toxoplasmosis, it is also possible to develop the disease in an immunocompetent state. Several cases of multisystemic toxoplasmosis including encephalitis in immunocompetent cats (Dubey et al. 1990b; Spycher et al. 2011; Nagel et al. 2013) and a case of pulmonary toxoplasmosis in an immunocompetent man (De Salvador-Guillouet et al. 2006) have been described.

The pathogenesis of the disease has been studied in immunodeficient athymic nude NMRI mice. As in other cases of apicomplexan infection, they exhibit granulomatous encephalitis with lymphocytic infiltrations in form of perivascular cuffs. Parasitic cysts could be found in the neuropil sometimes accompanied by granulomas surrounding the parasites (Schlüter et al. 1991).

Encephalitis caused by identified or unidentified *Sarcocystis* spp. in birds, apart from columbiformes and psittacines (Ecco et al. 2008; Godoy et al. 2009; Rimoldi et al. 2013; Olias et al. 2014) has been described in northern gannet (*Morus bassanus*; Spalding et al. 2002), golden eagle (*Aquila chrysaetos*; Dubey et al. 1991), northern goshawk (Aguilar et al. 1991), straw-necked ibis (*Carphibis spinicollis*, Dubey et al. 2001a), wild turkey (*Meleagris*

gallopavo; Teglas et al. 1998) and chicken (Mutalib et al. 1995). In mammals, it has been reported in cattle (Barr et al. 1990), sheep (Jeffrey et al. 1988), horses (Dubey et al. 2001b; Dubey and Hamir 2009), felidae (Forest et al. 2000; Bisby et al. 2010), canidae (Dubey et al. 1992; Dubey et al. 2006; Cooley et al. 2007; Dubey and Hamir 2009; Kubo et al. 2010), raccoons (*Procyon lotor*; Dubey et al. 1990a; Hamir and Dubey 2001; Dubey and Hamir 2009), mink (*Mustela vison*; Dubey and Hedstrom 1993; Dubey and Hamir 2009) and marine mammals (Dubey et al. 2003; Thomas et al. 2007). It has been suspected as a cause of encephalitis in humans (Mackie et al. 1992). All species share the characteristic inflammatory pattern and show a non-suppurative encephalitis pronounced in the cerebellum and less severely in the cerebrum with perivascular cuffs of mixed mononuclear cells. Multifocal to coalescing areas of malacia were often associated with intralesional parasitic structures consistent with schizonts and merozoites of *Sarcocystis* spp. occasionally accompanied by multinucleated giant cells (Jeffrey et al. 1988; Barr et al. 1990; Dubey et al. 1991; Aguilar et al. 1991; Mackie et al. 1992; Dubey and Hedstrom 1993; Mutalib et al. 1995; Teglas et al. 1998; Dubey et al. 2001a; Dubey et al. 2001b; Spalding et al. 2002; Dubey et al. 2003; Thomas et al. 2007; Ecco et al. 2008; Godoy et al. 2009; Dubey and Hamir 2009; Rimoldi et al. 2013; Olias et al. 2014).

The granulomatous meningoencephalitis in course of PPE in pigeons and cockatiels (Olias et al. 2009; Olias et al. 2014) shares the characteristic picture of an inflammatory reaction due to above mentioned apicomplexan parasites. However, several cases with identical histologic presentation have been described in mammals without identification of the etiological agent (O'Neill et al. 2005). Especially in dogs an idiopathic form of granulomatous meningoencephalitis with a very similar pathomorphology to a protozoan infection is well known (Schwab et al. 2007).

2.6 Non-Suppurative Central Nervous System Inflammation of Unknown Origin in Mammals

Non-suppurative inflammatory processes are frequent central nervous disorders in animals. Non-suppurative encephalitis is a nonspecific term that embraces a series of microscopic changes such as 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). It includes such entities as granulomatous meningoencephalitis (GME), necrotizing meningoencephalitis (NME), necrotizing leukoencephalitis (NLE; Talarico and Schatzberg 2010) and non-suppurative meningoencephalitis (Schwab et al. 2007). Their etiology and pathogenesis remain unclear, despite their recognition as clinical entities for over four decades in dogs. Genetic, autoimmune, infectious (especially viral), neoplastic and even toxic causes have been theorized (Talarico and Schatzberg 2010; Park et al. 2012).

Typical histologic lesions of these disorders include perivascular cuffs. These are usually composed of histiocytes and / or lymphocytes sometimes admixed with plasma cells, depending on the chronicity of the process. Additionally, areas of malacia may be overt. These histologically similar disorders are especially characteristic for many toy dog breeds such as Pugs (pug dog encephalitis; Levine et al. 2008), Yorkshire Terriers (necrotizing encephalitis in the Yorkshire Terrier; Sawashima et al. 1996; Lotti et al. 1999; Kuwamura et al. 2002; Lezmi et al. 2007), Chihuahuas (Higgins et al. 2008), Pekingese (Cantile et al. 2001), Papillons, Shih Tzu, Coton de Tulear and Brussels Griffons (Cooper et al. 2014), for many of which breed-specific terminology has been developed.

In contrast to NME and NLE, dogs with GME exhibit mostly granulomatous inflammation in the cerebellum and in the cerebral white matter. For this condition no specific breeds have been described. Cytokine profiling revealed an increased expression of IL-17 in these animals (Park et al. 2013). This cytokine produced by different innate immune cell types such as microglia, macrophages (Park et al. 2013) and Th1 cells (Aarvak et al. 1999), is thought to

play a crucial role in host defense and inflammatory diseases (Jin and Dong 2013; Park et al. 2013). In case of NME and NLE, a rat model has been established in order to thoroughly investigate the disease (Park et al. 2014).

Even though the dog is the most common species to diagnose non-suppurative meningoencephalitis of unknown origin, this disorder has been also diagnosed in cats (Hoff and Vandevelde 1981; Lundgren 1992; Quesnel et al. 1997; Bradshaw et al. 2004; Singh et al. 2005), cattle (Theil et al. 1998; Sanchez et al. 2013), pigs (Bukovsky et al. 2007), and goats (Allen et al. 2013). Common features in all cases are perivascular cuffs of different intensity with lymphocytes, plasma cells and macrophages as well as glia nodules in the neuropil. Occasionally, eosinophils and very rarely neutrophils are involved.

Because of the neuropathological similarity to viral infections in many cases of non-suppurative meningoencephalitis, retrospective screenings for viral agents have been performed on formalin-fixed, paraffin-embedded (FFPE) brain tissues (Theil et al. 1998; Schatzberg et al. 2005; Schwab et al. 2007; Allen et al. 2013; Sanchez et al. 2013). The screenings were usually performed by means of immunohistochemistry (IHC; Theil et al. 1998; Schwab et al. 2007; Allen et al. 2013; Sanchez et al. 2013) or polymerase chain reaction (PCR; Theil et al. 1998; Schatzberg et al. 2005; Sanchez et al. 2013). The number of investigated cases varied from 22 (Schatzberg et al. 2005) to 139 (Allen et al. 2013). The percentage of identified viral or other infectious agents associated with non-suppurative meningoencephalitis was low and varied between 0 % (Schatzberg et al. 2005) and 50 % (Allen et al. 2013), despite high efforts and sometimes large number of cases.

In case of protozoal meningoencephalitis which constitutes another major differential diagnosis for GME (Schwab et al. 2007) a severe inflammatory reaction is often present, but very rarely parasite specimen can be identified (Dubey et al. 2001b; Maier et al. 2015). The mechanism of parasite destruction by the immune cells is not known either. The extracellular forms are immediately affected by antibodies, but the intracellular forms are not (Dubey 2004). Experimental infections with *Sarcocystis neurona* of IFN-γ knockout mice resulted in lesions in different organs, but the parasite could be visualized only in some of them. Several

hypotheses concerning the pathophysiology of the lesions have been proposed. One of them assigns the damage directly to the parasitic infections; the other hypothesis suggests a delayed host autoimmune inflammatory response being the cause of the lesions in the course of the infection clearance (Sharon et al. 2003a). It may also be possible that parasite metabolites or even co-infections account for the severe neurological lesions, all of which would have to be determined in future investigations.

3 Hypothesis

Some avian *Sarcocystis* species such as *Sarcocystis neurona* and *Sarcocystis falcatula* are known for their vast variety of natural and aberrant host species (Dubey and Hedstrom 1993; Forest et al. 2000; Dubey et al. 2001a; Hamir and Dubey 2001; Luznar et al. 2001; Dubey et al. 2003; Siegal-Willott et al. 2005; Dubey et al. 2006; Cooley et al. 2007; Mansfield et al. 2008; Dubey and Hamir 2009; Godoy et al. 2009; Wunschmann et al. 2009). Similarly, avian species other than pigeons have been identified as intermediate hosts of *Sarcocystis calchasi* (Rimoldi et al. 2013; Olias et al. 2014). Therefore these *Sarcocystis* species not only share the characteristic polyxenous life cycle but are also phylogenetically very closely related. Given that *Sarcocystis calchasi* induces severe granulomatous meningoencephalitis without intralesional parasitic stages in pigeons and that a large number of meningoencephalitis of unknown origin in mammals exists, the following hypothesis for this work was formulated:

***Sarcocystis calchasi* may be involved in mammalian encephalitis of unknown origin.**

This hypothesis was tested in a retrospective study using mammalian FFPE brain tissue samples from previously identified cases of MUO.

4 Materials and Methods

4.1 Species Identification

As there are so many different *Sarcocystis* spp. morphologically similar to each other, some of them being pathogenic and others not (Pritt et al. 2008), a problem of species identification surges. In light microscopy there are no visible differences between these species. Thus, this tool fails to identify the different species of this genus (Dubey et al. 1988). For many years, transmission electron microscopy was used to differentiate the species based on the structure of their cyst wall. This tool is expensive, time-consuming and in case of closely related species, which do not exhibit cyst wall differences, not specific enough anymore (Pritt et al. 2008).

To establish phylogenetic relations in between particular species within the phylum Apicomplexa as well as for diagnostic species identification, molecular biology techniques have been developed. The most common molecular markers to differentiate *Sarcocystis* spp. are ribosomal desoxyribonucleic acid (DNA) sequences (Fig.4). For phylogenetic analysis, the genotyping of the small ribosomal subunit ribonucleic acid (SSU rRNA) is used (Tenter 1995). The sequence of this region is conservative for related organisms with a species-specific variable region, which makes it a good diagnostic marker. Another fragment of ribosomal DNA encoding for the large ribosomal subunit RNA (LSU rRNA), especially its D2 domain, has been successfully used to diagnose pathogenic *Sarcocystis* species (Stojecki et al. 2012). Two more DNA regions are considered good molecular markers in population genetic studies – internal transcribed spacer 1 and 2 (ITS1 and ITS2). They are adjacent to conservative genes encoding the small and the large ribosomal subunits (Stojecki et al. 2012). In this study the ITS1 and D2 domain of LSU were used for species identification (Olias et al. 2011).

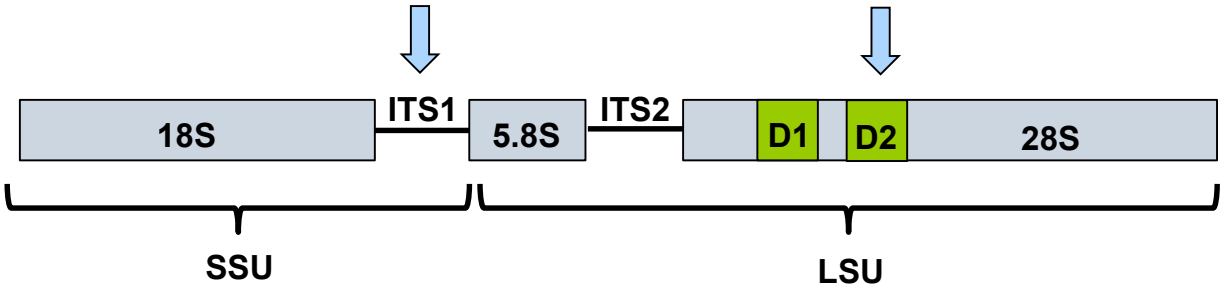

Figure 4: Ribosomal DNA sequence fragments used for *Sarcocystis* spp. identification in this study. SSU – fragment encoding for the small ribosomal subunit RNA, LSU – fragment encoding for the large ribosomal subunit RNA, ITS1 and 2 – internal transcribed spacer 1 and 2, D1 and D2 – domains of the fragment encoding for the large ribosomal subunit RNA, modified from Lafontaine and Tollervey (Lafontaine and Tollervey 2001)

4.2 Biological Samples

For this retrospective study FFPE brain tissue samples from brain stem, cerebellum and cerebral cortex from 143 animals with MUO of 15 mammalian species, necropsied in the period of 1989 – 2012, were selected from the archive of the Institute of Veterinary Pathology of the Freie Universität Berlin. As listed in Table 1, the sample pool included 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 (0.7 %), one raccoon (0.7 %) and one squirrel monkey (0.7 %). The preselection of samples was based on the archived digital necropsy reports. Paraffin blocks and corresponding hematoxylin and eosin (H&E) stained histological slides of brain tissues of animals with diagnosed non-suppurative meningitis, meningoencephalitis and / or encephalitis of unknown origin were selected from the archive. The slides were subsequently reexamined by light microscopy (Olympus BH2 BHS, Olympus, Tokyo, Japan) to confirm the diagnosis. All samples with dominating neutrophilic inflammatory components were sorted out and only the samples with lymphocytic, plasmacytic and / or histiocytic / granulomatous inflammation remained in the study (n = 143).

Table 1. Samples selected for the retrospective study

Species	**n = 143**	**100 %**
Dog	60	41.9 %
Cat	41	28.6 %
Pig	8	5.6 %
Cattle	7	4.9 %
Sheep	6	4.2 %
Guinea pig	6	4.2 %
Horse	4	2.8 %
Goat	3	2.1 %
Mouse	2	1.4 %
Ferret	1	0.7 %
Hamster	1	0.7 %
Mink	1	0.7 %
Maned wolf	1	0.7 %
Raccoon	1	0.7 %
Squirrel monkey	1	0.7 %

4.3 Sex and Age of the Animals

4.3.1 Sex

Of the 60 investigated dogs, 29 (48.3 %) were intact females, six (10.0 %) were spayed females, 22 (36.6 %) were intact males, two (3.3 %) were spayed males and in case of one dog (1.7 %) the sex was not specified. Of the 41 cats, 14 (34.1%) were intact females, nine (22.0 %) were spayed females, eleven (26.8 %) were intact males, five (12.2 %) were spayed males and in two cases (4.9 %) the sex was not specified. Out of the eight pigs, three

(37.5 %) were females, four (50.0 %) were intact males and one (12.5 %) was a spayed male. Of the four horses, three (75.0 %) were females and one (25.0 %) was intact male. Among the goats two were female and one was a spayed male. One of the two investigated mice was a spayed female and the other one was an intact male. The other species from which only one specimen was available for investigation were all intact (guinea pig, maned wolf) and spayed (hamster) males respectively (shown in Table 2).

Table 2. Sex of the animals selected for the retrospective study

Species	**Female**	**Spayed female**	**Male**	**Spayed male**	**Not specified**	**Total**
Dog	29	6	22	2	1	60
Cat	14	9	11	5	2	41
Pig	3	-	4	1	-	8
Cattle	5	-	2	-	-	7
Sheep	5	-	1	-	-	6
Guinea pig	4	-	2	-	-	6
Horse	3	-	1	-	-	4
Goat	2	-	-	1	-	3
Mouse	-	1	1	-	-	2
Ferret	-	-	1	-	-	1
Hamster	1	-	-	-	-	1
Mink	1	-	-	-	-	1
Maned wolf	-	-	1	-	-	1
Raccoon	-	-	-	1	-	1
Squirrel monkey	-	-	1	-	-	1

4.3.2 Age

The age of the animals ranged from six days to 19 years. In 18 animals the age was not specified, but in some cases judging on their weight or on the necropsy report information it could be assumed that they were either adult or juvenile.

The age of the dogs ranged from seven weeks to 16 years. In case of three individuals the age was unknown and two dogs were classified as adults in the necropsy reports, but no exact age was specified. The age of the cats ranged from 15 days to 19 years. Four cats were classified as adults and one as juvenile. Pigs were between six days and 15 years of age, one animal was juvenile and in one case the age was unknown and not further specified. The cattle samples were available from animals from 15 days to six years old. The sheep were from five months to four years old. In case of this species one animal was classified as adult and one was of unknown age. The guinea pigs were from two months to seven years of age. The age of the horses ranged from two years and six months to 17 years. Among the goats one specimen was one year and ten months old, the second one was four years old and the third one was classified as adult. One of the mice was five months old, the age of the second one was unknown. The age of the species from which only one specimen was available for investigation was as follows: ferret – one year old, hamster – adult, mink – two years old, maned wolf – four years old, raccoon – one year old, squirrel monkey – adult. The age of the particular animals is shown in Table 7.

4.4 General Measures for Prevention of Contamination

All handling was carried out in a sterile fashion to prevent contamination. Different operations such as slicing of paraffin blocks, DNA isolation, PCR and gel electrophoresis were carried out spatially separated. Wearing disposable gloves and clean overalls belonged to the hygiene standard. The working surfaces were disinfected with Megrosept (Megro GmbH & Co. KG, Wesel, Germany) or 10 % sodium hypochlorite solution prior to starting pipetting. Samples, working solutions and reagents were aliquoted in small quantities easy to handle. During the PCR preparation, the samples were placed on ice and stored at -20 °C immediately after preparation. In general, for all reactions nuclease-free and DNA- and RNA-free water was used. In order to monitor contamination and primer-dimer formation, which could produce false positive results, a no template control (NTC) was included in all reac-

tions. An equivalent volume of water was added to the NTC instead of DNA template. For all reactions a special set of pipettes (Eppendorf AG, Hamburg, Germany) and pipette tips (Sarstedt AG & Co., Nümbrecht, Germany) for molecular biological experiments was exclusively used.

4.5 DNA Isolation

4.5.1 Slicing of Paraffin Blocks

From each paraffin block 80 sections of 1 µm were cut with a rotary microtome (HM 350, Microm, Heidelberg, Germany) and placed in two separated 1.5 ml reaction vessels (Sarstedt AG & Co., Nümbrecht, Germany), 40 slices in each, for subsequent DNA isolation. In order to control the slicing procedure and avoid contamination, each FFPE tissue block was sliced with a new, clean, disposable, microtome blade (SEC 35, Microm International GmbH, Walldorf, Germany), and the microtome surface was cleaned with DNA-ExitusPlus IF™ (ApliChem GmbH, Darmstadt, Germany), a decontamination solution for DNA and RNA contamination removal. Every four tissue blocks, sections from one paraffin block without tissue were cut and later used as a negative control sample for slicing and DNA isolation procedure in terms of contamination.

4.5.2 DNA Isolation from FFPE Material

The samples were divided in batches of five (four FFPE tissue samples + one paraffin sample without tissue) to prevent possible contamination of other samples during DNA isolation. They were deparaffinized with xylene (Th. Geyer GmbH & Co, Renningen, Germany; 1 ml / sample), and centrifuged (Centrifuge 5417R, Eppendorf AG, Hamburg, Germany; 11 000 x *g*, 2 min). The supernatant was discarded, samples were subsequently washed with absolute ethanol (1 ml / sample) and centrifuged again. Following centrifugation the supernatant was discarded and the samples were incubated (Kühl-Thermomixer, HLC BioTech, Bovenden, Germany) for 10 minutes with an open lid at 60 °C to ensure evaporation of all residual ethanol. Subsequently lysis buffer FL (100 µl / sample) and Proteinase K (10 µl / sample)

were added and the samples were incubated overnight at 56 °C and shaken (400 shaking movements per minute). After incubation, to remove the crosslinks released during the previous lysis step, Decrosslink Buffer D-Link (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) was added (200 µl / sample) and the samples were incubated at 90 °C for 30 min. In the following incubation, to adjust binding conditions, absolute ethanol was once more added to the samples (400 µl / sample). In order to separate the undigested, solid parts of the samples from the liquid fraction, the tubes were gently centrifuged (Micro Centrifuge SD 220 VAC, Carl Roth GmbH, Karlsruhe, Germany). The liquid fraction of each preparation was loaded on one NucleoSpin®FFPE DNA silica membrane column (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). After centrifuging for 30 s at 11.000 x g the column was washed with B5 wash buffer (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and centrifuged twice (400 µl, 11.000 x g, 30 s). Finally the DNA was eluted by washing the column with elution buffer BE (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany; 30 µl, 30 s 11.000 x g).

4.5.3 Spectrophotometric Measurements of DNA Concentrations

The DNA concentrations of the samples was measured with a spectrophotometer (NanoDrop™, PEQLAB Biotechnlogie GmbH, Erlangen, Germany) at the absorption of 260 nm. The aim was to use 300 ng template in each PCR reaction, and depending on the concentration, the volume of PCR template was individually calculated.

4.6 DNA Amplification

4.6.1 Primers

4.6.1.1 *Sarcocystis calchasi*-Specific Primers

For the amplification of *Sarcocystis calchasi* DNA in the investigated samples a primer pair located in the ITS1 region (GeneBank Accesion Number: FJ232948) of the 18S ribosomal RNA (18S rRNA) gene (GeneBank Accesion Number: GQ245670) of this parasite, with a predicted amplicon size of 222 base pairs (bp) was used (Olias et al. 2011; Table 3).

4.6.1.2 Apicomplexa-Consensus Primers

For the amplification of *Sarcocystis* spp. or other parasites from the phylum Apicomplexa a second primer pair located in the D2 domain of the conserved 28S ribosomal RNA gene (GeneBank Accesion Number: FJ232949), with predicted amplicon size of 349 bp, was used (Wunschmann et al. 2011). Because the amplified domain is a region repeated in a number of apicomplexan species, the use of this primer pair allowed for a consensus PCR able to detect more species (Table 3).

4.6.1.3 Reference Gene Primers

In order to verify DNA integrity and check the level of degradation of DNA fragments present in the isolated samples, eukaryotic translation elongation factor 1 alpha (EF1α) was chosen as a reference gene. Primer pairs for two EF1α PCR products, 198 bp and 404 bp, respectively, were designed using Primer-BLAST online primer designing tool (http://www.ncbi.nlm. nih.gov/tools/primer-blast/).

The EF1a_198 rev. primer was designed as a common reverse primer for both EF1a_198 forward and EF1_404 forward primers. This enabled a duplex-PCR indicating the length of the DNA fragments present in the reaction. In order to avoid excessive amplification of the shorter DNA fragments, the volume of the shorter forward primer used in the reaction (EF1a_198 forw.) was reduced to 1 / 10 of the longer primer (EF1a_404) and the reverse primer (EF1a_198 rev.) volume.

All primers were synthesized by Biomers.net GmbH (Ulm, Germany), diluted at a concentration of 20 pmol / µl and aliquoted 50 µl in 1.5 ml Eppendorf tubes (Eppendorf AG, Hamburg, Germany).

Table 3. Primer pairs used for the study

	Primer name	**Sequence 5´ - 3´**	**Calculated melting temperature (Tm)**	**Predicted amplicon size (bp)**
S. calchasi-specific	WE8s (SCa1)	CTCCTTGCTCGAGAATGAACATGAG	58.0 °C	222
	WE9as (SCa2)	GATCATCTTTTCGACGACAATATCG	56.0 °C	
Apicom-plexa-consensus	WE2s (SAD 2F)	GGAAGCGATTGGAACC	45.0 °C	349
	WE5as (SAD 2R)	CCTTGGTCCGTGTTTCA	46.0 °C	
EF1α	EF1a_198 forw.	AGGCTCTTCCTGGGGACAAT	54.0 °C	198
	EF1a_198 rev.	GAGCTGTGTGACAATCCAGC	50.0 °C	
	EF1_404	GGATTGCATTCTGCCACCAA	56.0 °C	404

4.6.1.4 Confirmation of Primer Specificity

To test the specificity of the *Sarcocystis calchasi*-specific and Apicomplexa-consensus primers, their amplicons were purified using NucleoSpin®Gel and PCR Clean-up kit and sent for sequencing to Seqlab - Sequence Laboratories Göttingen GmbH (Göttingen, Germany). The obtained sequences were compared with the data base by using the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the consistency of the amplicons with the published genome fragments of *Sarcocystis calchasi* (GenBank: GQ245670.1 and FJ232949.2) was confirmed.

4.6.2 PCR Preparation

PCR was performed in 50 µl reaction mixtures containing 25 µl GoTaq®Hot Start Green Master Mix (Promega, Madison, USA), 1 µl of each primer or 0.1 µl of primer EF1a_198 forw., from 0.4 µl to 5 µl DNA template (approximately 300 ng DNA) and nuclease-free water was added to a total volume of 50 µl (18-22.6 µl; Tab. 3). For each reaction, the reagents were pipetted in the same order. First, the DNA was pipetted into 0.2 ml reaction vessels. The second step consisted of preparing three primer pair mixtures in three different 1.5 ml reaction vessels containing the adequate volume of primers, GoTaq®Hot Start Green Master Mix and nuclease-free water for all reactions. Subsequently, from 45.0 to 49.6 µl of the mixture, depending on the DNA template volume, were pipetted into the 0.2 ml reaction vessels. The reaction mixtures were briefly vortexed (1 s) to mix the DNA with the PCR solution, then centrifuged for 3 s at 1.200 x *g* (Heraeus Biofuge Stratos Centrifuge, Thermo Electron, Osterode, Germany) and placed in the thermal cycler TGradient 96 (Biometra, Göttingen, Germany).

Table 4. Composition of the 50 µl reaction volume

Component	**Concentration (stock solutions)**	**Volume [µl] per 1 reaction**
GoTaq®Hot Start Green Master Mix 2X	1X*	25
Forward primer	20 pmol / µl	1 / 0.1**
Reverse primer	20 pmol / µl	1
Template (DNA)	300 ng / reaction	0.5-5
H_2O	not applicable	ad 50 µl

DNA – desoxyribonucleic acid

* final concentration

** Primer EF1a_198 forw.

The thermal profile used for DNA amplification was 2 min at 94.0 °C for initial denaturation followed by 40 cycles of 30 s at 95.0 °C for denaturation, 30 s at 55 °C for annealing, and 1 min at 72.0 °C for DNA synthesis with a time increment of 2 s and finally 10 min at 72.0 °C for end annealing (Table 5).

Table 5. PCR thermal profile

Reaction step		**Time**	**Temperature**	**Comment**
1		2 min	94.0ºC	Initial denaturation
2	40 x	30 s	95.0ºC	Denaturation
3		30 s	55.0ºC	Annealing
4		1 min	72.0ºC	DNA synthesis
5		10 min	72.0ºC	End annealing

DNA – desoxyribonucleic acid

All reactions with *Sarcocystis calchasi*- and Apicomplexa-consensus primers were run as triplicates. The reactions with use of reference gene primers, as a control reaction for DNA quality, were performed once. In each PCR run, in order to verify contamination, an NTC with water instead of template DNA, and an isolation control with paraffin but without tissue that had undergone the same DNA isolation process, were included.

4.7 Gel Electrophoresis

To visualize the amplicons after the reaction, 10 µl of the PCR mixture was loaded on a 2.5 % agarose gel and gel electrophoresis was performed.

4.7.1 Agarose Gel Preparation

A 2.5 % gel was prepared as followed: to 100 ml of 0.5 x TBE buffer 2.5 g agarose (Bio&SELL e.K., Feucht, Germany) were added and shortly cooked in the microwave until it was completely dissolved and the solution became transparent. In order to later visualize the amplicons on the gel in the UV light a fluorescent tag, 2 µl of 1 % ethidium bromide, were added to the solution. The prepared mixture was poured into a gel tank. Subsequently, depending on the number of samples to be loaded in one run, a comb with an adequate number of slots was inserted into the liquid gel, and the gel was left at room temperature for 40 minutes to become solid.

4.7.2 Gel Electrophoresis

After the time necessary for the gel to become solid, the comb was taken out, the gel tank was placed in the TBE buffer-filled gel electrophoresis chamber (PEQLAB Biotechnlogie GmbH, Erlangen, Germany) and 10 µl of PCR mixture from each sample was loaded into each slot. FastRuler Low Range DNA Ladder (2 µl, Fisher Scientific-Germany GmbH, Schwerte, Germany) was used as a marker to identify the size (bp) of the amplicons. To separate the amplified DNA molecules, gel electrophoresis was performed. Electric potential difference of 100 V was applied (PowerPac™ Basic Power Supply, Bio-Rad, California, USA). After 1 hour the gel tank was taken out of the electrophoresis chamber, the gel itself

was taken out of the tank and the DNA was visualized through UV light with use of Bio-Rad Universal Hood II and Biorad Chemidox XRS detection system (Bio-Rad laboratories, Milan, Italy) and evaluated by using Quantity One analysis software (Bio-Rad laboratories).

4.8 Commonly Used Chemicals, Solutions and Enzymes

In Table 6. commonly used chemicals, solutions and enzymes are listed.

Table 6. Commonly used chemicals, solutions and enzymes

Used substance	**Company**
Agarose	Bio&SELL e.K., Feucht, Germany
Ethanol ≥ 99.5 %	Carl Roth GmbH, Karlsruhe, Germany
Ethidium bromide 1 %	Carl Roth GmbH, Karlsruhe, Germany
DNA isolation kit NucleoSpin®FFPE DNA containing: Decrosslink Buffer D-Link, Elution Buffer BE (5mM Tris / HCl, pH 8.5), Lysis Buffer FL, NucleoSpin®FFPE DNA silica membrane columns (green rings), Proteinase Buffer PB, Proteinase K, Wash Buffer B5	MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany
PCR clean-up kit NucleoSpin®Gel and PCR Clean-up containing: Binding buffer NTI, Elution Buffer NE, NucleoSpin®Gel and PCR Clean-up Columns (yellow rings), Wash Buffer NT3	MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany
TBE running buffer / stock solution 5x	54 g Tris-Base, 27.5 g boric acid, 20 ml 0.5 M EDTA, pH 8.0 ad 1 l Aqua destillata
TBE running buffer 0,5x	5x stock solution with Aqua destillata in 1:10 ratio
GoTaq® Green Master Mix	Promega, Madison, USA
Nuclease-free Water	Promega, Madison, USA

DNA – desoxyribonucleic acid; FFPE – formalin fixed paraffin embedded; PCR – polymerase chain reaction; TBE - tris / borate / ethylenediaminetetraacetic acid; EDTA - ethylenediaminetetraacetic acid

5 Results

5.1 Histopathological Findings

Sections of cerebral cortex, cerebellum and brain stem of all animals were examined in H&E staining for presence of non-suppurative inflammatory lesions and protozoal cysts. As shown in Table 7, animals had mild to severe chronic lymphohistiocytic encephalitis (eleven animals including the following species: cat, cattle, dog, guinea pig, mouse, pig), lymphohistiocytic meningoencephalitis (28 animals including the following species: cat, cattle, dog, goat, guinea pig, maned wolf, pig), lymphoplasmacytic encephalitis (39 animals including the following species: cat, cattle, dog, guinea pig, mink, pig, sheep, squirrel monkey) or lymphoplasmacytic meningoencephalitis (65 animals including following species: cat, dog, ferret, goat, guinea pig, hamster, horse, mouse, pig, raccoon, sheep). Examples of inflammatory changes are shown in Figures 5 and 6. In most of the cases (n =128 / 143, 89.5 %), inflammatory cells were aggregated around the vessels forming perivascular cuffs. There were neither protozoal cysts nor other structures of parasites detected in any of the samples.

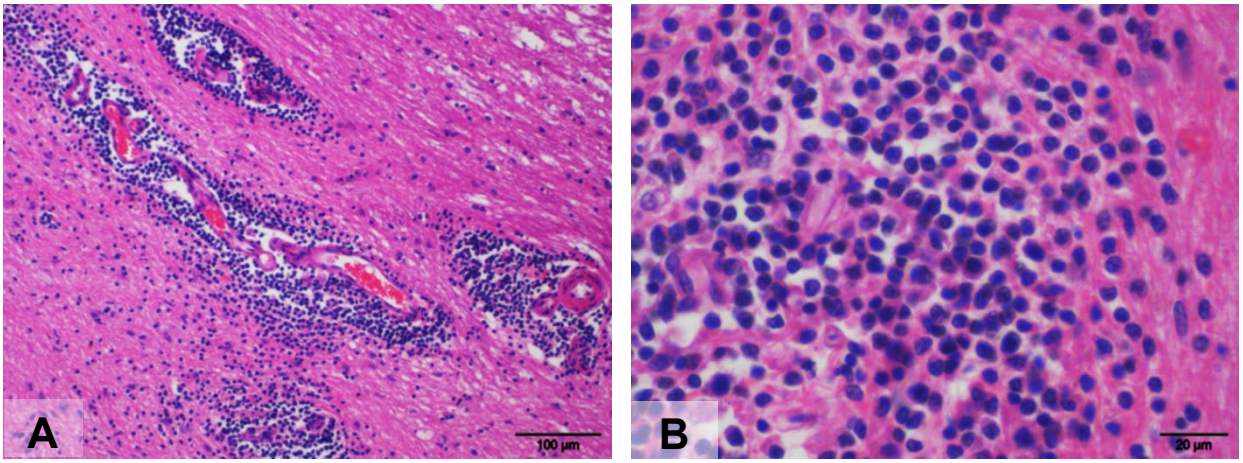

Figure 5. Example of inflammatory changes detected in a 17-year-old horse (sample No 78).

A. Severe, chronic, multifocal, lymphoplasmacytic encephalitis with multilayered perivascular cuffs (40x magnification; scale bar: 100 µm)

B. Higher magnification of a perivascular cuff reveals the presence of lymphocytes and plasma cells in the inflammatory infiltrate (400x magnification; scale bar: 20 µm)

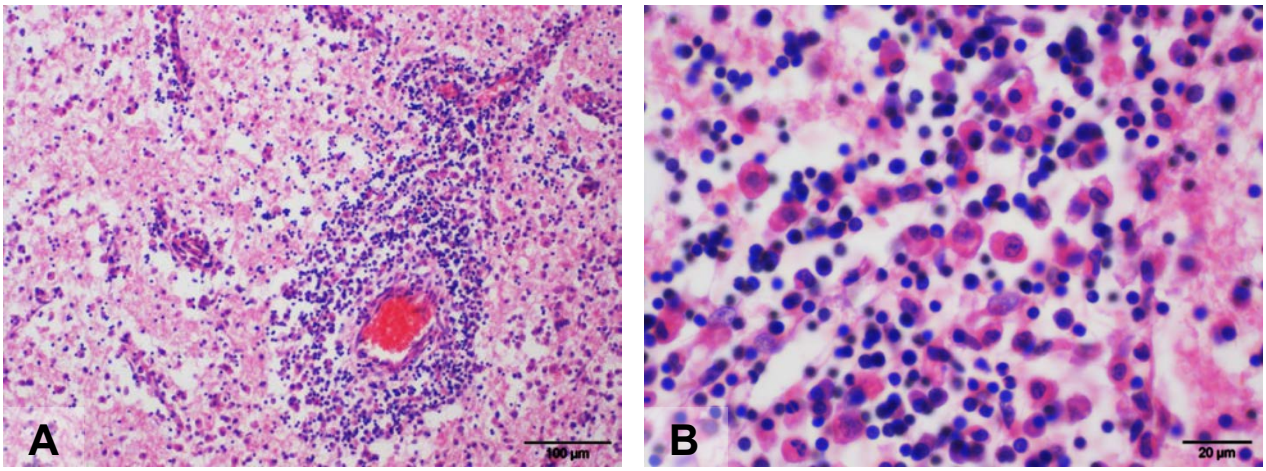

Figure 6. Example of inflammatory change detected in a one-year-old dog (sample No 140).

A. Severe, chronic, multifocal, lymphohistiocytic encephalitis with multilayered perivascular cuffs (40x magnification; scale bar: 100 µm)

B. Higher magnification of lymphocytes, macrophages and fewer plasma cells present in the inflammatory infiltrate (400x magnification; scale bar: 20 µm)

5.2 Molecular Biology Findings

5.2.1 Specific Amplification of *Sarcocystis calchasi* from Positive Control

In the first stage of the work all primer pairs to be used in the study were tested and established on two different positive controls – *Sarcocystis calchasi* cysts from fresh striated muscle tissue and FFPE brain tissue of experimentally infected pigeons (Olias et al. 2010a; Fig. 7).

For the amplification of *Sarcocystis calchasi* DNA a *Sarcocystis calchasi*-specific primer pair (WE 8s, 9as) located in the ITS1 region of the 18S rRNA gene of this parasite was used and generated a predicted amplicon size of 222 bp .

For the amplification of *Sarcocystis* spp. or other parasites from the phylum Apicomplexa an Apicomplexa-consensus primer pair (WE 2s, 5as) located in the D2 domain of the conserved 28S ribosomal RNA gene was used and generated a predicted amplicon size of 349 bp. In order to verify DNA integrity and length, EF1α was chosen as reference gene and primer pairs for two amplification products, 198 bp and 404 bp, respectively, from this gene were used.

Aditionally the Apicomplexa-consensus primer pair was tested on FFPE cardiac muscle tissue containing cysts from a sheep, in order to confirm the ability of these primers to amplify *Sarcocystis* species different from *Sarcocystis calchasi*. The reaction resulted in a specific band of the predicted size which after sequencing turned out to be consistent with *Toxoplasma gondii*.

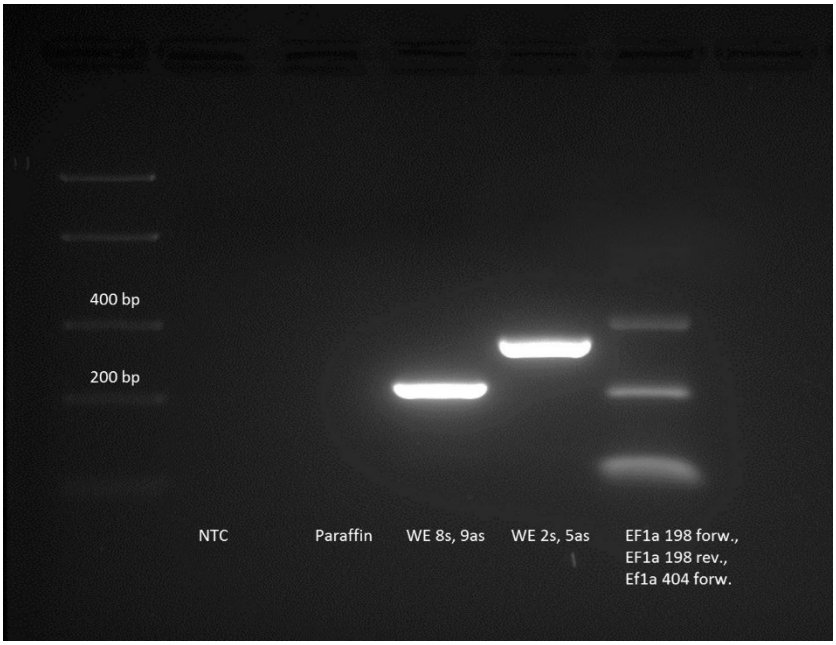

Figure 7. Amplicons from primer pairs established with the positive control (FFPE brain tissue of experimentally infected pigeons)

NTC – no template water control; Paraffin – isolation control, a paraffin block without tissue; WE 8s, 9as – *Sarcocystis calchasi*-specific primers; WE 2s, 5as – Apicomplexa-consensus primers; EF1a 198 forw., EF1a 198 rev., EF1a 404 forw. – reference gene primers (duplex PCR)

In order to minimize the influence of DNA amplification variability and PCR susceptibility to contamination, all reactions with *Sarcocystis calchasi*-specific and Apicomplexa-consensus primers were run as triplicates. A sample was considered positive if a band was visible in at least two out of three reactions (+, +, + and +, +, - = +), and negative if no band was visible in at least two out of three reactions (-, -, - and -, -, + = -).

The 143 samples were investigated in 35 PCR runs divided into groups of four samples and one PCR run of a group of three samples. In each of the runs, a NTC and an isolation control were included. NTC remained negative for all primer pairs in all runs. The isolation control remained negative for *Sarcocystis calchasi* and Apicomplexa in all runs, but in six out of the 36 runs, it turned out to be positive for the shorter (198 bp) and in two runs

for the longer (404 bp) fragment of the reference gene EF1α. This may be attributed to the fact that unspecific, non-target DNA got into the isolation control samples during processing which, however, had no influence on the study results.

In order to avoid contamination, the positive control used for establishing the primers was introduced to the actual screening PCR runs only when a new batch of primers was used, which happened twice. In both cases, it turned out positive confirming the efficacy of the primers.

5.2.2 *Sarcocystis calchasi* DNA Amplification

As shown in Table 7 the PCR results with *Sarcocystis calchasi*-specific primers were negative in all samples. In 133 (93.0 %) reactions three out of three repetitions were negative and in ten (7.0 %) reactions in one of the three repetitions a weak band was visible. Because these bands were weak, not reproducible and non identifiable when sequenced, these samples were considered negative as well.

5.2.3 Apicomplexa DNA Amplification

The PCR results with Apicomplexa-consensus primers were negative in all samples. In 137 (95.8 %) reactions three out of three repetitions were negative and in six (4.2 %) reactions there was a weak band visible, which was considered negative because of the above mentioned reasons.

Because neither *Sarcocystis calchasi* nor Apicomplexa DNA was detected in any of the samples, no statistical analysis of these data was performed.

5.2.4 DNA Integrity Control

A reaction with reference primers for EF1α amplifying products of two different lengths (198 bp and 404 bp) was performed for each investigated sample in order to verify its DNA integrity and to check the length of DNA fragments present in it. Because of a common reverse primer (EF1a_198rev.) for both forward primers (EF1a_198forw. and EF1a_404), a duplex PCR could be performed enabling visualization of the results for both primer pairs

after one reaction. Due to limited amount of isolated DNA and to the control character of these reactions, they were performed for each sample once only.

The PCR results for short reference gene primers were positive for 117 / 143 (81.8 %) and negative for n = 26 / 143 (18.2 %) samples.

The PCR results with long reference gene primers were positive for 76 (53.1 %) and negative for 67 (46.9 %) samples.

As shown in Table 7., most of the samples negative in the reaction with short reference gene primers (24, 92.3 %) were also negative in the reaction with long reference gene primers. Only two (1.4 %) samples were negative with short reference gene primers, but positive with long reference gene primers.

5.2.5 Samples with Low DNA Concentration

Taking into consideration the efficiency of GoTaq Polymerase being able to amplify one copy of DNA out of a genomic background of 50-500 ng of DNA template in one reaction and the inhibiting properties of DNA isolated from FFPE material (Dietrich et al. 2013), the desired template amount in one reaction was set at 300 ng (289.44-355.35; Table 7). In eight samples (No. 20, 32, 33, 34, 38, 39, 69 and 132; 5.6 %; Table 7), the DNA concentration was too low to provide the desired amount in the maximum template volume (5 µl). Nevertheless these reactions were performed anyway, with the total available amount of DNA (18.3 ng-276.3 ng; Table 7). As shown in Table 7, seven of these samples (87.5 %) were negative for the long reference gene fragment and four (50.0 %) were negative for the short reference gene fragment.

5.3 Summary of the Results

Table 7 summarizes all results sorted by species and gives an overview of the breed, necropsy year, age, sex, type of encephalitis, other diagnosed pathological conditions, PCR

results for each primer pair, necropsy number and the sample working number for each investigated animal.

Table 7: Summary of results (abbreviations see end of table)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation (see legend)	Other conditions and available data	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Dog	Dachshund	1989	2 y 6 m	F	+++ 2 pvc	None	313.52	-	-	+	-	S538/89	3
Dog	GSD	1989	1 y	M	++ 4 pvc	Dilated cardiomyopathy	307.17	-	-	+	-	S2143/89	6
Dog	Dachshund	1990	6 m	F	+ 4 pvc	Bronchopneumonia, interstitial nephritis, canine distemper virus antigen negative	300.20	-	-	+	+	S210/90	8
Dog	Yorkshire Terrier	1990	9 w	F	+ 4 pvc	Interstitial pneumonia, canine distemper virus antigen and canine parvovirus antigen negative	303.30	-	-	+	+	S453/90	10
Dog	Airedale Terrier	1990	6 y	F	++ 2 pvc	Dilated cardiomyopathy	294.80	-	-	+	+	S1036/90	12
Dog	Crossbreed	1989	n	F	+ 3 pvc	Acute cardiac myodegeneration, canine distemper virus antigen and rabies virus antigen negative in brain	298.95	-	-	+	-	S966/89	5
Dog	Pinscher	1990	7 w	M	+ 4 pvc	Interstitial pneumonia, acute cardiac myodegeneration, severe ascaridosis, canine distemper virus antigen negative	289.44	-	-	+	+	S2014/90	15

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Dog	Bolognese	1990	3 m	F	++ 4 pvc	Interstitial pneumonia, canine distemper virus antigen and canine parvovirus antigen negative	309.23	-	-	+	+	S2136/90	16
Dog	Crossbreed	1990	16 y	M	+ 1 pvc	End stage kidney, interstitial hepatitis, left testicle hemangiosarcoma	300.70	-	-	+	+	S2142/90	17
Dog	Pug dog	1991	adult	M	++ 3 pvc	Canine distemper virus and rabies virus antigen negative	302.44	-	-	+	+	S1552/91	25
Dog	Yorkshire Terrier	1991	12 y	F	++ 2 pvc	Eccentric cardiac hypertrophy, chronic pulmonary congestion	301.00	-	-	+	+	S1602/91	26
Dog	West Highland White Terrier	1992	16 y	F	+ 3 pvc	Diphtheroid rhinitis	299.46	-	-	-	-	S139/92	27
Dog	Maltese	1992	5 m	F	++ 1 pvc	Interstitial pneumonia	316.88	-	-	+	+	S180/92	28
Dog	Crossbreed	1993	4 m	F	++ 2 pvc	Canine distemper virus antigen and parvovirus antigen negative	18.30	-	-	-	-	S281/93	33

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Dog	Crossbreed	1993	6 m	F	+ 4 pvc	Catarrhal and hemorrhagic duodenitis and jejunitis; acute invagination of the caudal duodenum and cranial jejunum; mild ascaridiosis; canine parvovirus antigen and canine distemper virus antigen negative	32.7	-	-	-	-	S283/93	34
Dog	Dalmatian	1993	5 m	M	+++ 4 pvc	Interstitial pneumonia, acute cardiac myodegeneration, Canine distemper virus antigen and canine parvovirus antigen negative	303.48	-	-	+	+	S571/93	36
Dog	Miniature Poodle	1993	4 y	M	+++ 2 pvc	Catarrhal laryngotracheobronchitis; lymphadenitis simplex	301.08	-	-	+	-	S676/93	37
Dog	n	1993	n	F/S	+ 4 pvc	Global cardiac hypertrophy	188.85	-	-	+	-	S1158/93	39
Dog	n	1993	n	n	+ 4	Interstitial pneumonia, acute cardiac myodegeneration, canine distemper virus antigen and rabies virus antigen negative	301.82	-	-	+	+	S1192/93	40

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Dog	Crossbreed	1993	3 m 2 w	M	++ 2 pvc	Canine distemper virus antigen negative	305.95	-	-	+	-	S1308/93	41
Dog	French Bulldog	1993	2 y	F	+ 3 pvc	Suppurative bronchointerstitial pneumonia	304.66	-	-	+	-	S1489/93	42
Dog	Dalmatian	1993	7 y	M	+ 3 pvc	Canine distemper virus antigen and canine parvovirus antigen negative	295.13	-	-	+	-	S1668/93	44
Dog	Poodle	1994	7 y	M	+ 3 pvc	Suppurative pancreatitis, left ventricular cardiac hypertrophy with right atrial dilation	299.78	-	-	+	-	S1724/94	48
Dog	Golden Retriever	1995	1 y	F	++ 3 pvc	Dilated cardiomyopathy	297.57	-	-	+	+	S692/95	55
Dog	West Highland White Terrier	1996	1 y 7 m	F	+ 3 pvc	None	309.41	-	-	+	+	S1664/96	66
Dog	Crossbreed	1997	6 y	M	+ 3 pvc	Dilated cardiomyopathy	302.03	-	-	+	+	S2176/97	71
Dog	Jack Russel Terrier	1998	5 y	F	+++ 4 pvc	Membrano-proliferative glomerulopathy	295.06	-	-	+	+	S484/98	76
Dog	Cocker Spaniel	1998	12 y	M	+ 4 pvc	Spinal cord astrocytoma	294.12	-	-	+	+	S499/98	77

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Dog	West Highland White Terrier	1999	5 y	M	+++ 2 pvc	Otitis	313.37	-	-	+	+	S1344/99	85
Dog	Saluki	1999	12 y	F	++ 3 pvc	None	308.39	-	-	+	+	S1832/99	90
Dog	Poodle	2000	11 y	F	+ 4 pvc	Left ventricular cardiac hypertrophy with right ventricular dilation, mixed glioma	302.85	-	-	+	-	S675/00	94
Dog	Poodle	2002	15 y	M	+ 4 pvc	Uremia, dilated cardiomyopathy, interstitial nephritis, catarrhal enteritis, thyroid adenomas, trichoepitheliomas	298.00	-	-	+	-	S217/02	100
Dog	Cocker Spaniel	2003	6 y	M	+ 1 pvc	Dilated cardiomyopathy, hepatic dystrophy, bronchopneumonia	301.58	-	-	+	+	S1747/03	104
Dog	Husky Crossbreed	2003	4 y	F	+ 4 pvc	None	293.03	-	-	+	+	S1843/03	105
Dog	Long Hair Dachshund	2003	12 y	M	+ 4 pvc	Eccentric cardiac hypertrophy with right ventricular dilation	296.93	-	-	+	+	S1887/03	107
Dog	Flat Coated Retriever	2003	8 y	M/S	+ 4 pvc	Valvular endocardiosis, dilated cardiomyopathy	298.95	-	-	+	+	S1906/03	108

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Dog	Yorkshire Terrier	2004	11 y	M	+ 4 pvc	Global cardiac hypertrophy, cardiac myodegeneration	301.29	-	-	+	+	S40/04	109
Dog	Crossbreed	2004	13 y	M	++ 2 pvc	Global cardiac hypertrophy, chronic urocystitis	298.98	-	-	+	+	S192/04	110
Dog	Boxer	2004	7 y 7 m	F	+ 3	Dilated cardiomyopathy, chronic pulmonary congestion, chronic nephropathy	303.37	-	-	+	+	S483/04	111
Dog	Collie Crossbreed	2004	adult	F/S	+ 4 pvc	Anemia, icterus, mammary gland adenocarcinoma with metastasis in all organs	297.24	-	-	+	+	S678/04	112
Dog	Rottweiler	2004	9 y	M	+ 2 pvc	Interstitial nephritis, dilated cardiomyopathy, beta-cell adenocarcinomas	301.10	-	-	+	+	S727/04	114
Dog	Crossbreed	2004	12 y	F	+ 4 pvc	Interstitial nephritis, dilated cardiomyopathy, mammary gland adenocarcinomas	305.28	-	-	+	+	S1075/04	116
Dog	Golden Retriever	2004	12 y	M	+ 3 pvc	Uremia, cardiac hypertrophy	294.97	-	-	+	+	S1365/04	118
Dog	Jack Russell Terrier	2004	3 m	F	+ 4 pvc	Anemia, bronchointerstitial pneumonia	306.78	-	-	+	+	S1415/04	119
Dog	Golden Retriever	2004	8 m	F/S	+ 4 pvc	Tumor cell emboli in the lungs, dilated cardiomyopathy	308.95	-	-	+	+	S1454/04	122

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Dog	Chihuahua	2004	2 m 2 w	M	+ 4	Anemia, canine distemper virus antigen and rabies virus antigen negative	312.36	-	-	+	+	S1805/04	123
Dog	Chihuahua	2006	6 y	F	++ 4 pvc	Interstitial nephritis	301.85	-	-	+	+	S274/06	127
Dog	Giant Schnauzer	2006	10 y	F/S	+ 4 pvc	None	311.06	-	-	+	+	S309/06	128
Dog	Pug dog	2006	2 y	F	+++ 2 pvc	Left ventricular cardiac hypertrophy	300.61	-	-	+	+	S548/06	130
Dog	Labrador	2007	3 w	M	+ 4	Necrotizing pneumonia	305.83	-	-	+	-	S82/07	131
Dog	Cavalier King Charles Spaniel	2007	4 y	F	+ 3 pvc	Suppurative bronchopneumonia	276,30	-	-	+	+	S95/07	132
Dog	Terrier mix	2007	2 y	F/S	+ 4 pvc	Left ventricular cardiac hypertrophy	301.59	-	-	+	+	S697/07	134
Dog	Miniature Pinscher	2008	2 y 5 m	F	+ 1 pvc	Left ventricular cardiac hypertrophy with right ventricular dilation	305.41	-	-	+	+	S104/08	135
Dog	Beagle	2009	10 y	M/S	+++ 4 pvc	Left ventricular cardiac hypertrophy with right ventricular dilation	304.02	-	-	+	+	S406/09	136

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Dog	Siberian Husky	2010	12 y	F/S	++ 4 pvc	Canine distemper virus antigen negative	301.55	-	-	+	+	S20/10	137
Dog	Wire-haired Dachshund	2010	5 y	F	+ 2	None	299.33	-	-	+	-	S99/10	138
Dog	Pug dog	2011	1 y	M	+++ 2 pvc	None	294.40	-	-	+	+	S262/11	140
Dog	Pekingese	2011	2 y	F	+ 2 pvc	None	303.05	-	-	+	+	S384/11	141
Dog	Yorkshire Terrier	2011	2 y	F	+ 3 pvc	Hemothorax, hemoabdomen	302.03	-	-	+	+	S393/11	142
Cat	DSH	1989	12 y	M/S	+ 3	Interstitial nephritis, pancreatitis, concentric cardiac hypertrophy	299.23	-	-	+	-	S126/89	1
Cat	DSH	1991	15 y	F	+ 2 pvc	None	306.60	-	-	+	-	S89/91	4
Cat	DSH	1990	11 y	F/S	+ 3 pvc	Right eye panophthalmitis (trauma)	297.74	-	-	+	-	S1669/90	7
Cat	DSH	1990	11 y	F	+++ 4 pvc	None	295.44	-	-	+	+	S1434/90	14
Cat	DSH	1991	2 y	F	+ 3 pvc	Interstitial pneumonia	292.54	-	-	+	-	S538/91	18
Cat	DSH	1991	4 y	F	+ 4 pvc	None	199.75	-	-	-	-	S765/91	20
Cat	Maine Coon	1991	5 m	F	+ 4 pvc	Feline leukemia virus antigen negative	301.29	-	-	+	+	S1440/91	23
Cat	DSH	1992	2 y 6 m	F/S	+ 4 pvc	None	308.64	-	-	+	-	S570/92	31
Cat	DSH	1993	8 y	F/S	+ 2 pvc	Interstitial pneumonia	309.51	-	-	-	-	S551/93	35

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Cat	DSH	1993	9 y	F/S	+ 2 pvc	Dilated cardiomyopathy, hepatic lipidosis	307.52	-	-	+	-	S1604/93	43
Cat	DSH	1994	10 y	F/S	+++ 4 pvc	Lymphocytic pancreatitis	310.69	-	-	+	-	S160/94	46
Cat	DSH	1995	adult	F	+ 2 pvc	Anemia, uremia, interstitial nephritis, cardiac myodegeneration	296.93	-	-	-	-	S383/95	54
Cat	DSH	1995	2 m	F	+ 4 pvc	None	293.89	-	-	+	+	S902/95	56
Cat	DSH	1995	12 y	F	+ 1 pvc	Anemia	297.96	-	-	-	-	S909/95	57
Cat	DSH	1996	adult	F/S	+ 4 pvc	Anemia, suppurative and necrotizing bronchopneumonia, pleuritis and pericarditis, rabies virus antigen negative	306.26	-	-	+	-	S40/96	61
Cat	DSH	1996	14 y	F/S	+ 3 pvc	Interstitial nephritis	306.88	-	-	-	-	S104/96	62
Cat	DSH	1996	11 y	M/S	++ 4 pvc	Left ventricular cardiac hypertrophy with right ventricular dilation, lung adenocarcinoma, interstitial nephritis	295.11	-	-	-	-	S408/96	63
Cat	DSH	1996	8 y	F/S	+ 4 pvc	Interstitial nephritis	302.38	-	-	+	+	S1210/96	64

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Cat	DSH	1997	2 m	M	++ 2 pvc	Interstitial pneumonia, myocarditis, ulcerative glositis	302.58	-	-	+	-	S1786/97	70
Cat	DSH	1997	6 m	M	++ 2 pvc	Global cardiac hypertrophy	303.99	-	-	+	-	S2207/97	73
Cat	DSH	1998	19 y	F	+ 3 pvc	Cachexia, icterus, thyroid adenocarcinoma, interstitial nephritis and pancreatitis, suppurative and necrohemorrhagic cholangiohepatitis	300.94	-	-	+	-	S702/98	79
Cat	DSH	1998	adult	M/S	+ 4 pvc	Anemia, interstitial nephritis, left ventricular cardiac hypertrophy, rabies virus antigen negative	309.00	-	-	+	-	S2153/98	82
Cat	DSH	1999	11 y	M	++ 4 pvc	None	309.84	-	-	+	+	S972/99	84
Cat	DSH	1999	6 y	F/S	+ 4	None	301.51	-	-	+	-	S1379/99	86
Cat	DSH	1999	13 y	F	+ 4 pvc	Hepatic dystrophy	316.93	-	-	+	+	S1540/99	88
Cat	DSH	1999	3 m	M	+ 3 pvc	None	318.53	-	-	+	+	S1597/99	89
Cat	DSH	1999	1 m	M	+ 2 pvc	Corneal ulcera, interstitial pneumonia, massive nematode infestation	308.42	-	-	+	+	S1911/99	91
Cat	Maine Coon	1999	juvenile	M	+++ 4 pvc	Interstitial pneumonia	298.65	-	-	+	-	S1953/99	92
Cat	DSH	2000	11 y	M/S	++ 4 pvc	Dilated cardiomyopathy, interstitial pneumonia	301.19	-	-	+	-	S172/00	93

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Cat	DSH	2000	5 y	F	+ 3 pvc	Granulomatous pneumonia	306.57	-	-	+	-	S1269/00	95
Cat	n	2000	2 y	F	+ 4 pvc	Interstitial pneumonia, suppurative endometritis	297.76	-	-	+	-	S2067/00	96
Cat	DSH	2000	adult	M	+ 3 pvc	Myeloid leukemia, meningioma	308.29	-	-	+	+	S2140/00	97
Cat	DSH	2000	7 m	n.	+ 3 pvc	Pyothorax due to a perforated lung abscess, catarrhal enteritis, ascaridiosis	303.14	-	-	+	+	S2311/00	98
Cat	DSH	2001	3 y	n	+ 3 pvc	Myeloid leukemia, interstitial pneumonia	305.90	-	-	+	-	S42/01	99
Cat	Devon Rex	2002	8 m	M	+ 3 pvc	Anemia, icterus	338.84	-	-	-	+	S1351/02	101
Cat	DSH	2003	3 y	M/S	+ 4	Catarrhal jejunitis	315.1	-	-	+	+	S1856/03	106
Cat	DSH	2004	11 y	F	+ 4	Interstitial nephritis, right ventricular cardiac dilation	299.73	-	-	+	+	S711/04	113
Cat	DSH	2004	1 m	M	+ 4	Interstitial pneumonia	297.18	-	-	+	+	S788/04	115
Cat	DSH	2004	15 d	M	+ 4	Anemia, interstitial pneumonia	295.68	-	-	+	+	S1278/04	117
Cat	DSH	2004	4 m	F	+ 4	Interstitial pneumonia	309.67	-	-	+	+	S1448/04	121
Cat	ESH	2007	2 y	M	++ 4 pvc	None	305.86	-	-	-	-	S531/07	133
Pig	Angeln Saddleback	1991	4 w	M	++ 4 pvc	Interstitial pneumonia, follicular hyperplasia of the lymphatic tissue, non-suppurative hepatitis	302.84	-	-	+	+	S749/91	19

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Pig	n	1993	juvenile	M	+ 4 pvc	Eosinophilic enteritis, interstitial nephritis	309.30	-	-	-	-	S2058/93	45
Pig	Hybrid	1994	12 d	F	++ 4 pvc	Suppurative bronchopneumonia, catarrhal enteritis, *Salmonella typhimurium*, var. Kopenhagen and porcine respiratory coronavirus antigen positive, classical swine fever antigen negative	308.55	-	-	-	-	S1878/94	49
Pig	n	1994	15 y	M	+ 2 pvc	Anemia, suppurative bronchopneumonia, nonsuppurative enteritis	303.99	-	-	-	-	S1971/94	50
Pig	German Landrace	1995	4 m	F	++ 4 pvc	Cachexia, anemia, interstitial pneumonia, suppurative rhinitis and sinusitis	305.11	-	-	-	-	S128/95	51
Pig	n	1995	n	M	+ 2 pvc	Dilated cardiomyopathy, catarrhal gastroenteritis, interstitial pneumonia	300.60	-	-	-	+	S935/95	58

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Pig	Hybrid	1997	6 d	F	+ 3	Cachexia, anemia, polyarthritis, apostematous myositis, catarrhal rhinitis and bronchopneumonia	301.72	-	-	-	-	S829/97	68
Pig	Hybrid	1999	3 m	M/S	+ 1 pvc	Catarrhal enteritis	314.87	-	-	-	-	S119/99	83
Cattle	Deutsche Rotbunte	1990	6 w	M	++ 2 pvc	Suppurative and necrotizing bronchopneumonia, congenital internal hydrocephalus, bovine viral diarrhea virus immunoserology negative	297.90	-	-	+	-	S1431/90	13
Cattle	n	1992	10 m	F	+ 2 pvc	Catarrhal enteritis, BVDV negative	302.87	-	-	+	-	S327/92	30
Cattle	German Black Pied	1995	15 d	M	+ 4	Cachexia, anemia, fibrinous pleuropneumonia, interstitial nephritis	300.10	-	-	+	+	S267/95	53
Cattle	German Black Pied	1996	4 y	F	+ 3 pvc	Diphtheroid rhinitis, pharyngitis, tracheitis, fibrinonecrotic bronchopneumonia, vegetative endocarditis, infectious bovine rhinotracheitis virus antigen positive	311.65	-	-	+	+	S1527/96	65

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Cattle	German Black Pied	1998	4 y 6 m	F	+ 1 pvc	Fibrinous and exudative peritonitis	303.78	-	-	+	+	S480/98	75
Cattle	German Black Pied	1998	6 y	F	+ 1 pvc	Proventricular and abomasal atonia	302.04	-	-	+	+	S1141/98	81
Cattle	Holstein Friesian	2004	2 y	F	+ 3 pvc	Anemia, pericarditis, epicarditis, endocarditis valvularis thromboticans	355.35	-	-	+	+	S1984/04	124
Sheep	Merino	1990	n	F	++ 4 pvc	Rabies virus antigen negative, *Listeria* antigen negative	301.50	-	-	+	+	S415/90	9
Sheep	German Whitehead	1990	2 y	F	+ 3 pvc	Interstitial pneumonia	300.19	-	-	+	-	S692/90	11
Sheep	Texel sheep	1992	4 y	F	++ 3 pvc	Catarrhal rhinitis, laryngotracheitis, bronchitis, enteritis, interstitial pneumonia, suppurative endometritis, *Chlamydia* antigen positive in the uterus	262.95	-	-	+	-	S980/92	32
Sheep	Heidschnucke	1995	5 m	M	+ 3 pvc	Cachexia, anemia, icterus, dilated cardiomyopathy, calcified myocardial sarcosporidial cysts	299.48	-	-	+	+	S1367/95	59

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Sheep	Black Dairy Sheep	1997	11 m	F	+ 3 pvc	Right ventricular dilation	263.00	-	-	-	-	S938/97	69
Sheep	East Friesian Sheep	1998	adult	F	+ 3 pvc	Cachexia, anemia	299.00	-	-	+	+	S136/98	74
Guinea pig	n	1995	2 m	M	+ 2 pvc	Cachexia, interstitial pneumonia, catarrhal enteritis	292.58	-	-	-	-	S265/95	52
Guinea pig	n	1997	1 y	M	++ 1	Interstitial pneumonia, mycotic hepatitis	329.17	-	-	-	-	S179/97	67
Guinea pig	n	1998	2 m	F	+ 2 pvc	Interstitial pneumonia	316.32	-	-	-	-	S761/98	80
Guinea pig	n	1999	7 y	F	+ 4 pvc	Interstitial pneumonia	305.63	-	-	+	-	S1516/99	87
Guinea pig	n	2002	2 y	F	+ 3	Liver cirrhosis, interstitial nephritis, ovarian cysts, endocrine dermatosis	304.91	-	-	-	-	S1962/02	103
Guinea pig	n	2004	6 y	F	+ 4 pvc	Interstitial nephritis, interstitial pneumonia, catarrhal enteritis	309.47	-	-	+	-	S1416/04	120
Horse	Moritzburger	1998	17 y	M	+++ 4 pvc	Fibrinous pneumonia	314.71	-	-	+	-	S640/98	78
Horse	German Riding Pony	2002	5 y	F	+++ 4 pvc	Suppurative bronchitis	306.57	-	-	+	+	S1957/02	102
Horse	Warmblood	2005	2 y 6 m	F	++ 4 pvc	None	295.74	-	-	-	-	S268/05	125

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Horse	Warmblood	2006	8 y	F	++ 4 pvc	Ulcerative gastritis, suppurative and necrotizing tracheitis with foreign material	293.80	-	-	+	+	S473/06	129
Goat	German Premium Goat	1991	1 y 10 m	M/S	+ 2 pvc	Interstitial nephritis, internal hydrocephalus	302.17	-	-	+	+	S1436/91	22
Goat	Pygmy Goat	1991	adult	F	+ 2 pvc	Subacute cardiac myodegeneration	301.75	-	-	+	-	S1489/91	24
Goat	White German Premium Goat	1992	4 y	F	++ 4 pvc	Fibrinous gonitis	309.30	-	-	+	-	S218/92	29
Mouse	n	1995	n	M	+ 1 pvc	Ceruminous gland adenoma, periauricular dermatitis	328.30	-	-	-	-	S1678/95	60
Mouse	Fancy Mouse	2010	5 m	F/S	+ 4 pvc	Bronchointerstitial pneumonia	295.11	-	-	+	-	S144/10	139
Ferret	Not applicable	1989	1 y	M	++ 4 pvc	Interstitial pneumonia, canine distemper virus antigen negative	295.74	-	-	+	+	S478/89	2
Hamster	n	1991	adult	F	+ 4 pvc	Protozoal infestation of cecum and large intestine, lymphohistiocytic hepatitis with giant cells	307.33	-	-	+	-	S1061/91	21

Table 7: Summary of results (continued)

Species	Breed	Necropsy year	Age	Sex	Type of inflammation	Other conditions and investigations	DNA / reaction (ng)	PCR 8s, 9as	PCR 2s, 5as	PCR EF1a 198	PCR EF1a 404	Necropsy number	Sample working number
Maned wolf	Not applicable	1994	4 y	M	+++ 2 pvc	Valvular endocarditis	294.95	-	-	+	+	S616/94	47
Mink	Not applicable	1993	2 y	F	+ 3 pvc	Interstitial pneumonia, canine distemper virus antigen, canine parvovirus antigen and pseudorabies virus antigen negative	76.70	-	-	-	-	S1087/93	38
Raccoon	Not applicable	2012	1 y	M/S	+ 4 pvc	Interstitial pneumonia	298.40	-	-	+	+	S419/12	143
Squirrel monkey	Not applicable	1997	adult	M	+ 3 pvc	Jejuno – ileal invagination	299.20	-	-	+	-	S2189/97	72

y – year; m – month; w – week; n – not specified; F – female; F/S – spayed female; M – male; M/S – spayed male; DSH – Domestic Shorthair;

GSD – German Shepherd; + - mild; ++ - moderate; +++ - severe; 1 – lymphohistiocytic encephalitis; 2 – lymphohistiocytic meningoencephalitis;

3 – lymphoplasmacytic encephalitis; 4 – lymphoplasmacytic meningoencephalitis; pvc – perivascular cuff

6 Discussion

Sarcocystis calchasi is a new parasitic species first discovered and described in Berlin (Olias et al. 2010b). Recently, it has also been identified on the American continent (Wunschmann et al. 2011; Rimoldi et al. 2013) and in Japan (Ushio et al. 2015). Its morphology and life cycle have already been described (Olias et al. 2010a; Olias et al. 2010b). Northern goshawk and pigeon seem to be its natural definitive and intermediate hosts, respectively (Olias et al. 2010c; Olias et al. 2011). Furthermore, several intermediate host species, such as cockatiels (Olias et al. 2014) and parrots (Rimoldi et al. 2013) have been identified.

Albeit Sarcocystidae are usually host specific (Tenter 1995), there are many polyxenous organisms within this family that have the potential to infect more than one host. The best known example, spread world-wide with zoonotic potential, is *Toxoplasma gondii* (Tenter et al. 2000). Another important pathogen able to cause meningoencephalitis in many different aberrant mammalian hosts is *Sarcocystis neurona* (Dubey et al. 2000). This parasite, likewise *Sarcocystis calchasi,* is most probably of avian origin (Mansfield et al. 2008). Both species are genetically closely related (Olias et al. 2013).

Even though several aspects of the *Sarcocystis calchasi* biology have already been explained, there are still important questions unanswered. One question, vital to understand the parasite's epidemiology, is whether this species, like many genetically closely related parasites (Tenter et al. 2000; Dubey et al. 2001b; Cooley et al. 2007; Bisby et al. 2010) may infect mammals. Although some mammalian species including dogs, ferrets, rats and mice have experimentally been excluded as definitive hosts (Olias et al. 2010b), their role and the role of other mammals as intermediate or aberrant hosts of the parasite have not been studied so far. One way to approach this question was to search for the presence of *Sarcocystis calchasi* DNA in mammals with pathological lesions similar to those presented in the parasite's intermediate hosts suffering from PPE.

Even though the first phase of PPE may have a mild course and manifests in various organs, the second phase is always characterized by an encephalitis (Olias et al. 2013). This is why we decided to investigate brain samples with meningoencephalitis of unknown origin, given that other organs might have not been affected or the parasites would be clearly visible and allow for previous diagnosis of a protozoal inflammation. After selecting adequate material from the archive and thorough histopathological reexamination of the samples a PCR screening was performed.

The samples were screened for *Sarcocystis calchasi* DNA as technical triplicates. For quality control in each screening round for each of the triplicates a negative control without DNA template and DNA isolation control from a paraffin block without tissue were included. All investigated samples turned out to be negative and no evidence of *Sarcocystis calchasi* involvement in meningoencephalitis of unknown origin in mammals could be stated.

The investigated samples came from animals necropsied in the period between 1989 and 2012. It was suggested that *Sarcocystis calchasi* is a novel species (Olias et al. 2009) which emerged in the area of Berlin between 2006 and 2008 (Olias et al. 2009). In that context, the absence of *Sarcocystis calchasi* DNA in the samples before 2006 may be explained by the general absence of this species in the area of Berlin at that time. Nevertheless, also the samples from the later period were negative. This might have been due to several causes that will be discussed in the following paragraphs.

6.1 Host Spectrum of *Sarcocystis calchasi*

The absence of *Sarcocystis calchasi* DNA in the investigated samples indicates that even though this parasite has several avian species as intermediate hosts, it appears not to play a role in meningoencephalitis in mammals. This finding is consistent with the biological behavior of another related *Sarcocystis* sp. – *Sarcocystis falcatula*. This parasite also has various avian species as intermediate hosts, a mammal – opossum – as the definitive host (Luznar et al. 2001), but in an infectious challenge, it failed to infect horses (Cutler et al. 1999). *Sarcocystis falcatula* also forms skeletal muscle cysts, but in course of the disease pneumonia is

present, due to the schizogony in the pulmonary capillaries (Box et al. 1984), but no encephalitis. The only known definitive host is the opossum. Rats, cats, dogs and ferrets fed tissue cysts could not be infected experimentally (Box et al. 1984) which is consistent with the experimental infection of rats, dogs, ferrets and mice with *Sarcocystis calchasi* cysts (Olias et al. 2010c). The results of these infectious trials may suggest that it is unlikely for a human to become a definitive host of *Sarcocystis calchasi* and transmission of the parasite by ingesting muscle cysts from pigeon meat is rather unlikely. However the results of this study are far from proof that *Sarcocystis calchasi* may infect mammals.

The specificity in parasitic interactions, defined as certain parasite genotypes being able to infect only certain host genotypes, is thought to have a genetic basis but it can also be modulated by phenotypic plasticity. The genetic adaptation requires a minimum of two generations of organisms to occur, but usually this process takes longer. Nevertheless many parasitic species can rapidly adapt their phenotype to the host. A good example would be *Plasmodium spp*. which is able to change membrane protein expression in order to adapt to the host immune system (Little et al. 2006). The results of our study suggest that there may be no genetic adaptation between *Sarcocystis calchasi* and the mammalian hosts. The question whether there is a potential of phenotypic plasticity of this parasite and future adaptation to mammals remains unanswered and requires further investigations.

In our study, neither *Sarcocystis calchasi* nor other apicomplexan parasites could be detected as a cause of meningoencephalitis of unknown origin in mammals. Because the study was focused on finding this particular group of pathogens, no other agents were sought for. However, other authors searching for various infectious agents involved in non-suppurative meningoencephalitis of unknown origin in FFPE samples in most cases could not state the cause of the disease either. Thus, it still remains unknown in a great proportion of cases (Theil et al. 1998; Schwab et al. 2007; Sanchez et al. 2013).

6.2 Histopathological Detection of Pathogens

Certain methodical drawbacks might have lowered the detectability or prevented the detection of the parasites in this study. Histopathology is a very precise method to confirm parasitic structures, if present and visible, but it is not that reliable as an exclusion method when the pathogens are not visible. Because each slide always represent only one 5 µm cross-section of the sampled area, the absence of parasitic structures in that particular cross section does not always mean that they are absent in the whole sample. Even though the histological examination does not always enable pathogen visualization, this method served well for sample selection based on inflammation classification. Suppurative inflammation was excluded from the potential sample pool, and only non-suppurative processes were taken into consideration, because this is the inflammatory pattern consistent with *Sarcocystis calchasi* infection.

Another histopathological method that could be taken into consideration for parasite detection would be immunohistochemical staining. If specific antibodies were available the brain cysts, schizonts and merozoits of the parasite could be visualized more clearly than in the H&E stain. This method would also constitute a good complementation for the PCR screenings as sometimes the antigen proteins targeted by the IHC in the archived FFPE samples are preserved better than the DNA fragments (Scicchitano et al. 2009; Ramos-Vara and Miller 2014). Unfortunately the available and previously used antibodies (Olias et al. 2013) turned out not be specific enough to distinguish between different species of the Sarcocystidae family and there was a strong background staining affecting the interpretation of the results. Because of these drawbacks and the fact that parasitic structures, when present, are also visible in the H&E staining IHC was not performed.

6.3 DNA Integrity in FFPE Samples

Being a sensitive method frequently used for infectious agent detection (Yang and Rothman 2004; Bukovsky et al. 2007; Pritt et al. 2008; Sanchez et al. 2013), PCR was se-

lected as the method of choice for detection of parasites. Even though it is one of the most reliable methods, there are several challenges that have to be overcome when dealing with FFPE tissues. A general problem in PCR-screenings from FFPE material is the different state of DNA integrity in the samples due to their formalin fixation. Formalin fixation causes detrimental changes to DNA, like its chemical modification, trapping and fragmentation (Lenze et al. 2012; Maes et al. 2014).

The chemical modification consists in direct cross-linking of proteins to DNA as well as DNA trapping in protein networks and causes difficulties in DNA isolation from FFPE tissues. These problems are most prominent when DNA isolation methods for fresh tissues are applied to FFPE tissues (Maes et al. 2014). In our case, a special DNA isolation kit for FFPE tissue was used. Nevertheless the mentioned chemical changes might have still contributed to obtaining insufficient DNA concentration in seven out of the 143 investigated samples (4.9 %).

DNA fragmentation takes place especially when inadequately buffered formalin is used for fixation (Maes et al. 2014). This leads to formic acid formation and DNA depurination which, in turn, increases its susceptibility for cleavage. Fixation time influences the DNA integrity as well (Maes et al. 2014). Proper fixation time ranges from 12 to 36 hours. Fixation beyond 36 hours has a negative impact on the DNA quality. High temperatures are also detrimental to nucleic acids. This is why the embedding temperature should be kept below 60 °C (Maes et al. 2014).

All the mentioned aspects constitute a general problem for retrospective studies involving FFPE tissues. Usually the quality of formalin and the fixation time of the samples is poorly standardized (Nirmalan et al. 2008) and mostly unknown for the samples studied.

Because so many factors that can affect the quality of investigation are independent of the investigator and cannot be influenced at the point of performing a retrospective study, it is essential to maximize the number of examined samples which was intended in our case.

Typical DNA fragments extracted from FFPE tissues range from 300 to 400 bp. This is why the length of PCR amplicons to be obtained from the samples should not exceed this

range (Maes et al. 2014). In case of our study the first primer pair used for screening, which was *Sarcocystis calchasi*-specific, had an amplicon length of 222 bp. The second primer pair, amplifying a gene region conserved for a wider range of Apicomplexa, had an amplicon length of 349 bp. Theoretically, both primer pairs would amplify products within the range of fragmented DNA size. Regardless of that, DNA integrity was investigated with reference gene primers for EF1α of two different amplicon lengths (198 bp and 404 bp), in order to test for the availability of DNA fragments of adequate length for the screening. It turned out that in case of the reference gene primers in 26 samples (18.2 %) the shorter target sequence (198 bp) could not be amplified which may be associated with the fact that these samples did not contain DNA fragments of adequate length. In case of the longer target sequence (404 bp) in 67 samples (46.9 %) it was impossible to amplify DNA, which supports the hypothesis that longer DNA fragments are present in much lesser amount in FFPE tissue sample isolates than the shorter ones (Maes et al. 2014).

In case of *Sarcocystis calchasi*-specific primers 81.8 % (117) of the samples exhibited sufficient DNA integrity for the primers to function and for the test to be reliable. In case of Apicomplexa-consensus primers in as much as 53.1 % (76) of the samples DNA was preserved well enough to state the absence of apicomplexan DNA in them. Taking into consideration the total number of investigated cases it still leaves a large number of samples with amplifiable reference genes but negative for the sought parasites, which implies a meaningful result. In the remaining 46.9 % (67) of samples screened for Apicomplexa, due to insufficient DNA integrity, the presence of these organisms could neither be confirmed nor excluded. This leaves the question unanswered whether Apicomplexa other than *Sarcocystis calchasi* can play a role in MUO.

In the literature, it is stated that even in experimental infections with *Sarcocystis calchasi* very few schizonts were present in only about half of the brains of infected pigeons (Olias et al. 2013). It also occurred that a sample remained negative in PCR despite experimental infection of the pigeon and severe cerebral lesions (Olias et al. 2013). This fact might explain a low detection rate of the parasite in light microscopy but also in the PCR. Even in

potentially positive samples (117 samples with sufficient DNA integrity to detect *Sarcocystis calchasi*), given that parasitic structures were present in the sliced tissue block fragments, the amount of protozoal DNA in relation to the total DNA amount used for reaction might have been too low for amplification of the target genes (Debnath et al. 2010).

6.4 Non-Reproducible PCR Results

In 7 % (10 / 143) reactions for *Sarcocystis calchasi* amplification and in 4.2 % (6 / 143) reactions for the Apicomplexa amplification a weak band was visible in the gel electrophoresis in one of the three PCR repetitions. At the beginning of the study several of these bands were sequenced, but the quality of the sequence and the query coverage were insufficient to identify the product. Because of this and due to the fact that these bands were not reproducible the mentioned samples were considered negative in the study.

6.5 Protozoal Evasion Strategies

Protozoal parasites are known for being able to escape the host humoral and cellular activity (Sacks and Sher 2002). In the first place these evasion strategies include the intracellular life style of many parasites and the formation of parasitophorous vacuole, but there is also evidence that they can regulate the T cell response and suppress their effector functions (Sacks and Sher 2002). This kind of protective mechanism probably applies in case of *Sarcocystis calchasi* as well. It has been proven that in course of PPE the IL-12 / IL-18 / IFN- γ axis, crucial for cellular response activation (Netea et al. 2005), was down-regulated during the first, schizogonic phase of the disease leading to evasion of the strong cellular response of the host. The severe lymphohistiocytic encephalitis observed in the second phase of PPE might be due to up-regulation of IFN-γ and TNF-α – related cytokines (TNF-like cytokine 1A and lipopolisacharide induced TNF-α factor) which causes an exaggerated cellular response, inadequate to the parasite load in the brain, and also occurring in case of absence of intralesional parasitic structures (Olias et al. 2013). Such evasion mechanisms have been suspected in other related Apicomplexa such as *Sarcocystis neurona* (Dubey et al. 2001b) and *Tox-*

oplasma gondii (Melo et al. 2011). It is also possible that in animals infected with *Sarcocystis calchasi* the parasite is not detectable in the brain tissue and the inflammatory changes may be caused by its metabolites.

6.6 Outlook

Sarcocystis calchasi in the area of Berlin constitutes a continuous threat to the pigeon population and possibly to the pigeon breeders and has to be taken into consideration as a differential diagnosis in case of neurological disease in these animals. Our results fail to provide evidence that *Sarcocystis calchasi* may infect mammals. Thus no zoonotic potential is evident. Nevertheless, obtained data are preliminary and this supposition has to be verified with other scientific methods.

DNA detection by means of PCR is the first and very general screening modality that was undertaken. To obtain more solid data concerning this problem, experimental animal infections could be conducted. Given the difficulty and expense of experimental host infection models for *Sarcocystis* spp., much of our current knowledge of pathogenesis and immune responses is extrapolated from murine models of infection (Schlüter et al. 1991; Rohlman et al. 1993; Lindsay et al. 1995b; Sharon et al. 2003b, 2003a; Sellon et al. 2004). They have been extensively studied for *Sarcocystis neurona*, a protozoan species closely related to *Sarcocystis calchasi*, which is the predominant causative agent of equine protozoal myeloencephalitis (Dubey et al. 2001b). These models allow for a structured, thoroughly planned infectious disease trial. In the infectious trials with *Sarcocystis neurona* immune competent BALB/c mice did not develop neurologic abnormalities after oral infection with sporocysts and parasites were not detectable in these mice (Dubey et al. 1997; Marsh et al. 1997a; Dubey et al. 2001b). Mice with a similar BALB/c genetic background that have been genetically altered to knock out the gene for IFN-γ developed fulminant neurologic disease after infection with *Sarcocystis neurona* (Dubey et al. 1998; Dubey et al. 2001b). Analogous trials with *Sarcocystis calchasi* would allow for assessment whether this parasite may interact with the host in a similar way and whether mammals have to be expected as potential hosts of this emerging

parasite under certain conditions. This would also allow for a detailed investigation of infection strategies and mechanisms of the parasite.

In order to gain further insights into pathophysiology, *in vitro*-cultivation could be established. One of the biggest advantages of *in vitro*-culture, especially axenic culture, is obtaining a continuous supply of pure organisms without any bacterial and fungal contamination for experimental infectious trials in living animals. It is also important for the production of antigens used to prepare specific antibodies against the organisms for use in immunologic tests such as IHC and immunofluorescence, identification of specific proteins that may enhance the invasive properties of the parasite and investigation of the parasite's influence on the cells on the microscopic and molecular level. *In vitro*-culture would be capable of supplying a number of different developmental phases of the parasite (schizonts, merozoites) for use in studies involving immunity, antigen analysis, biochemistry, and molecular biology. A similar strategy has been successfully employed for *Sarcocystis neurona* (Davis et al. 1991; Marsh et al. 1997b). So far, different *Sarcocystis* spp. have been cultured through part or all of their life cycles *in vitro* (Fayer 1970, 1972; Hughes et al. 1986). There were several cell lines tested for their *in vitro*-cultivation including VERO (Hughes et al. 1986; Evans et al. 1999; Speer et al. 2000), human fetal lung fibroblasts (MRC-5), human epithelial type 2 cells, African green monkey primary kidney (Hughes et al. 1986) and many others (Fayer 1970, 1972; Speer et al. 2000). Because *Sarcocystis calchasi* is a novel emerging pathogen, it would be of great importance to identify and establish a sustainable cell line and culture method which could continuously provide a sufficient quantity of different developmental stages of this parasite to serve the needs of further investigation and to reduce the need of laboratory animals for experimental studies.

The immune response of animals to infection with apicomplexan parasites is poorly understood. Studies of related apicomplexan protozoa, such as *Toxoplasma gondii*, have shown that cell-mediated immune responses are essential for the host to control intracellular infections (Denkers 1999). In recent years, it has become clear that Th1 and Th2 cytokine responses must be tightly regulated by the host for the optimal control of infection. Further-

more, cytokine imbalances resulting from a loss of control can play a role in the pathological changes associated with infections by intracellular organisms (Pusterla et al. 2012). However, the mechanism of neuropathogenesis associated with meningoencephalitis caused by *Sarcocystis calchasi* is not fully understood. Few or no organisms are usually visible in neural tissues of affected pigeons, even when there are extensive histopathological lesions, suggesting that cytokines or metabolites may be responsible for those lesions (Olias et al. 2013). In diseases caused by other protozoal agents, e.g. *Toxoplasma gondii*, it has been shown that they are capable of suppressing the host immune response (Shapira et al. 2002). *Toxoplasma gondii* is able to directly modulate the NF-κB signaling pathway, which is a family of transcription factors that plays an important role in the regulation of genes associated with the development of innate and adaptive responses required for the recognition and immunologic control of pathogens (Shapira et al. 2004). It remains to be elucidated whether *Sarcocystis calchasi* may influence the host in a similar way. As described, there are many ways in which protozoan parasites evade host immune responses and these include changes in cytokine patterns that require further investigation (Spencer et al. 2004) especially in novel species such as *Sarcocystis calchasi.*

In order to do so, a comparative study of metabolites and gene expression of *Sarcocystis calchasi* and *Sarcocystis neurona* should be conducted. After establishing a cell line suitable for both species and cultivating them for a definite period of time the gene expression of the cells used in the cell line could be examined and compared by means of DNA microarray. This could clarify the molecular basis of such parasite actions as modification of host's cytoskeleton organization, inhibition of host cell apoptosis, up-regulation of proinflammatory cytokines and deprivation of nutrients from the host cells (Brown and Blader 2009). These processes in general are common ways of parasite adaptive strategies, but they are being evoked differently by each species, by up- and down-regulation of different genes. The understanding of the way of action of *Sarcocystis calchasi* and its comparison to the infectious strategies of *Sarcocystis neurona* would bring us closer to the explanation of the pathogene-

sis of the diseases caused by these protozoans and thus, to the future possibilities of their therapy.

Apart from the mentioned possibilities of further research the new occurring cases of MUO should be monitored in a prospective study and screenings should be performed on fresh material with optimal DNA quality.

7 Summary

Is there Evidence of *Sarcocystis calchas*i Involvement in Meningoencephalitis of Unknown Origin in Mammals? A Retrospective Study

Aleksandra Żuraw

Sarcocystis calchasi is a novel intracellular protozoan parasite belonging to the phylum Apicomplexa with an obligatory two-host, predator-prey life cycle. The northern goshawk (*Accipiter gentilis*) is its definitive host and the domestic pigeon (*Columba livia f. domestica*) its intermediate host. It has been identified as the causative agent of Pigeon Protozoal Encephalitis (PPE) during an outbreak in Berlin in the period between 2006 and 2008. PPE is an ongoing threat as new cases are continuously diagnosed in pigeons in the Berlin area.

Since many *Sarcocystis* spp. are polyxenous, being capable of infecting more than one species, and birds as well as mammals may usually serve as their intermediate hosts, a retrospective study was conducted to determine whether *Sarcocystis calchasi* may be involved in cases of meningoencephalitis of unknown origin (MUO) in mammals.

Formalin fixed paraffin embedded (FFPE) samples of 143 brains with MUO of different mammalian species (dog, cat, pig, cattle, sheep, guinea pig, horse, goat, mouse, raccoon, ferret, hamster, mink, mane wolf) from the time period between 1989 and 2012 were reexamined histologically using H&E stain. DNA was isolated from FFPE material and screened by polymerase chain reaction (PCR) with primers specific for the 18S rRNA and the Internal Transcribed Spacer region 1 (ITS1) to detect *Sarcocystis calchasi* or other apicomplexan parasites, respectively.

In all samples the diagnosis of non-suppurative (lymphoplasmacytic and / or granulomatous) meningoencephalitis was histologically confirmed but no parasitic structures were found in the histopathological investigation. DNA of *Sarcocystis calchasi* or other apicomplexan parasites could not be detected in any of the samples. However due to formalin fixation the DNA quality of the samples might not have been optimal in all cases, which is why the results should be interpreted carefully.

Even though the prevalence of PPE in pigeons remains constant and *Sarcocystis calchasi* constitutes a persistent threat to pigeons in the Berlin area, no evidence was found here for a role of this parasite in MUO in mammalian species. Nevertheless, taking into consideration the methodical drawbacks of analyzing FFPE retrospective cases, a prospective study using new emerging cases with optimal DNA quality and / or immunohistochemical studies with specific antibodies would be advisable.

8 Zusammenfassung

Gibt es Hinweise auf eine Beteiligung von *Sarcocystis calchasi* an Meningoenzephalitiden unbekannter Genese bei Säugetieren? Eine retrospektive Studie

Aleksandra Żuraw

Sarcocystis calchasi ist ein intrazellulärer, einzelliger Parasit, der zum Stamm Apicomplexa gehört. Der Parasit hat einen obligatorischen zwei-wirtigen Lebenszyklus. Der Habicht (*Accipiter gentilis*) ist der Endwirt und die Taube (*Columba livia f. domestica*) ist der Zwischenwirt. *Sarcocystis calchasi* ist als Erreger der *Pigeon Protozoal Enzephalitis* (PPE) während eines Ausbruchs dieser Krankheit in Berlin in der Zeit zwischen 2006 und 2008 identifiziert worden. Seit 2008 konnte eine konstante Inzidenz der Krankheit beobachtet werden. Diese Beobachtungen sprechen für eine dauerhafte Belastung der Taubenpopulation in dieser Region.

Da viele *Sarcocystis* spp. mehrwirtig und in der Lage sind mehrere Tierarten zu infizieren, könnten sowohl Vögel als auch Säugetiere grundsätzlich als Zwischenwirte dienen. Deswegen wurde eine retrospektive Studie durchgeführt, um festzustellen, ob *Sarcocystis calchasi* in Fällen von Meningoenzephalitis unbekannter Genese (meningoencephalitis of unknown origin, MUO) bei Säugetieren beteiligt sein könnte.

Formalinfixierte und in Paraffin eingebettete (FFPE) Proben von 143 Gehirnen mit MUO verschiedener Säugetierspezies (Hund, Katze, Schwein, Rind, Schaf, Meerschweinchen, Pferd, Ziege, Maus, Waschbär, Frettchen, Hamster, Nerz, Mähnenwolf) aus dem Zeitraum zwischen 1989 und 2012 wurden histologisch erneut untersucht. DNA wurde aus FFPE-Material isoliert und mittels Polymerase-Kettenreaktion (PCR) mit spezifischen Primern für die 18S rRNA und die *Internal Transcribed Spacer-Region* 1 (ITS1) untersucht, um *Sarcocystis calchasi* oder andere Apicomplexa nachzuweisen.

In allen Proben wurde die Diagnose einer nicht-eitrigen (lymphoplasmazytären und / oder granulomatösen) Meningoenzephalitis bestätigt, jedoch konnten keine parasitären Strukturen in der histopathologischen Untersuchung festgestellt werden. DNA von *Sar-*

cocystis calchasi oder von anderen Apicomplexa konnte in keiner der Proben nachgewiesen werden. Alle Kontrollproben dagegen verliefen einwandfrei. Aufgrund Formalinfixierung könnte jedoch die DNA Qualität der Proben beeinträchtigt gewesen sein, weshalb die Ergebnisse vorsichtig interpretiert werden sollten.

Auch wenn die Prävalenz der PPE konstant bleibt und *Sarcocystis calchasi* eine anhaltende Bedrohung für die Tauben im Berliner Raum darstellt, wurden keine Hinweise für eine Rolle dieses Parasiten bei MUO von Säugetieren festgestellt. Unter Berücksichtigung der methodischen Nachteile einer retrospektiven Analyse von FFPE-Proben wären jedoch eine prospektive Studie der neu auftretenden Fälle mit optimaler DNA-Qualität und / oder immunohistochemischen Untersuchungen mit spezifischen Antikörpern empfehlenswert.

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Yang S, Rothman RE (2004) PCR-based diagnostics for infectious diseases: Uses, limitations, and future applications in acute-care settings. Lancet Infect Dis 4:337-348

10 List of Own Publications

Oral presentations:

Żuraw, A.; Plog, S.; Klopfleisch, R.; Olias, P.; Gruber, A. D. (2014):
No Evidence of *Sarcocystis calchasi* Involvement in Meningoencephalitis of Unknown Origin in Mammals. Second Joint European Congress of the European Society of Toxicologic Pathology, the European Society of Veterinary Pathologists and the European College of Veterinary Pathologists, Berlin, Germany, 27.08.-30.08.2014 (Oral Presentation Award of the European Society of Veterinary Pathology). Abstract published in: *Journal of Comparative Pathology*, Volume 152: 1, 2015, Page 53, doi:10.1016/j.jcpa.2014.10.052

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Archivprävalenz von *Sarcocystis calchasi* bei Tauben im deutschsprachigen Raum sowie bei Säugern mit Meningoenzephalitis. 58th Annual Conference of the German Veterinary Medical Society, Section Veterinary Pathology, Fulda, Germany, 07.03.-08.03.2014. Abstract published in: *Tierärztliche Praxis / Ausgabe K, Kleintiere, Heimtiere*; Volume 43: 3, 2015, Page 6; ISSN: 1434-1239

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Declaration of Originality

Hereby, I declare that the present thesis has been prepared by myself. I assure that I exclusively used the mentioned sources and facilities.

Berlin, 2015 Aleksandra Żuraw