



Article/Artigo

Detection and molecular analysis of *Toxoplasma gondii* and *Neospora caninum* from dogs with neurological disorders

Detecção e análise molecular de *Toxoplasma gondii* e *Neospora caninum* em cães com distúrbios neurológicos

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ABSTRACT

Introduction: *Toxoplasma gondii* and *Neospora caninum* are related Apicomplexa parasites responsible for systemic diseases in many species of animals, including dogs. **Methods:** This study aimed to determine the occurrence of *T. gondii* and *N. caninum* infections in 50 dogs with neurological signs that were admitted to the Veterinary Hospital of Universidade Estadual Paulista, City of Botucatu, Brazil. All animals were screened for antibodies using an immunofluorescent antibody test for both parasites. Tissues of positive animals were bioassayed in mice (*T. gondii*) and gerbils (*N. caninum*), and DNA was analyzed using the polymerase chain reaction (PCR). Positive samples for *T. gondii* by PCR were typed using restriction fragment length polymorphism-PCR for 11 markers: SAG1, SAG2 (5'-3'-SAG2 and alt.SAG2), SAG3, Btub, GRA6, L358, c22-8, c29-6, PK1 and Apico, and CS3 marker for virulence analysis. **Results:** Specific antibodies were detected in 11/50 (22%; 95% confidence interval (CI95%), 12.8-35.3%) animals for *T. gondii* and 7/50 (14%; CI95%, 7.02-26.3%) for *N. caninum*. In the bioassay and PCR, 7/11 (63.6%; CI95%, 34.9-84.8%) samples were positive for *T. gondii* and 3/7 (42.9%; CI95%, 15.7-75.5%) samples were positive for *N. caninum*. Three different genotypes were identified, but only 1 was unique. **Conclusions:** These data confirm the presence of *T. gondii* and *N. caninum* in dogs from Brazil, indicating the importance of this host as a sentinel of *T. gondii* for human beings, and the genotypic variation of this parasite in Brazil.

Keywords: *Toxoplasma gondii*. *Neospora caninum*. Genotypes. Dogs. RFLP-PCR. Zoonosis.

RESUMO

Introdução: *Toxoplasma gondii* e *Neospora caninum* são parasitas Apicomplexa responsáveis por doenças sistêmicas em muitas espécies de animais, incluindo cães, o que representa grande importância em animais de estimação. **Métodos:** Este estudo teve como objetivo determinar a prevalência da infecção de *T. gondii* e *N. caninum* em 50 cães com sinais neurológicos internados no Hospital Veterinário da Universidade Estadual Paulista (UNESP) na Cidade de Botucatu, Brasil. Todos os animais foram examinados para detecção de anticorpos por IFAT para ambos os parasitas. Tecidos de animais positivos foram analisados por bioensaio em camundongos (*T. gondii*) e gerbilos (*N. caninum*) e o DNA foi pesquisado por PCR. Amostras positivas para *T. gondii* por PCR foram analisadas por meio de análise de restrição de fragmentos polimórficos (restriction fragment length polymorphism-polymerase chain reaction - RFLP-PCR), utilizando-se 11 marcadores: SAG1, SAG2 (5'-3'-SAG2 e, alt.SAG2), SAG3, Btub, GRA6, L358, c22-8, C29-6, PK1 e Apico e o marcador CS3 para análise de virulência. **Resultados:** Os anticorpos específicos foram detectados em 11/50 animais (22%; IC95% 12,8-35,3%) para *T. gondii*, e 7/50 (14%; IC95% 7,0-26,3%) para *N. caninum*. No bioensaio e PCR, 7/11 (63,6%; IC95% 34,9-84,8%) das amostras foram positivas para *T. gondii*, e 3/7 (42,9%; IC95% 15,7-75,5%) para *N. caninum*. Três diferentes genótipos foram identificados. Apenas um foi único. **Conclusões:** Estes dados confirmam a presença de *T. gondii* e *N. caninum* em cães do Brasil, e demonstra a importância do *T. gondii* como sentinela para a infecção e a variação genotípica deste parasita no Brasil.

Palavras-chaves: *Toxoplasma gondii*. *Neospora caninum*. Genótipos. Cães. RFLP-PCR. Zoonoses.

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INTRODUCTION

Neospora caninum and *Toxoplasma gondii* are related coccidian intracellular protozoan parasites that infect many warm-blooded vertebrates causing systemic disease in many species of animals, including production animals and dogs^{1,2}. Clinical canine toxoplasmosis rarely results from a primary infection; instead, most dogs that die of toxoplasmosis have a distemper virus infection or other immunosuppressive conditions^{2,4}. Toxoplasmosis is recognized as an opportunistic disease in dogs, which is characterized by neuromuscular, respiratory, and gastrointestinal signs or by generalized infection, in addition to its most common neurological impairments, e.g., ataxia, behavioral changes, circling, seizures, paralysis, paraplegia, twitching, and tremors^{5,6}. *T. gondii* comprises different clonal lineages that may influence the progression and severity of the disease in animals and humans⁷.

Dogs are the definitive hosts of *N. caninum* and play a pivotal role in its transmission to other animals, including cattle. Neosporosis has polymorphic clinical signs in dogs. In general, the clinical findings in dogs are similar to those of toxoplasmosis, but neurologic deficits and muscular abnormalities predominate. It also causes myocardial, pulmonary, and dermal disease in dogs⁸.

The similarity between symptomatic toxoplasmosis and neosporosis in dogs reinforces the importance of the differential diagnosis of these diseases. Therefore, this study aimed to determine the occurrence of toxoplasmosis and neosporosis in naturally infected dogs with neurological symptoms and the genotypes circulating in this species.

METHODS

This study was conducted in the ambulatory attendance clinic of the Infectious Disease Section, School of Veterinary Medicine and Animal Science (FMVZ), São Paulo State University (UNESP), São Paulo, Brazil.

Studied animals

Fifty dogs admitted to the Veterinary Hospital from September 2009 to July 2010 presenting with neurological symptoms, such as ataxia, seizures, behavioral changes, paralysis and paraplegia of limbs, and tremors, were studied.

For the bioassay of *T. gondii*, 30-day-old, non-isogenic, Swiss albino female mice (*Mus musculus*) were used. For the bioassay of *N. caninum*, 30-40-day-old, non-isogenic, female gerbils (*Meriones unguiculatus*) were used.

Serology

Dog serum samples were screened for *T. gondii* and *N. caninum* antibodies. Serum samples were diluted 4-fold in phosphate-buffered saline (0.01 mol/L, pH 7.2) from a 1:16 dilution and promptly tested using an immunofluorescent antibody test (IFAT) for *T. gondii* immunoglobulin G (IgG) antibodies, as described by Camargo⁹, and for *N. caninum* IgG antibodies under a fluorescence microscope (Zeiss SG250), as described by Dubey et al.³, using 1:25 as a cut-off point. Both tests were performed using a commercially available mouse fluorescein isothiocyanate (FITC)-labeled IgG Fc antibody conjugate (Bethyl Laboratories Inc., USA). Positive sera controls for toxoplasmosis were obtained by the chronic infection of mice inoculated with the RH strain by the subcutaneous (s.c.) route; while for neosporosis, they were obtained by the chronic infection of gerbils inoculated with the NC-1 strain by the s.c. route. Negative control sera for toxoplasmosis and neosporosis were obtained from mice and gerbils, respectively, inoculated with sterile saline solution by the s.c. route.

For the serological test, tachyzoites of *N. caninum* (NC-1 strain) and *T. gondii* (RH strain) were used as antigens. Dog serum samples showing complete (non-polar or bipolar) parasite fluorescence at dilutions $\geq 1:16$ or $\geq 1:25$ for toxoplasmosis and neosporosis, respectively, were classified as positive.

Bioassay in the experimental models

Brain samples from all seropositive animals were bioassayed by s.c. injection with 1 mL of each tissue sample in 4 mice for the isolation of *T. gondii* and 4 gerbil for the isolation of *N. caninum*, on the basis of its specific serology¹⁰. Peritoneal fluid from all of the mice or gerbils that died was examined for *T. gondii* and *N. caninum* tachyzoites. From the surviving animals, blood was collected by retro-orbital sinus puncture on day 30 post-inoculation. The animals were considered positive when tachyzoites or tissue cysts were found in the brain or when showing positive serology in IFAT³.

Polymerase chain reaction

The extraction and purification of DNA from brain samples were carried out by using the Illustra Tissue and Cells GenomicPrep Mini Spin Kit (GE Healthcare Life Sciences do Brasil Ltda, Brazil).

Polymerase chain reaction was performed by using the primers TOX4 and TOX5 described by Homan et al.¹¹, which amplify a 529-base pair (bp) fragment, AF146527 (GenBank), and repeated 200- to 300-times in the *T. gondii* genome. PCR for *N. caninum* was performed using the species-specific primer pair Np21-Np6, directed to the genomic Nc-5 region, which amplify a 328-bp fragment¹².

TABLE 1 - Genotypic profile of *Toxoplasma gondii* isolates from dog samples.

Isolates	Virulence	Survival (days)*	Genetic markers ^a											Genotype	Reference	
			SAG1	5'-3'SAG2	alt-SAG2	SAG3	Btub	GRA6	c22-8	c29-2	L358	PK1	Apico			CS3
TgDogBr(btu)1	avirulent	30	I	III	III	III	III	III	I	I	I	II	III	I	unique	this study
TgDogBr(btu)2	avirulent	30	I	I	II	III	III	III	I	III	I	II	III	I	TgCatBr1	Su et al. ¹³
TgDogBr(btu)3	avirulent	30	I	III	III	III	III	III	II	III	III	III	III	III	P89	Dubey et al. ²⁶

*maximum observation period = 30 days post-inoculation. ^aSAG: surface antigen; Btub: b-tubulin; GRA: dense granule; Apico: apicoplast; PK: protein kinase.

Genotyping

Strain typing was performed using 11 genetic markers: SAG1, SAG2 (5'-3'SAG2, and alt-SAG2), SAG3, Btub, GRA6, c22-8, c29-2, L358, PK1, Apico, and CS3, as previously described¹³⁻¹⁶. CS3 marker was included in the present study to evaluate virulence¹⁷. Reference strains (GT1, PTG, CTG, TgCgCa1, MAS, and TgCatBr5) were used for control reactions. The target DNA sequences were first amplified by multiplex-PCR using external primers for all markers, followed by nested-PCR for individual markers for genotyping, as previously described¹³⁻¹⁶. All products were visualized through electrophoresis in a 2.5 or 3% agarose gel, depending on the marker, stained with ethidium bromide, and recorded using the digital Gel-Doc-it system (UVP, USA).

Statistical analysis

Statistics associated with the results from the bioassay and PCR were calculated by adopting antibody detection as a standard screening test using the spreadsheet described by Mackinnon¹⁸. The associations between the epidemiological variables and the serological results were analyzed by the chi-square or Fisher's exact tests, considering $\alpha = 0.05$ ¹⁹. For the analysis of the results, concordance testing was performed among the serology, bioassay, and PCR data using the McNemar test²⁰. All tests were carried out using the EpiInfoTM v.3.5.1 program²¹.

Ethical considerations

This study was approved by the Ethics Committee for Animal Experimentation, FMVZ, UNESP, Botucatu Campus (65/2006-CEEA).

RESULTS

Of the 50 animals studied, 11 (22%; 95% confidence interval (CI95%), 12.8-35.3%) were positive for toxoplasmosis, 4 at titer of 1:16, 4 at 1:64, 1 at 1:256, 1 at 1:1,024, and 1 at 1:4,096. Seven animals (14%; CI95%I, 7.0-26.3%) were positive for neosporosis, 2 dogs at a titer of 1:25, 2 at 1:50, and 3 at 1:100. In the bioassay and PCR, 7/11 (63.6%; CI95%, 34.9-84.8%) samples were positive for toxoplasmosis. For neosporosis, 2/7 (28.6%; CI95%, 8.52-65.09) samples were positive in the bioassay and 3/7 (42.9%; CI95%I, 15.7-75.5%) were positive in PCR. Two dogs were positive for both diseases. The bioassay was also carried out on 11 animals that tested negative for both diseases.

Both parasitological tests (i.e., bioassay and PCR) had a 100% agreement for toxoplasmosis and neosporosis. Comparing the results obtained by IFAT and PCR for toxoplasmosis, or IFAT and the bioassay, the tests had an 82% agreement. For the comparison of the 3 diagnostic tests for neosporosis, PCR and the bioassay had an agreement of 82.4%, IFAT and the bioassay test had an agreement of 88.2%, and IFAT and PCR had an agreement of 94.1%.

No variables presented with significant differences for the IFAT results for the analysis of *T. gondii* and *N. caninum* antibodies. Significant associations were observed for both parasites in IFAT [$p = 0.038$; odds ratio (OR) = 6.8571 (CI95%, 1.2503-37.6068)] and PCR [$p = 0.037$; OR = 21.5000 (CI95%, 1.5810-292.3818)]. The genotyping results and the detection rates in other studies are presented in Table 1.

DISCUSSION

Neurological signs of toxoplasmosis, neosporosis, and distemper are similar, emphasizing the importance of the differential diagnosis of these diseases. Toxoplasmosis is recognized as one of the most common diseases in dogs with neurological signs⁵, and has been related to combined infections with distemper²². *N. caninum* infections have been described in animals affected with toxoplasmosis and it should be considered in the differential diagnosis or with concurrent detection in cases like the present one²³.

In the present study, *T. gondii* was isolated from the brain tissue of 7 of 11 dogs with IFAT titers of $\geq 1:16$. *N. caninum* was isolated from the brain tissue of 2 of 7 dogs with IFAT titers of $\geq 1:25$. These data are in agreement with those of Cavalcante et al.²⁴ who identified *N. caninum* in dogs fed with masseter, heart, liver, and brain from infected cattle.

In the detection of *T. gondii* infection, PCR presented with a higher sensitivity than the conventional diagnostic methods. Two dog samples with negative results for serology and the bioassay generated positive PCR results. In this case, one can possibly suggest that these dogs had a chronic previous infection with non-detectable antibody at the 1:16 dilution, which was associated with a parasite load that was too low to be detected in the bioassay, but enough to be detected by PCR. Additionally, the sensitivity of PCR depends on whether the chosen tissue sample contains parasite DNA.

For neosporosis diagnosis, serology demonstrated a greater sensitivity; from 7 positive dogs, 2 were positive in the bioassay and 3 were positive with PCR. Additionally, serological studies using IFAT performed in different hosts have shown that there is little cross-reactivity with the coccidia of other parasites; therefore, IFAT has been considered the standard test for the diagnosis of neosporosis. PCR with the primer pair Np21/Np6 can be an efficient tool for large-scale epidemiological studies using brain tissue obtained at necropsy¹². Despite the sensitivity and specificity of PCR, the brain tissue used may not have contained parasite DNA. This is a limitation of DNA detection, not only for *N. caninum* but also for other microorganisms. Hůrková & Modrý²⁵ detected *N. caninum* DNA in the brains of only 4.6% (7/152) red foxes (*Vulpes vulpes*).

Three genotypes were identified in the present study, TgDogBr1-3. All of them were avirulent in mice. Only one TgDogBr1 presented with a unique genotype. TgDogBr2 presented with a genotype identical to the reference strain TgCatBr1, which was previously identified by Su et al.¹³ in cats from the State of Paraná, Brazil. TgDogBr3 was identical to the reference strain P89, which was previously reported by Dubey et al.²⁶ in a pig from Iowa State, USA.

Phenotypic differences are observed between P89 and TgDogBr3. TgDogBr3 was avirulent, while P89 was classified as virulent for mice²⁶, where only 1 oocyst is lethal to mice by any route of administration. TgDogBr3, as with the other genotypes, presented with the type I or III allele at the CS3 locus. Da Silva et al.²⁷ observed that 5 (62.5%) of 8 strains with the type II allele at the CS3 locus were avirulent in mice, while 3 (37.5%) strains with the type II allele were virulent. In other reports, 82% of strains with the type II allele at the CS3 locus were virulent in mice¹⁶, and 85.5% of strains with the type II allele at the CS3 locus were virulent in mice from another study in Brazil²⁸. According to the classification formulated by Pena et al.¹⁶, TgDogBr2 was classified in the Type BrII group

(intermediate virulence), while TgDogBr3 was classified in the Type BrIII group (avirulent), which correlates with the virulence observed in the present study. TgDogBr1 did not match with any of the types proposed by Pena et al.¹⁶.

These data confirm both types of infection in dogs, the importance of this host as a reservoir for these pathogens for humans, and the genotypic variation of this parasite in Brazil.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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