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Toxoplasma gondii, Neospora caninum, and *Sarcocystis* spp. in brain samples of dogs with and without neurological signs in Southern Brazil (2008-2023)

Toxoplasma gondii, Neospora caninum e Sarcocystis spp. em amostras de cérebro de cães com e sem sinais neurológicos no Sul do Brasil (2008-2023)

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Highlights

Protozoan are linked to encephalitis in dogs in Rio Grande do Sul.
Protozoan DNA detection in the brains of dogs.
Molecular analysis enhances diagnosis.

Abstract

Encephalitis can be associated with protozoa of the phylum Apicomplexa. *Sarcocystis* spp., *Toxoplasma gondii,* and *Neospora caninum* share morphological similarities that complicate differentiation in histopathology, leading to inaccurate diagnoses without complementary techniques. Clinical manifestations often associate with host immunosuppression secondary to other diseases. This study examined brain samples from dogs undergoing necropsy in Rio Grande do Sul, Brazil, from 2008 to 2023, using two groups: dogs with neurological signs caused by protozoa (G1) and dogs without neurological signs (G2). G1 aimed to verify if protozoa identified histopathologically matched molecular techniques. G2 observed the frequency of protozoa detection using PCR in dogs without neurological signs. A total of 230 samples were analyzed: 11 paraffin-embedded in G1 and 219 frozen in G2. DNA extraction used commercial kits, and all samples underwent PCR for the 18S rRNA gene. G1 samples also underwent

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Restriction Fragment Length Polymorphism (RFLP) analysis. Protozoal DNA was detected in six samples (6/11) from G1 with varying molecular identifications. In G2, 82 samples (82/219; 37.45%) were PCR-positive. These findings highlight the importance of molecular techniques for accurate etiological confirmation and diagnosis, extending epidemiological knowledge and emphasizing *Toxoplasma gondii, Neospora. caninum,* and *Sarcocystis* spp. as differential diagnoses for central nervous system diseases in dogs.
Key words: Apicomplexa. Histopathology. Molecular diagnosis. PCR. RFLP.

Resumo

Encefalites podem estar relacionadas a protozoários do filo Apicomplexa. *Sarcocystis* spp., *Toxoplasma gondii* e *Neospora caninum* compartilham semelhanças morfológicas que dificultam a diferenciação na histopatologia, o que pode levar a diagnósticos imprecisos sem técnicas complementares. As manifestações clínicas geralmente estão associadas à imunossupressão do hospedeiro, secundária a outras doenças. Este estudo examinou amostras de cérebro de cães submetidos à necropsia no Rio Grande do Sul, Brasil, entre 2008 e 2023, utilizando dois grupos: cães com sinais neurológicos causados por protozoários (G1) e cães sem sinais neurológicos (G2). O G1 teve como objetivo verificar se os protozoários identificados histopatologicamente correspondiam aos encontrados por técnicas moleculares. O G2 observou a frequência de detecção de protozoários por PCR em cães sem sinais neurológicos. Um total de 230 amostras foram analisadas: 11 em parafina no G1 e 219 congeladas no G2. A extração de DNA foi realizada com kits comerciais, e todas as amostras passaram por PCR para o gene 18S rRNA. As amostras do G1 também foram submetidas à análise de Polimorfismo de Fragmento de Restrição (RFLP). O DNA de protozoários foi detectado em seis amostras (6/11) do G1, com diferentes identificações moleculares. No G2, 82 amostras (82/219; 37,45%) foram positivas para PCR. Esses achados destacam a importância das técnicas moleculares para a confirmação etiológica precisa e o diagnóstico, ampliando o conhecimento epidemiológico e enfatizando *Toxoplasma gondii, Neospora caninum* e *Sarcocystis* spp. como diagnósticos diferenciais para doenças do sistema nervoso central em cães.
Palavras-chave: Apicomplexa. Histopatologia. Diagnóstico molecular. PCR. RFLP.

Introduction

Neurological diseases in dogs can have various causes, including infectious agents (parasites, bacteria, viruses, and fungi), as well as vascular, metabolic, degenerative, and neoplastic alterations (Chaves et al., 2014; Giraldi et al., 2002). There are several reports that protozoa of the phylum Apicomplexa, such as *Sarcocystis* spp., *Toxoplasma gondii,* and *Neospora caninum,* are potential causes of diseases involving the nervous system in different animal species, including dogs (Dubey et al., 2014; Giraldi et al., 2002; Henderson et al., 1997; Moretti et al., 2002).

Toxoplasmosis is one of the most common zoonoses caused by protozoa, usually related to immunosuppression (Graham et al., 2021), and is often associated with infection by other agents, such as canine distemper virus (Headley et al., 2013; Aguiar

et al., 2012; Moretti et al., 2002). Clinical manifestations in dogs can vary, presenting signs such as ataxia, seizures, behavioral changes, paralysis, limb paraplegia, and tremors (A. V. da Silva et al., 2005; Langoni et al., 2012).

Neosporosis in dogs was first reported in boxer puppies presenting with paresis (Bjerkas et al., 1984). *Neospora caninum* is described as morphologically similar to *T. gondii,* although they have structural and antigenic differences (Uggla et al., 1989). Unlike toxoplasmosis, where dogs act as intermediate hosts, in neosporosis, dogs are definitive hosts (McAllister et al., 1998). Although it occurs in dogs of different ages, neurological neosporosis tends to occur more severely in congenitally infected puppies, who may present with hind limb paralysis, difficulty swallowing, mandibular paralysis, flaccidity, and muscle atrophy (Didiano et al., 2020; Gaitero et al., 2006; Barber et al, 1996).

Sarcocystis spp. can parasitize different animal species and are often found in the skeletal and cardiac muscles of hosts, but can also be detected in the brain (Miller et al., 2001; Ravi et al., 2015; Dubey, Lindsay & Speer, 1998; Fitzgerald et al., 1993). When infecting the central nervous system, they can cause paraparesis or hind limb paresis (Cooley et al., 2007; Gerhold et al., 2014). *Sarcocystis neurona* is one of the most well-known species for causing fatal neurological disease in horses (equine protozoal myeloencephalitis - EPM), and it can cause neurological manifestations in other animals, including dogs (Vashisht et al., 2005; Kubo et al., 2010; Dubey et al., 2014; Oliveira et al., 2020).

The presence of clinical signs is normally associated with a greater quantity of protozoa in tissues (Dubey, Lindsay & Speer, 1998). The formation of cysts containing bradyzoites characterizes the chronic form of infection caused by these protozoa. In this stage, they remain distributed throughout different tissues indefinitely, as they are poorly immunogenic and have very slow development (Skariah et al., 2010; Neupane et al., 2023). The rupture of cysts and reactivation of the rapidly replicating forms of these protozoa (tachyzoites and merozoites), usually associated with host immunological compromise, can cause acute disease episodes with severe clinical signs and even death (Blanchard et al., 2015; Didiano et al., 2020).

Methods of morphological identification in paraffin-embedded tissues are commonly used and offer cost benefits compared to molecular techniques. However, histopathology has limitations, especially regarding specificity, since morphological similarity between protozoa can hinder confirmation of the involved agent. In this sense, molecular biology can be implemented to obtain a definitive diagnosis for epidemiological and clinical studies. Additionally, molecular methods allow for the detection of protozoa in their different developmental stages, increasing the sensitivity and specificity of diagnosis (Barker, 1989; Tavares et al., 2011; Elbert et al., 2022).

Thus, this study aimed to investigate the presence of *Sarcocystis* spp., *N. caninum,* and *T. gondii* in the brains of dogs with and without neurological signs, and to verify the agreement between molecular diagnosis and histopathology in cases of protozoal encephalitis in dogs.

Material and Methods

Sample origin

Brain samples from dogs with and without a history of neurological disease, undergoing necropsy at the Laboratório de Patologia Veterinária of the Universidade Federal de Santa Maria (UFSM) and the Laboratório Axys Análises in Porto Alegre, RS, Brazil, from 2008 to 2023, were analyzed. Eleven cases of animals with neurological signs (G1) and two hundred and nineteen cases without neurological signs (G2) were included in the study. Brain tissue fragments from G1 were stored in paraffin blocks, while those from G2 were kept frozen. For animals in G1, cases were included where the pathologist described inflammatory lesions in the central nervous system associated with the presence of protozoa and where paraffin blocks containing brain tissue fragments were available in the archive.

Histopathological findings

Only the brains of animals with neurological signs (G1) were subjected to histological evaluation. Information regarding histopathological findings, including the morphological diagnosis and etiology attributed by the responsible pathologist, was collected. Subsequently, the cases underwent histological reassessment. For this, blocks containing brain tissue samples were sectioned into new 3 µm-thick slices and stained with hematoxylin and eosin. Periodic Acid-Schiff (PAS), Toluidine Blue (TB), Alcian Blue (AB), and Grocott-Gomori methenamine silver stains were also used. TB and AB techniques were adapted from Behmer et al. (1976) and involved deparaffinization, dehydration, exposure to the dye for two minutes, and rinse in running water for one minute for AB and distilled water for TB, followed by dehydration, clarification, and mounting. PAS and Grocott techniques were performed based on the protocols provided in the respective commercial kits (EasyPath®). The reassessments were conducted by two veterinary pathologists.

DNA extraction

Excess paraffin was removed, and subsequently, using a scalpel blade, approximately 30mg to 40mg of material was obtained and transferred to a 2000µL microtube. DNA extraction was carried out using the commercial QIAmp DNA FFPE Tissue Kit (Qiagen®), following the manufacturer's specifications, and then stored at -20°C until analysis. Since these were known samples with protozoal encephalitis, those that tested negative in the *nested*-PCR were subjected to an additional attempt at DNA extraction using the same kit as previously described.

Nested-PCR

DNA from all samples (G1 and G2) was subjected to nested PCR for the detection of *T. gondii, N. caninum,* and/or *Sarcocystis* spp. through the amplification of the region corresponding to the 18S ribosomal RNA (rRNA) gene, as described by da R. C. da Silva et al. (2009). In the primary reaction, the primers used were Tg18s48F forward (5′-CCATGCATGTCTAAGTATAAGC-3′) and Tg18s359R reverse (3′-GTTACCCGTCACTGCCAC-5′).

In the secondary reaction, the primers were Tg18s58F forward (5′-CTAAGTATAAGCTTTTATACGGC–3′) and Tg18s348R reverse (5′-TGCCACGGTAGTCCAATAC-3′). At the end of the second reaction, products of 290 base pairs (bp) were amplified for *S. neurona, N. caninum, H. hammondi,* and *T. gondii,* and 310 bp for other *Sarcocystis* spp. Both nested-PCR reactions were performed with a final volume of 25 µl containing 100-200 ng of total DNA, 1.25 mM $MgCl_2$, 2.5mM 10x Taq buffer (2.5 µl), 1U Taq DNA polymerase® (0.2 µl) (Thermo Fisher Scientific®, Massachusetts, USA), 20 µM of deoxyribonucleotides (dNTPs), 1 µM of each primer, and ultrapure water q.s.p. The samples were subjected to denaturation at 94ºC for 5 min, followed by 35 cycles of 94ºC for 45 s, 55ºC for 45 s, and 72ºC for 45 s, with a final extension at 72ºC for 5 min. Milli-Q water was used as a negative control in all reactions, and a previously sequenced sample corresponding to each of the agents studied (*Sarcocystis* spp., *S. neurona, N. caninum,* and *T. gondii*) was used as a positive control.

Restriction fragment length polymorphism (RFLP)

Only positive samples from G1 were subjected to RFLP. The positive products in nested-PCR were incubated with two restriction enzymes: *Dde-I*® (Thermo Fisher Scientific, Massachusetts, USA) and *Hpa-II*® (Promega, Madison, WI, USA). Seven microliters of DNA product from each sample, along with 2 U (0.2 µl) of each enzyme, were homogenized and incubated for 1 hour at 37 ºC. Subsequently, the product was subjected to electrophoresis on a 2% agarose gel. The expected restriction pattern for samples positive for *T. gondii* was two DNA fragments for the *Hpa-II* enzyme (173 bp and 119 bp) and two fragments for the *Dde-I* enzyme (182 bp and 110 bp); for *N. caninum/H. hammondi,* a pattern of two fragments for Hpa-II (173 bp and 120 bp) and a single 290 bp fragment for Dde-I was expected; for *Sarcocystis neurona/S. falcatula,* a pattern of two fragments for the *Dde-I* enzyme (184 bp and 111bp) was expected, while for *Hpa-II,* a single 298 bp fragment was expected; and for *Sarcocystis* spp., both *Hpa-II* and *Dde-I,* a pattern of only one 310 bp fragment were expected (Figure 1). Positive and negative controls were included in all reactions.

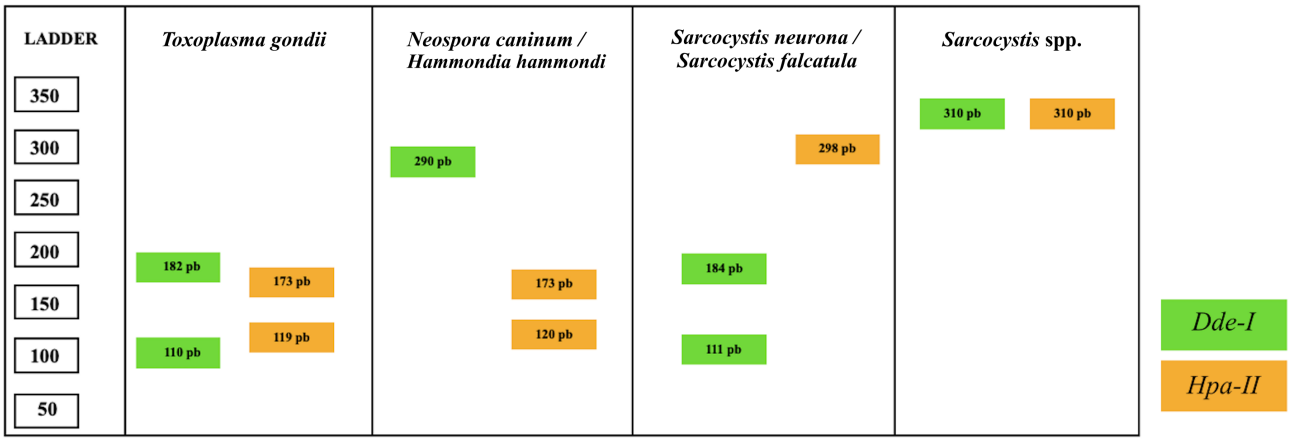

Figure 1. Restriction sites obtained in silico for the Dde-I and Hpa-II enzymes used in the RFLP technique for differentiation between *Toxoplasma gondii, Neospora caninum/Hammondia hammondi, Sarcocystis neurona/falcatula,* and *Sarcocystis* spp.

Positive samples to *N. caninum/H. hammondi* in PCR and RFLP were submitted to a specific PCR to *N. caninum,* and to differentiate both protozoa. PCR was based on the Nc5 gene, according to described by Yamage et al. (1996).

Results and Discussion

Out of the 11 samples from G1, DNA amplification was achieved in six (6/11 – 54.54%) cases. Among these, two were identified as *N. caninum* (33.33%), two as *T. gondii* (33.33%), one as *S. neurona/ falcatula* (16.67%), and one as Sarcocystis spp. (16.67%). Positive samples for *N. caninum/Hammondia* hammondi in RFLP were confirmed as *N. caninum* using specific primers for the Nc5 gene of *N. caninum.* DNA amplification was unsuccessful in five cases, likely due to the prolonged storage time of the samples. Although paraffin embedding allows better morphological preservation of the sample structures, formalin fixation can cause DNA fragmentation, reducing PCR sensitivity (Perkins & Kjeldsberg, 1993; Scorsato & Telles, 2011).

In samples from dogs without neurological signs (G2), 37.45% (82/219) tested positive in the PCR reaction for the 18S rRNA gene for protozoa of the phylum Apicomplexa. Unfortunately, RFLP was not performed due to the low quality/quantity of amplified DNA, thus preventing genus identification. In these cases, the infection was confirmed to be caused by a protozoan agent of the phylum Apicomplexa. The samples from group G2 were not subjected to histochemical staining, as the animals showed no neurological signs, the brains were stored only frozen, and, due to the absence of clinical suspicion, they were not submitted for histopathological examination. Toxoplasma in the CNS has two phases: acute and chronic. During the acute phase, tachyzoite replication occurs, and during the chronic phase, cysts with bradyzoites are formed (Lüder & Rahman, 2017; Dubey, Lindsay &

Speer, 1998). In the histopathological analysis of the nervous system in G1, concerning the amplified samples, multiple foci of necrosis were observed, as well as the presence of protozoa (tachyzoites and merozoites), most of which were morphologically interpreted as *T. gondii* according to necropsy reports. The tachyzoites and merozoites were negative for all histochemical stains applied.

The necrotizing and granulomatous inflammatory pattern observed in the encephalitis cases in this study was similar, regardless of the protozoa identified. This morphological pattern is consistent with what is described by other authors, where encephalitis is caused by *T. gondii, N. caninum,* and *Sarcocystis* spp. usually presents with nervous system necrosis and the presence of epithelioid macrophages associated with intralesional protozoa (Giraldi et al., 2002; Dubey et al., 2014; Uggla et al., 1989; Patitucci et al., 1996; Elbert et al., 2022; Cooley et al., 1987; Robson & Smith, 2011; Frade et al., 2018). In animals 1, 2, and 5, multiple areas of malacia with neutrophil infiltration and Gitter cells (foamy macrophages) were identified (Table 1). Vasculitis was also a frequent finding in the studied cases (samples 3, 4, and 5) and is characterized by inflammation of the vascular wall (Hajj-Ali & Calabrese, 2014), which is often accompanied by fibrinoid necrosis with abundant fibrin exudation (Brandt et al., 2007). Vasculitis can be related to various infectious and non-infectious factors (Lotti et al., 1999; Frade et al., 2018; Morita et al., 2020) and is frequently described in encephalitis caused by *T. gondii* and *N. caninum* (Dubey & Lappin, 2005).

Histopathology, although an excellent diagnostic tool, regarding the different cyst-forming coccidia, does not provide sufficient elements to allow definitive differentiation between the protozoa (Shannon et al., 2015; Speer et al., 1999). Morphological differences between the parasitic forms of *N. caninum* and *T. gondii* are minimal, as both forms are nearly identical tissue bradyzoites and tachyzoites. It is known that tissue cysts of *T. gondii* have a thinner wall (<0.5 µm) and measure from 5 to 70 µm in diameter, while the cyst wall of *N. caninum* ranges from 1 to 4 µm thick, and cysts can reach up to 107 µm in diameter. Additionally, *N. caninum* tachyzoites are larger (4 to 7 µm in length) compared to *T. gondii* tachyzoites (2 to 6 µm). Although there are some more marked differences between *T. gondii* and *N. caninum,* certain tissue phases of *Sarcocystis* can appear very similar to these two coccidia. *Sarcocystis merozoites,* for example, have a shape and size (*S. neurona,* 5µm in length and *S. canis,* 5 to 7 µm) similar to the tachyzoites of *T. gondii* and *N. caninum* (Cantile & Youssef, 2016). These differences are too subtle to be noticed under a common optical microscope. Furthermore, the measurements between tachyzoites and merozoites are very and have overlapping areas, making differentiation of these protozoa by the pathologist difficult.

PCR is an essential tool for detecting DNA or RNA from viruses, bacteria, fungi, and protozoa in central nervous system tissues, even in small quantities, allowing for rapid and accurate diagnosis (Su et al., 2010). When combined with the restriction fragment length polymorphism technique (PCR-RFLP), this molecular approach becomes even more robust, enabling the differentiation of closely related protozoa in clinical and preserved samples, such as paraffin-embedded tissue blocks or frozen specimens. This significantly broadens its applicability in

diagnostic investigations and retrospective studies (Freitas et al., 2023; Ajzenberg et al., 2009; Ferreira, 2003). The inclusion of these molecular techniques is especially valuable in cases where histopathological features alone do not allow for a reliable identification of the etiological agent, particularly due to the morphological similarities between *T. gondii, N. caninum,* and *Sarcocystis* spp.

Table 1
Relationship between results found in PCR and RFLP with the etiology indicated in the necropsy reports and histological lesions observed in the central nervous system of the six amplified samples in the PCR

Sample/ Year	Molecular analysis	Etiology suggested in the necropsy report	Histological lesions observed in the central nervous system
1 / 2012	*Sarcocystis* spp.	*Toxoplasma gondii*	Necrotizing and granulomatous meningoencephalitis with the presence of merozoites. The area of malacia was infiltrated by neutrophils and Gitter cells, accompanied by epithelioid macrophages, fibrin exudation, and hemorrhage. Lympoplasmacytic infiltration was observed in the Virchow-Robin spaces of some blood vessels adjacent to the malacia.
2 / 2008	*Sarcocystis neurona/ falcatula*	Protozoan with morphology consistent with *Toxoplasma gondii*	Necrotizing and granulomatous meningoencephalitis associated with merozoites. The areas of malacia were infiltrated by neutrophils and Gitter cells, accompanied by epithelioid macrophages, fibrin exudation, and hemorrhage. There was lymphoplasmacytic infiltration in the Virchow-Robin spaces of some blood vessels near the malacia.
3 / 2009	*Neospora caninum*	Protozoan with morphology consistent with *Toxoplasma gondii*	Necrotizing and granulomatous encephalitis with vasculitis associated with intracellular and extracellular tachyzoites. The areas of malacia were accompanied by epithelioid macrophages and fibrin exudation. There was lymphoplasmacytic infiltration in the Virchow-Robin spaces of some blood vessels near the malacia.
4 / 2013	*Toxoplasma gondii*	Protozoan with morphology consistent with *Toxoplasma gondii*	Necrotizing and granulomatous encephalitis with vasculitis associated with intracellular and extracellular tachyzoites. The areas of malacia were accompanied by epithelioid macrophages and fibrin exudation. There was lymphoplasmacytic infiltration in the Virchow-Robin spaces of some blood vessels near the malacia.

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5 / 2013	*Neospora caninum*	*Toxoplasma gondii*	Necrotizing and granulomatous encephalitis with vasculitis predominantly in gray matter, associated with intracellular and extracellular tachyzoites. The areas of malacia were infiltrated by a large number of neutrophils, in smaller quantities, epithelioid macrophages, and accompanied by fibrin exudation. Lymphoplasmacytic infiltrate in the Virchow-Robin spaces of some blood vessels near the malacia.
6 / 2022	*Toxoplasma gondii*	Observation of undefined protozoa	Necrotizing and granulomatous meningoencephalitis with vasculitis associated with intralesional and extracellular tachyzoites. The areas of malacia were accompanied by epithelioid macrophages and fibrin exudation. There was a lymphoplasmacytic infiltrate in the Virchow-Robin spaces of some blood vessels near the malacia. Perivascular cuffs of more than 10 layers of inflammatory cells containing histiocytes, plasma cells, lymphocytes, and neutrophils.

Thus, additional tests, such as immunohistochemistry, which uses the identification of specific protozoal antigens, or molecular methods such as PCR, can be associated with histopathological diagnosis (Shannon et al., 2015; Patitucci et al., 1996; Cooley et al., 2007; Elbert et al., 2022). The results presented in this study are not intended to diminish the importance of histopathological diagnosis in detecting *Sarcocystis* spp., *N. caninum*, and *T. gondii* but rather to demonstrate that the morphological similarity among them does not allow for reliable differentiation between the genera through microscopy (Speer et al., 1999). Thus, for the diagnosis of encephalitis etiology, it is essential to associate histopathological analysis with other techniques, such as and enzyme restriction, as confirming the etiological agent enables the establishment of prevention and intervention measures, as well as the epidemiological importance of correctly determining the sources of infection.

The distribution and circulation of *T. gondii, N. caninum,* and *Sarcocystis* spp. in the dog population in Brazil and worldwide are widely reported (Kubo et al., 2010; Caramalac et al., 2021; Arruda et al., 2021; Rodrigues et al., 2016). Generally, although variations occur depending on the study location, in Brazil, there is a higher frequency of detection of *T. gondii,* followed by *N. caninum* and *Sarcocystis* spp., respectively (Caramalac et al., 2021; Arruda et al., 2021; Oliveira et al., 2020; V. L. B. Silva et al., 2022). However, a recent study by Caramalac et al. (2023) investigated the seroprevalence of *T. gondii* and *N. caninum* in dogs with and without neurological signs in Mato Grosso do

Sul, Brazil, and observed that among animals with neurological signs, *N. caninum* was more prevalent (30%) compared to T. gondii (23.3%), while in the group of dogs without neurological signs, 16.7% and 13.3% were seropositive for *T. gondii* and *N. caninum,* respectively.

Sarcocystis spp., *N. caninum,* and *T. gondii* can distribute through different tissues and systems of their hosts, such as the nervous, digestive, reproductive, and ocular systems, among others (Ferreira et al., 2006). It is known that they can be detected in the nervous system of healthy animals without causing clinical signs since they use mechanisms such as cyst formation (chronic phase of infection) to suppress the immune response and remain viable in the organism (Toscan et al., 2012). On the other hand, host immunological vulnerability favors the rupture of these cysts, leading to rapid replication of the protozoa (acute phase of infection), causing clinical signs according to their location (Ferreira et al., 2006; Vashisht et al., 2005; Gondim, 2006; Dubey et al., 2014).

Conclusion

Although histopathological diagnosis is essential for confirming protozoal encephalitis in dogs, the etiological agent must be determined through specific techniques, as the microscopic changes are often indistinguishable among *Toxoplasma gondii, Neospora caninum,* and *Sarcocystis* spp. The findings of this study suggest that the circulation of *N. caninum* and *Sarcocystis* spp. may be underestimated in the dog population, particularly in the central region of Rio Grande do Sul. This may help identify subclinical cases and improve our understanding of the epidemiology of these pathogens. Although the prognosis may remain unchanged from a clinical standpoint, it is important that both veterinary clinicians and epidemiological studies recognize that protozoal encephalitis is not exclusively associated with *T. gondii,* and may involve other protozoa such as *N. caninum* and *Sarcocystis* spp. Therefore, in cases based solely on histopathological findings, it is advisable to describe the lesions as protozoal encephalitis or encephalomyelitis, without specifying the genus.

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Competing interests

The authors have no financial or proprietary interests in any material discussed in this article.

Ethics approval

No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with laboratories stocked samples from diagnosis routine.

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