

Fatal toxoplasmosis in an immunosuppressed domestic cat from Brazil caused by *Toxoplasma gondii* clonal type I

Toxoplasmose fatal em um gato doméstico imunossuprimido do Brasil causada por *Toxoplasma gondii* clonal tipo I

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Received February 4, 2017

Accepted April 10, 2017

Abstract

The objective of the study was to report on a fatal case of feline toxoplasmosis with coinfection with the feline leukemia virus (FeLV). A domestic cat (*Felis silvestris catus*) presented intense dyspnea and died three days later. In the necropsy, the lungs were firm, without collapse and with many white areas; moderate lymphadenomegaly and splenomegaly were also observed. The histopathological examination showed severe necrotic interstitial bronchopneumonia and mild necrotic hepatitis, associated with intralésional cysts and tachyzoites of *Toxoplasma gondii* that were positive by anti-*T. gondii* immunohistochemical (IHC) evaluation. The bone marrow showed chronic myeloid leukemia and the neoplastic cells were positive by anti-FeLV IHC evaluation. DNA extracted from lungs was positive for *T. gondii* by PCR targeting REP-529. *T. gondii* was characterized by PCR-RFLP and by the microsatellites technique. ToxoDB-PCR-RFLP #10, i.e. the archetypal type I, was identified. Microsatellite analysis showed that the strain was a variant of type I with two atypical alleles. This was the first time that a *T. gondii* clonal type I genotype was correlated with a case of acute toxoplasmosis in a host in Brazil.

Keywords: *Felis silvestris catus*, FeLV, genotyping, immunohistochemistry, microsatellite markers.

Resumo

O objetivo deste estudo foi relatar um caso de toxoplasmose felina fatal com coinfeção com o vírus da leucemia felina (FeLV). Um gato doméstico (*Felis silvestris catus*) apresentou intensa dispnéia e morreu três dias depois. Na necropsia, observaram-se pulmões firmes, não colabados e com múltiplas áreas brancas, além de linfadenomegalia e esplenomegalia moderadas. No exame histopatológico, evidenciaram-se broncopneumonia intersticial necrótica acentuada e hepatite necrótica discreta associada a cistos e taquizoítas de *T. gondii* intralésionais positivos na imuno-histoquímica (IHC) anti-*T. gondii*. Evidenciou-se ainda, na medula óssea, leucemia mieloide crônica com IHC anti-FeLV positiva nas células neoplásicas. O DNA extraído dos pulmões foi positivo para *T. gondii* por meio da PCR-REP-529. *T. gondii* foi caracterizado por PCR-RFLP e pela técnica de microssatélites. Foi identificado o genótipo ToxoDB-PCR-RFLP #10, i.e., o arquétipo tipo I. A análise por microssatélites mostrou que a cepa era uma variante do tipo I, com dois alelos atípicos. Esta é a primeira vez que *T. gondii* clonal tipo I foi relacionado com um caso agudo de toxoplasmose em um hospedeiro no Brasil.

Palavras-chave: *Felis silvestris catus*, FeLV, genotipagem, imunohistoquímica, marcadores microssatélites.

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Introduction

Toxoplasmosis is a cosmopolitan infection disease caused by the obligate intracellular protozoon *Toxoplasma gondii*. Domestic cats and wild felids are the only definitive hosts and they can excrete the oocysts in their feces after infection. All homeothermic animals serve as intermediate hosts, including humans (DUBEY, 2010).

In Brazil, the prevalence of anti-*T. gondii* antibodies varies between different regions, with reports of 19.5% in the state of Rio de Janeiro (GONÇALVES et al., 2003), 35.4% in the state of São Paulo (PENA et al., 2006), 16.3% in Paraná (CRUZ et al., 2011) and 14.33% in Santa Catarina (ROSA et al., 2010). There are many reasons for these variations, including the cat population sampled (stray or owned), sampling, age of cats, density of cats, environmental contamination with oocysts, *T. gondii* seroprevalence in small animals (such as rodents), serological test and cutoff titers used. In France, seroprevalence in cats was correlated with the weather, such that it was the highest with hot and humid weather or moderate-temperature and drier weather (AFONSO et al., 2006).

Concomitant infection with viral agents from cats has been correlated with the seroprevalence of *T. gondii*. Lucas et al. (1998) demonstrated that there is a strong association ($p < 0.05$) between serological findings of infection by the feline immunodeficiency virus (FIV) and the presence of *T. gondii*. Dorny et al. (2002) found that cats seropositive for FIV had higher antibody titers than did FIV-negative cats, but these associations were not found with the feline leukemia virus (FeLV). A recent review on *T. gondii* and concurrent infection with FIV and FeLV concluded that there is no evidence for this kind of association (DUBEY et al., 2009).

Infection by *T. gondii* in cats is generally subclinical and most cats do not develop clinical abnormalities (LAPPIN, 2010). However, fatal systemic cases have been reported in immunocompetent cats (SPYCHER et al., 2011; JOKELAINEN et al., 2012; NAGEL et al., 2013; COHEN et al., 2016) and in immunocompromised cats after administration of immunosuppressant drugs (LAST et al., 2004) and kidney transplantation (BERNSTEEN et al., 1999). Coinfection with immunosuppressant retroviruses such as FIV has also been reported to be a risk factor for development of a clinical condition of toxoplasmosis (DAVIDSON et al., 1993). Nonetheless, experimental FeLV infection before *T. gondii* challenge in cats did not cause acute toxoplasmosis or excretion of oocysts (PATTON et al., 1991).

In South American countries, including Brazil, the *T. gondii* population has high genetic diversity (AJZENBERG et al., 2004; LEHMANN et al., 2006; PENA et al., 2008) and the globally distributed archetypal clonal genotypes types I, II and III (HOWE & SIBLEY, 1995) occur at low frequencies, particularly types I and II (SHWAB et al., 2014). From 106 previously identified genotypes, corresponding to 385 Brazilian strains, which were included in the geographical patterns of *T. gondii* studied by Shwab et al. (2014), two were type I clonal, one was type II clonal, five were type II variant, six were type III clonal (ToxoDB RFLP #10, #1, #3 and #2) and 92 were non-archetypal genotypes. Besides being highly diverse, most of the strains isolated in Brazil are virulent towards mice, thus differing from those in North America and Europe (PENA et al., 2008).

It is of epidemiological interest to report on *T. gondii* genotypes that are associated with clinical disease in different hosts and regions. The objective of the present study was to report on the clinical, anatomopathological, immunohistochemical and molecular characterization findings relating to *T. gondii* in a fatal case of toxoplasmosis in an immunosuppressed domestic cat that presented leukemia due to infection by the feline leukemia virus.

Material and Methods

A short-furred five-year-old male domestic cat (*Felis silvestris catus*) without any defined breed, which had not been vaccinated or dewormed, was attended at the Veterinary Clinical Hospital of the University of Santa Catarina State (UDESC), state of Santa Catarina, southern Brazil, in March 2015. It had respiratory signs and subsequently died. Epidemiological data were obtained during the clinical consultation. The animal was necropsied and samples were collected from organs, including encephalon, lung, heart, skeletal muscle, liver, kidney, bladder, adrenal gland, thyroid, spleen, lymph nodes (mandibular, axillary, mediastinal, tracheobronchial, epigastric, hepatic, perirenal, mesenteric and popliteal), bone marrow, stomach and intestines. They were fixed in 10% buffered formalin and routinely processed for histological evaluation, with staining using the hematoxylin-eosin (HE) method. Lung fragments were frozen at -20°C for subsequent molecular study.

Immunohistochemical (IHC) evaluation was performed on the lung tissue to detect *T. gondii* using a polyclonal antibody (VMRD[®], at dilution 1:1000) with 0.1% trypsin for 10 minutes for antigen retrieval, and also a modified avidin-biotin peroxidase complex method (LSAB-HRP kit, DakoCytomation[®]) using diaminobenzidine (DakoCytomation[®]) as the chromogen; lung tissue from a dog with toxoplasmosis was used as the positive control. IHC analysis was performed on the bone marrow to detect the feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) using a monoclonal antibody (Serotec[®], at dilution 1:500 and 1:100, respectively) with Tris EDTA buffer pH 9.0 (FeLV) and 0.01 M citrate buffer pH 6.0 (FIV), for 40 min at 100°C for antigen retrieval, and also streptavidin biotin alkaline phosphatase (LSAB-AP kit, DakoCytomation[®]) using permanent red (DakoCytomation[®]) as the chromogen. Previously confirmed positive FeLV and FIV positive controls were analyzed simultaneously with the tested samples.

For molecular detection of the agent, PCR targeting a repeated 529 bp fragment of the genome of *T. gondii* was used in accordance with the protocol of Homan et al. (2000). The positive control consisted of DNA extracted from tachyzoites of the RH reference strain of *T. gondii*. PCR-RFLP was performed using 11 distinct markers: SAG1, SAG2 (5'3'SAG2 and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico and CS3. DNA from tachyzoites from archetypal clonal strains (Type I-GT1, Type II-PTG and Type III-CTG) and reference strains (TgCgCa1-Cougar, MAS and TgCatBr5) were used as positive controls. The protocols for amplification, enzymatic digestion and analysis of the fragments were as previously described (SU et al.,

2010; PENA et al., 2008). The causative strain was classified in accordance with the genotypes present on the ToxoDB platform (GAJRIA et al., 2007) and in recent publications, from 2014 to March 2017, that were retrieved from the PubMed (NCBI, 1988) and ScienceDirect® (SCIENCE DIRECT, 2017) databases.

The strain analyzed was also genotyped by means of 15 microsatellite (MS) markers: TUB2, W35, TgMA, B18, B17, M33, IV.1 and X1.1 (typing markers); and N60, N82, AA, N61, N83, M48 and M102 (fingerprinting markers), in accordance with the protocols of Ajzenberg et al. (2010), so as to increase the resolution of the genotyping. The positive control used was the DNA from tachyzoites of the PTG strain. The analysis on the results was done using the Genemapper® 4.1 software (Applied Biosystems). The Neighbor-Joining tree was reconstructed from microsatellite data only. The unrooted tree was reconstructed using Populations 1.2.32 (LANGELLA, 1999) based on Cavalli-Sforza and Edwards chord-distance estimator (CAVALLI-SFORZA & EDWARDS, 1967) and generated with MEGA version 7.0.18 (KUMAR et al., 2016). In order to verify the phylogenetic relationships among the strain studied and others that have been reported, and analyzed by MS markers, clonal type I strains from South and Central America and one from a human patient in Europe were chosen.

Results

The clinical signs presented by the cat were severe dyspnea and tachypnea, which evolved over a 24-hour period. However, the cat had been prostrated and hyporexic for a week, with episodes of sneezing. It had been living with several other cats, with free access to the streets and had the habit of hunting rodents. In view of the patient's condition, chest radiography and hematological evaluation were requested after stabilization had been achieved. The radiograph revealed bronchoalveolar pneumopathy and alveolar opacification in cranial pulmonary lobes. The hematological evaluation demonstrated mild leukopenia and marked lymphopenia

(Table 1). From then onwards, the suspected diagnosis became feline asthma or chronic bronchitis in association with secondary pneumonia. The cat was then interned and subjected to oxygen therapy, intravenous antibiotic therapy (ampicillin with sulbactam) and administration of bronchodilators (terbutaline sulfate). No clinical improvement occurred and the cat died two days after internment.

In the necropsy examination, the cat presented pallid mucosa and moderate dehydration. The lungs were firm, without collapse, and had many white areas interspersed with reddened areas. Upon cutting, serous fluid came out of the bronchi (Figure 1A). The mediastinal lymph nodes and spleen were moderately enlarged, and the parenchyma of the spleen presented many whitened points.

The histological lesions observed in the lungs were due to diffuse interstitial pneumonia with hyperplasia of type II pneumocytes. There was multifocal fibrinoid necrosis of alveoli and bronchioles associated with severe diffuse infiltrate of macrophages and neutrophils in the lumen of the alveoli and bronchioles, accompanied by a large quantity of basophilic oval to piriform structures. These were suggestive of cysts and tachyzoites of *T. gondii*, which were free, or surrounded by a capsule, or inside intralésional macrophages (Figure 1B). Multifocal moderate hyperplasia of the bronchial and bronchiolar epithelium and hypertrophy of the smooth musculature of the alveolar wall were also observed.

In the liver, there was mild multifocal fibrinoid necrosis with a small quantity of free cysts and tachyzoites of *T. gondii*. The spleen presented hyperplasia of lymphoid follicles with rare cysts of *T. gondii*. In the mediastinal lymph node, there was diffuse infiltrate of macrophages in the capsular sinus and medullary region with severe erythrophagocytosis.

The bone marrow presented total absence of adipocytes, whereas there was strong predominance of cells of granulocytic lineage, forming parenchyma of high cellular heterogeneity, in which no organized or delimited erythroblast cell colonies were observed (this cell type was already scarce). Many of the blastic granulocytic cells

Table 1. Hemathological values from a cat with fatal toxoplasmosis associated with infection by the feline leukemia virus (FeLV).

Parameters	Cat values	Reference range*	Conclusion
Erythrocytes (x10 ⁶)	7.37	5.0-10	WNR [#]
Hemoglobin (g/dL)	10.8	8.0-15.0	WNR
Hematocrit (%)	35	24-45	WNR
Mean corpuscular volume (fL)	47.5	39-55	WNR
Mean corpuscular hemoglobin concentration (%)	30.9	31-35	WNR
Platelets (x10 ³)	414	300-800	WNR
Total plasma protein (g/dL)	6.8	6.0-8.0	WNR
Total leukocytes (/μL)	5350	5500-19500	Decreased
Band neutrophils (/μL)	0	0-300	WNR
Segmented neutrophils (/μL)	4441	2500-12500	WNR
Lymphocytes (/μL)	856	1500-7000	Decreased
Eosinophils (/μL)	54	0-1500	WNR
Monocytes (/μL)	0	0-850	WNR
Basophils (/μL)	0	rare	Decreased

*Feldman et al. (2000); [#]Within Normal Range.

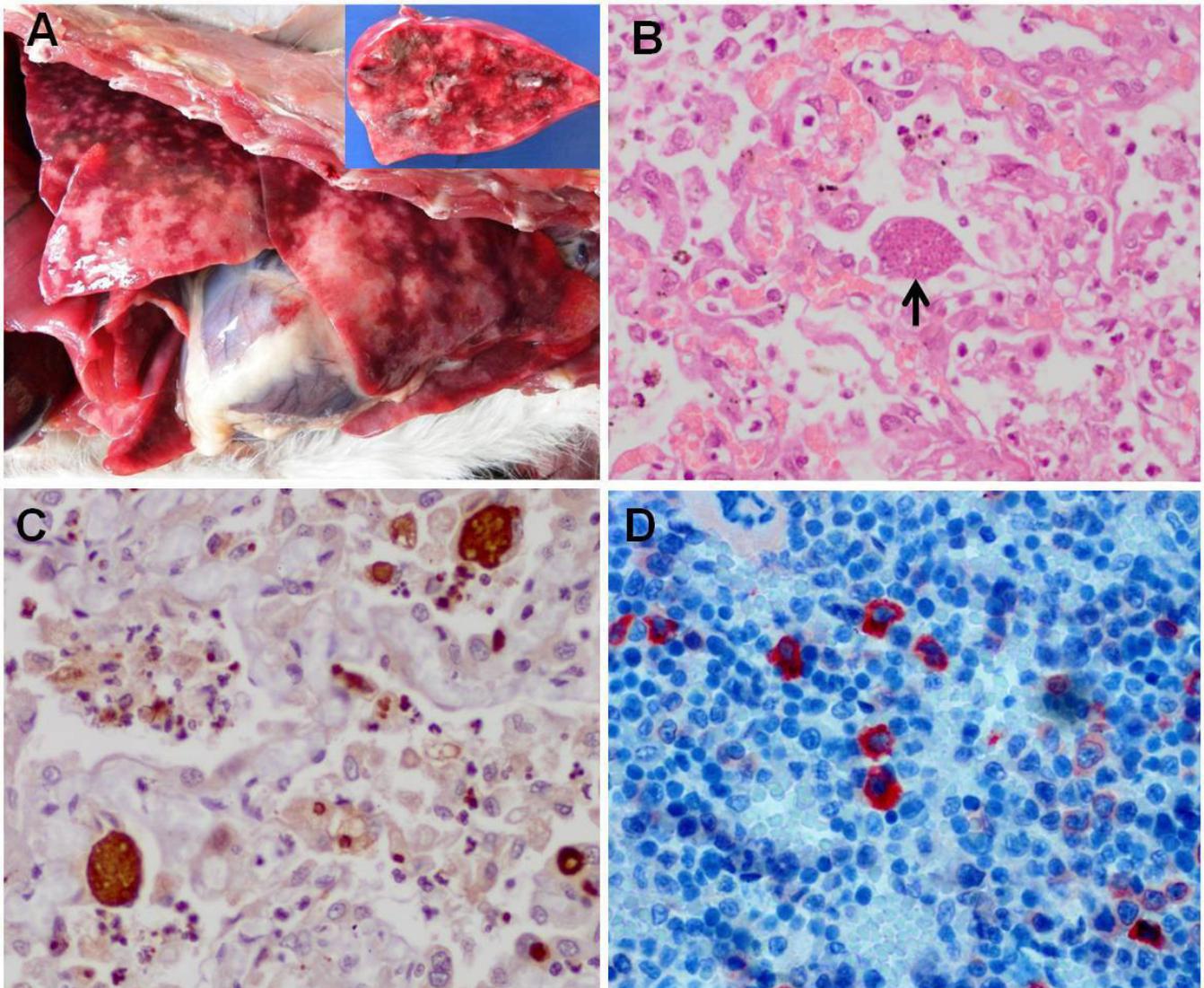


Figure 1. Toxoplasmosis in a cat. A: Non-collapsed lung tissue showing many white areas interspersed with reddened areas. In the detail, the cut surface. B: Interstitial pneumonia associated with *Toxoplasma gondii* cyst (arrow). HE; obj 40x. C: Anti-*T. gondii*-positive immunohistochemical evaluation on cysts and tachyzoites in lung tissue. Streptavidine-biotine ligated to peroxidase; obj 40x. D: Anti-FeLV-positive immunohistochemical evaluation on neoplastic bone marrow cells. Streptavidine-biotine ligated to phosphatase; obj 40x.

showed slight nuclear polymorphism, with moderate quantities of atypical megakaryocytes, without nuclear multilobulation, and smaller numbers of micromegakaryocytes that sometimes formed small groupings dispersed throughout the parenchyma. Around two to three typical and atypical mitoses were observed for every ten high-magnification (40x) fields. These neoplastic cells were seen to infiltrate the lymph nodes and portal region of the liver.

In the anti-*T. gondii* IHC examination, there was intense positive marking of cysts and tachyzoites relating to inflammatory foci inside the pulmonary alveoli (Figure 1C). The anti-FeLV IHC examination on the bone marrow was positive in the neoplastic cells (Figure 1D) and the anti-FIV examination was negative. Through the anatomopathological and IHC findings, the conclusion was a diagnosis of severe diffuse necrotic interstitial bronchopneumonia

due to toxoplasmosis, associated with chronic myeloid leukemia due to FeLV. It was not possible to determine whether the cat was primarily immunocompromised due to other causes.

Lung fragments from the cat were *T. gondii*-positive according to REP-529 PCR. This strain was called PS-TgCatSCBr1. ToxoDB-PCR-RFLP #10, i.e. the archetypal type I was identified. All the RFLP markers, including CS3, had type I alleles. However, through microsatellite analysis it could be seen that the strain identified was a variant of type I that showed atypical alleles with the typing markers B17 and M33 (Table 2). The neighbor-joining analysis based on microsatellite data showed that the strain PS-TgCatSCBr1 is divergent from the other reported Brazilian type I strains that have been reported and completely genotyped with MS markers (Figure 2).

Table 2. Microsatellite genotyping of *Toxoplasma gondii* clonal type I genotype from a cat from Brazil and comparison with other reported type I isolates. Three reference archetypal strains (GT1, ME49 and NED) are included.

Isolate ID	MS Type [#]	Origin	Microsatellite markers*														
			TUB2	W35	TgM-A	B18	B17	M33	IV.1	XI.1	M48	M102	N60	N82	AA	N61	N83
PS-TgCatBrSC1 [§]	I variant	Cat, southern Brazil	291	248	209	160	334	167	274	358	223	166	145	129	269	087	306
TgCkBr146 [§]	I	Chicken, southern Brazil	291	248	209	160	342	169	274	358	211	166	147	121	265	087	306
S11 ^b	I variant	Pig, southern Brazil	291	248	209	160	344	169	274	358	209	166	147	119	267	087	306
TgCatPr01 ^c	I variant	Cat, Puerto Rico	291	248	209	160	336	169	274	356	213	166	145	111	263	087	306
HDC ^e	I	Human, Colombia	291	248	209	160	342	169	274	358	209	166	145	125	265	087	306
TgCtCo07 ^d	I	Cat, Colombia	291	248	209	160	342	169	274	358	209	166	147	127	265	087	306
MOR ^e	I	Human, France	291	248	209	160	342	169	274	358	209	166	147	119	265	087	306
GT1	I	Goat, USA	291	248	209	160	342	169	274	358	209	166	145	119	267	087	306
ME49	II	Sheep, USA	289	242	207	158	336	169	274	356	215	174	142	111	265	091	310
NED	III	Human, France	289	242	205	160	336	165	278	356	209	190	147	111	267	091	312

*Bold indicates atypical alleles; [#]All type I and type I variant samples were classified as RFLP genotype #10; [§]This study; ^aDubey et al. (2007a); ^bMartins et al. (1990); ^cDubey et al. (2007b); ^dDubey et al. (2006); ^epersonal communication.

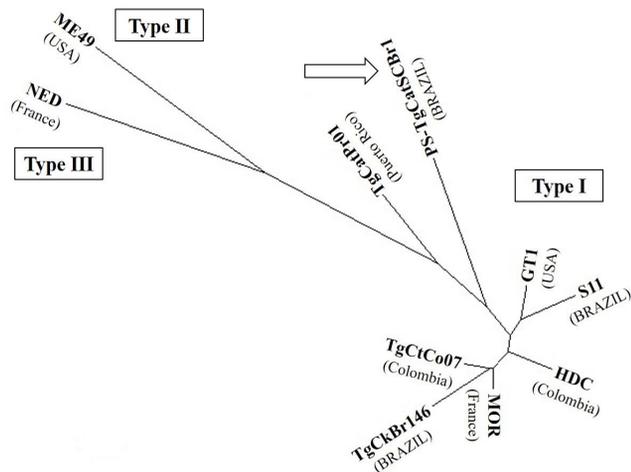


Figure 2. Neighbor-joining clustering of *Toxoplasma gondii* type I strains based on 15 microsatellite markers. Arrow is for the strain analyzed in the present study. Origin of isolates is also indicated. GT1, ME49 and NED are reference strains.

Discussion

Fatal clinical toxoplasmosis has been sporadically reported among cats. Studies conducted by Henriksen et al. (1994) and Jokelainen et al. (2012) demonstrated that 3.2% (5/155) and 3.1% (6/193) of the cats evaluated, respectively, died of toxoplasmosis.

In cats, after the intestinal phase of the infection, *T. gondii* tends to form cysts in various tissues, frequently in the liver, lungs, CNS, muscles and pancreas, and this is the main form of presentation in immunocompetent individuals (LAPPIN et al., 1989; LAPPIN, 2010). The acute form of the infection could be very important in immunocompromised cats, such as in those infected by FeLV and FIV (SVOBODOVÁ et al., 1998). In these, the cysts may become reactivated, thus making it possible for *T. gondii* to disseminate and cause disease (DAVIDSON et al.,

1993). In the present study, it is likely that the concomitant infection by FeLV contributed towards development of the disease, but it was not possible to determine whether the cat's infection was primary or whether it was a reactivation of latent infection. FeLV is a retrovirus that is known to cause several clinical syndromes, including immunosuppression, thereby facilitating opportunistic infections. This virus has the ability to cause thymus and bone marrow atrophy and, consequently, lymphopenia, neutropenia and depletion of CD4+ and CD8+ lymphocytes (OGILVIE et al., 1988; LUTZ et al., 2009). In the case reported here, increased lymphopenia was observed. Patton et al. (1991) reported that concurrent FeLV infection in experimentally infected cats did not interfere with the clinical outcome of *T. gondii*. However, differences in the ability of FeLV isolates to cause immunosuppression, or differences in *T. gondii* isolates, cannot be ruled out (OVERBAUGH et al., 1988).

The main clinical signs encountered related to the respiratory system, which is one of the main sites for dissemination of *T. gondii* (HARTMANN et al., 2013). The presentation and magnitude of the clinical signs will depend on the location and degree of tissue destruction caused by dissemination of the protozoan (LAPPIN et al., 1989; LAPPIN, 2010; COHEN et al., 2016). This explains the severe respiratory signs manifested by this cat, which were caused by severe diffuse necrotic bronchopneumonia. Furthermore, toxoplasmosis may present nonspecific signs such as fever, depression, anorexia and weight loss to varying degrees (HARTMANN et al., 2013; COHEN et al., 2016). These signs were also presented by the cat studied here.

The definitive diagnosis was obtained after the patient's death, by means of histopathological analysis, like in most other cases that have been reported (HENRIKSEN et al., 1994; LAST et al., 2004; SPYCHER et al., 2011; JOKELAINEN et al., 2012; NAGEL et al., 2013; COHEN et al., 2016). However, cytological analysis on the bronchoalveolar lavage to directly detect tachyzoites might have elucidated the clinical case (HARTMANN et al., 2013). In the necropsy, the main macroscopic lesion seen was pulmonary consolidation. This was also the most significant lesion observed in these other reports and also in an experimental study on cats

with coinfection caused by FIV (DAVIDSON et al., 1993). Lymphadenomegaly and splenomegaly, which were seen in the present case, are also frequent lesions in cases of feline toxoplasmosis (DAVIDSON et al., 1993; HENRIKSEN et al., 1994; LAST et al., 2004; SPYCHER et al., 2011; JOKELAINEN et al., 2012; COHEN et al., 2016).

The main histological lesions in the present case were necrotic interstitial pneumonia and necrotic hepatitis, which were similar to the lesions described in the reports from the authors cited above. However, in some cases, necrosis was described in the spleen and lymph nodes, along with myocarditis, pancreatitis, adrenalitis, meningoencephalitis and uveitis (DAVIDSON et al., 1993; HENRIKSEN et al., 1994; LAST et al., 2004; SPYCHER et al., 2011; JOKELAINEN et al., 2012; NAGEL et al., 2013; COHEN et al., 2016). These lesions were not observed in the present study.

The diagnosis of toxoplasmosis in the present case was confirmed by means of IHC and PCR analysis. With HE staining, cysts and tachyzoites could be seen, especially in the lung tissue, which was found to be positive in anti-*T. gondii* IHC analysis.

The clinical outcome of toxoplasmosis depends on a variety of factors relating to the infectious agent and to the host. Among these, the host's immune status and genetic factors relating to host susceptibility and resistance stand out, along with the inoculum dose of the parasite, its infective stage and the genetic background of the parasite strain (MAUBON et al., 2008). It is unclear whether the strain of *T. gondii* that was identified as type I contributed to the severity of toxoplasmosis in this immunosuppressed cat. Khan et al. (2005) genotyped *T. gondii* strains from human immunodeficiency virus-positive patients and found high prevalence of type I strains or strains with type I alleles.

Among hundreds of isolates already genotyped in Brazil by means of PCR-RFLP (taking successful genotyping to relate to at least 10 markers), type I had only previously been isolated also in southern Brazil, from a chicken from the state of Rio Grande do Sul (DUBEY et al., 2007a) and from a pork sausage intended for human consumption, in the same state (MARTINS et al., 1990), in the southern Brazil. This region of Brazil is one of the regions with fewer strains genotyped, most studies have been conducted in the southeastern region, where the non-archetypal Brazilian clonal genotypes BrI, BrII and BrIII predominate (PENA et al., 2008; SHWAB et al., 2014). There is no information on *T. gondii* genotypes circulating in the state of Santa Catarina, but genotyping of *T. gondii* strains in the states of Rio Grande do Sul and Paraná, which are in the same southern region, have shown the predominance of non-archetypal genotypes and the great diversity that is characteristic of mainland areas of Brazil (SHWAB et al., 2014).

Shwab et al. (2014) reviewed the geographical patterns of *T. gondii* diversity by means of PCR-RFLP based on almost 1,500 samples that had already reported from Africa, Asia, Europe, Central/South America and North America. They showed that genotype type I (#10) was the tenth most frequently identified type, accounting for 2.1% of the samples (originating from cats, chickens, humans, cows, pigs and tree sparrows). This genotype was most prevalent in East Asia and absent in Africa. It was interesting that while PCR-RFLP showed a typical type I clonal profile,

microsatellite analysis (which has higher genotyping resolution) revealed atypical alleles in two typing markers, thus emphasizing the diversity of this parasite in Brazil.

For the first time in Brazil, this genotype was correlated with clinical disease in a host. Genotype characterization of *T. gondii* in all cases of toxoplasmosis from different hosts and regions will contribute towards knowledge of the epidemiology of this parasite.

Acknowledgements

This study was supported by the Coordination Office for Improvement of Higher Education Personnel (CAPES/PROEX, Brazil, grant 1841/2016).

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