

4.3 Estudo III

Detection of IgG antibodies to *Neospora caninum* and *Toxoplasma gondii* in dogs examined at a veterinary hospital from Brazil

Um outro parasito protozoário do filo Apicomplexa, *Neospora caninum*, tem sido reconhecido como agente causal de doenças neuromusculares em cães e, até 1988, era erroneamente diagnosticado como *T. gondii*, devido à estreita similaridade morfológica e biológica entre ambos (DUBEY et al., 1988a). Entretanto, estudos recentes têm mostrado que *N. caninum* é filogeneticamente, ultraestruturalmente, biologicamente e antigenicamente distinto de *T. gondii* (SPEER et al., 1999; SU et al., 2003; DUBEY, 2003) e que há pouca ou nenhuma reatividade cruzada em algumas análises sorológicas (BJÖRKMAN; UGGLA, 1999). Como toxoplasmose e neosporose estão associadas com distúrbios neuromusculares, gastrointestinais e respiratórios em cães (DUBEY, 1999), o diagnóstico clínico torna-se difícil e, portanto, um diagnóstico diferencial entre esses dois parasitos é de fundamental importância.

Neste contexto, o estudo III investigou a ocorrência de anticorpos IgG anti-*N. caninum* (Nc) e anti-*T. gondii* (Tg) em 163 cães com distúrbios neuromusculares, respiratórios e/ou gastrointestinais, atendidos no Hospital Veterinário/UFU. Testes sorológicos para *T. gondii* incluíram IHAT, IFAT e ELISA e os resultados discordantes entre os testes foram confirmados por *immunoblotting*, considerando a reatividade ao antígeno SAG-1 (p30) de *T. gondii* como marcador da infecção. Sorologia para *N. caninum* foi realizada por IFAT e confirmada por imunoprecipitação.

Baseado nos resultados sorológicos para *T. gondii*, os 163 soros foram divididos em dois grupos: 59 (36%) amostras Tg-soropositivas e 104 (64%) amostras Tg-soronegativas. Anticorpos anti-*N. caninum* foram detectados em 11 (6,7%) do total de soros analisados, com 5 (3,1%) amostras reativas a ambos parasitas e 6 (3,7%) reativas somente a *Neospora*. Sorologia positiva apenas para *T. gondii* foi observada em 54 (33%) amostras (Tabela 1).

IFAT tem sido utilizado como teste de referência para anticorpos anti-*N. caninum* com a diluição de 1:50 considerada como o título de *cut off* mais freqüente (BJÖRKMAN; UGGLA, 1999), mas neste estudo IV, nós avaliamos duas diluições como títulos de *cut-off* (1:25 e 1:50) em IFAT, sendo consideradas como fraca e forte reatividade, respectivamente, mas sempre apresentando coloração periférica uniforme dos taquizoítas. Entretanto, um grande número de soros mostrou forte coloração polar ou apical dos taquizoítas em ambos

grupos Tg-soropositivos e Tg-soronegativos, refletindo possíveis reatividades cruzadas com outros parasitos Apicomplexa (GANLEY; COMSTOCK, 1980).

Das 11 amostras soropositivas para *N. caninum* analisadas em *immunoblotting* para *T. gondii* (Fig. 1A), somente as 5 amostras Tg-soropositivas mostraram forte reatividade a vários antígenos de *T. gondii*, especialmente SAG1(p30), enquanto nenhuma reatividade a SAG1 foi observada entre as 6 amostras Tg-soronegativas, embora outros antígenos foram fracamente reconhecidos. Em contrapartida, as 11 amostras soropositivas para *N. caninum* quando analisadas por imunoprecipitação reconheceram fortemente pelo menos duas proteínas (29 e 35 kDa) do parasito, embora com reatividade mais fraca pelas amostras de soros com baixo título de anticorpos (1:25), confirmando assim a especificidade dos testes (Fig 1B). Estas duas proteínas são consideradas antígenos imunodominantes de *N. caninum* reconhecidos por antisoros de várias espécies de animais e são provavelmente relacionadas a NcSAG1(p29) e NcSRS2(p35), as quais apresentam um certo grau de homologia com as proteínas de *T. gondii* (TgSAG1 e TgSRS2) (HOWE et al., 1998).

Como para *T. gondii*, há uma grande variação nas freqüências de anticorpos anti-*N. caninum* em cães, devido aos diversos fatores já destacados, particularmente à variedade de testes sorológicos empregados e diferentes títulos de *cut off*, de modo que comparações entre soroprevalências tornam-se inadequadas. Nossos resultados de soropositividade para *N. caninum* não foram altos (6,7%), mas estes dados podem estar subestimados e não representar a real prevalência da infecção na população canina, uma vez que há relatos mostrando que alguns cães experimentalmente infectados com *N. caninum* podem eliminar oocistos nas fezes e não apresentar soroconversão detectável por IFAT (McALLISTER et al., 1998; LINDSAY; DUBEY; DUNCAN, 1999).

Os resultados do estudo III demonstraram que a infecção por *N. caninum* pode estar concomitantemente presente com a infecção por *T. gondii* em cães desta região, embora com menores taxas de soropositividade e, portanto, deve ser considerada no diagnóstico clínico diferencial com *T. gondii* em cães apresentando sintomas neuromusculares, respiratórios e/ou gastrointestinais.



Detection of IgG antibodies to *Neospora caninum* and *Toxoplasma gondii* in dogs examined in a veterinary hospital from Brazil

T.W.P. Mineo^a, D.A.O. Silva^a, G.H.N. Costa^b,
A.C.B. von Ancken^b, L.H. Kasper^c, M.A. Souza^a, D.D. Cabral^b,
A.J. Costa^d, J.R. Mineo^{a,*}

^a Laboratório de Imunologia, Universidade Federal de Uberlândia, Av. Pará 1720,
Campus Umuarama, 38400-902 Uberlândia, MG, Brazil

^b Laboratório de Parasitologia, Universidade Federal de Uberlândia, Av. Pará 1720,
Campus Umuarama, 38400-902 Uberlândia, MG, Brazil

^c Dartmouth Medical School, Hanover, NH, USA

^d Centro de Pesquisas em Sanidade Animal, CPPAR/FCAVJ/UNESP,
14870-000 Jaboticabal, SP, Brazil

Received 3 October 2000; received in revised form 5 February 2001; accepted 12 April 2001

Abstract

A total of 163 dogs with neuromuscular, respiratory and/or gastrointestinal disorders, was admitted at the Veterinary Hospital, Federal University of Uberlândia, Brazil, and submitted to serology for *Toxoplasma gondii* and *Neospora caninum*. Assays for *T. gondii* included indirect haemagglutination (IHA), indirect fluorescent antibody (IFAT-Tg), immunoenzymatic (ELISA), and immunoblotting (IB-Tg). Assays for *N. caninum* included IFAT-Nc and immunoprecipitation (IP-Nc). Based on concordant results by three serological tests (IHA, IFAT-Tg and ELISA) for *T. gondii*, and divergent results further confirmed by IB-Tg for reactivity to TgSAG1, the 163 sera were divided into two groups: 59 (36%) Tg-seropositive samples and 104 (64%) Tg-seronegative samples. Antibodies to *Neospora* were detected in 11 (6.7%) out of 163 analyzed dog sera, with 5 (3.1%) samples reactive to both parasites (Tg+/Nc+), and 6 (3.7%) reactive only to *Neospora* (Tg–/Nc+). Antibodies only to *T. gondii* were found in 54 (33%) samples. Among the 11 *Neospora*-positive sera analyzed by IB-Tg, the five sera Tg+/Nc+ showed strong reactivity to *Toxoplasma* antigens, especially to TgSAG1 (p30). No reactivity was observed to TgSAG1 in the six samples Tg–/Nc+. By IP-Nc, two highly immunodominant antigens (29 and 35 kDa proteins) were recognized by all 11 IFAT-Nc positive sera.

*Corresponding author. Tel.: +55-34-3218-2195; fax: +55-34-3218-2333.
E-mail address: jrmineo@ufu.br (J.R. Mineo).

Our results suggest that the infection by *N. caninum* can be concomitantly present in dogs from this area, although less common, and therefore should be considered in the differential clinical diagnosis with *T. gondii* in dogs presenting neuromuscular, respiratory and/or gastrointestinal disorders. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Neospora caninum*; *Toxoplasma gondii*; Dog; IgG antibodies

1. Introduction

Toxoplasma gondii and *Neospora caninum* are intracellular protozoan parasites of the sub-family Toxoplasmatinae, a group composed of *Toxoplasma*, *Neospora*, *Besnoitia* and *Hammondia*. A broad range of naturally infected hosts has already been reported worldwide, including dogs (Dubey and Lindsay, 1993), which were found to be the definitive hosts for *N. caninum* (McAllister et al., 1998), by shedding oocysts in their feces.

Toxoplasmosis and neosporosis are associated with neurological, gastrointestinal, respiratory, and muscular disorders in dogs (Dubey, 1999; Ahmed et al., 1983). Due to the similar symptoms, the differential clinical diagnosis becomes difficult, as observed by Bjerkas et al. (1984) who first identified a different parasite causing clinical signs almost identical to those induced by *T. gondii* in dogs.

A family of glycosylphosphatidylinositol (GPI)-anchored proteins has been identified on the surface of *T. gondii* (Manger et al., 1998) and *N. caninum* (Howe et al., 1998) tachyzoites and designated as surface antigens (SAGs) and SAG1-related sequences (SRSs). Due to the homology between the SAG1/SRS2 antigens of both parasites, Howe and Sibley (1999) have recommended the nomenclature TgSAG1/TgSRS2 and NcSAG1/NcSRS2 for the major surface antigens of *T. gondii* and *N. caninum*, respectively. However, *N. caninum* appears to be antigenically distinct from *T. gondii*, since no apparent cross-reactivity between the two parasites was found in different serological assays (Dubey et al., 1996). Accordingly, TgSAG1 and TgSAG2 are absent in *N. caninum* (Brindley et al., 1993), and NcSRS2 is not found in *T. gondii* (Hemphill and Gottstein, 1996).

The aim of the present study was to investigate the occurrence of antibodies to *N. caninum* and *T. gondii* in sera from dogs presenting neuromuscular, respiratory and/or gastrointestinal disorders.

2. Materials and methods

2.1. Animals and serum samples

A total of 163 serum samples was obtained from dogs admitted at the Veterinary Hospital, Federal University of Uberlândia, Brazil, with neuromuscular symptoms as ataxia and convulsions, associated to dyspnea, pneumonia, diarrhea and other gastrointestinal disorders. Serum samples were submitted to serology for *T. gondii* and *N. caninum*.

2.2. Serology for *T. gondii*

An indirect haemagglutination test (IHA) was carried out using a HAP-Toxoplasmosis commercial kit (Salck Ind. Com. Prod. Biológicos Ltd., São Paulo), according to the manufacturer instructions. Serum samples with titers ≥ 64 were considered positive.

The indirect fluorescent antibody test (IFAT-Tg) for detecting IgG antibodies to *T. gondii* was used as described by Silva et al. (1997), using as secondary antibody, a fluorescein isothiocyanate-labeled protein A (Sigma Chem. Co., USA). Titer 16 was considered as cut off of the reaction.

An immunoenzymatic test (ELISA) for the detection of *T. gondii* IgG antibodies was performed as described (Silva et al., 1997) using peroxidase-labeled protein A (Sigma) as secondary antibody. Serum samples were tested at single dilution (1:64) in duplicate. Positive and negative serum controls were included in each assay. Cut off was determined as the mean absorbance values of the negative controls plus three standard deviations.

Immunoblot (IB-Tg) was carried out in order to verify the reactivity to TgSAG1 in dog sera with divergent results obtained by IHA, IFAT and ELISA. In addition, *Neospora*-positive serum samples were also analyzed by IB-Tg. Thus, whole extract of *T. gondii* lysed in sample buffer containing 4% sodium dodecyl sulfate (SDS) was submitted to electrophoresis in 12% polyacrylamide gel (SDS-PAGE) as described by Laemmli (1970). Colored high molecular weight markers (Rainbow, Amersham Life Science, USA) were included in each gel. Proteins electrophoretically separated were transferred to nitrocellulose membranes according to Towbin et al. (1979). After blocking, nitrocellulose strips were incubated with dog sera (1:100) and further with protein A-peroxidase (1:2500). The reaction was developed by adding enzyme substrate (1% H₂O₂ and 3,3'-diaminobenzidine tetrahydrochloride, DAB; Sigma). Samples that showed reactivity to TgSAG1 antigen were considered positive.

2.3. Serology for *N. caninum*

IFAT for IgG antibodies to *Neospora* (IFAT-Nc) was carried out using antigen slides of formalized tachyzoites of the NC-1 strain (VRMD Inc., Pullman, WA, USA). Slides were incubated with serum samples and then with protein A-FITC (1:100). The following steps were similar to IFAT-Tg. Only a bright, linear peripheral fluorescence of the tachyzoites was considered a positive result. Cut-off titers in IFAT-Nc were established at 1:25 and 1:50 dilutions, corresponding to weak and strong reactive sera, respectively.

Immunoprecipitation (IP) was performed using biotinylated antigen of *N. caninum*, as described by Pearce and Sher (1989), with some modifications. *Neospora*-positive serum samples in IFAT-Nc were previously incubated with biotinylated antigen and further precipitated with protein A sepharose-4B (Sigma). Immunoprecipitated material was separated by SDS-PAGE, transferred to nitrocellulose and the proteins detected as described in IB-Tg.

3. Results and discussion

Based on concordant results obtained by the three serological tests (IHA, IFAT-Tg and ELISA) for *T. gondii* antibodies, and divergent results further confirmed in IB-Tg for

Table 1

IgG antibodies to *N. caninum* by IFAT-Nc in *T. gondii*-seropositive and -seronegative dogs

Group ^a	Samples No.	IgG antibodies to <i>N. caninum</i>		
		IFAT-Nc dilutions		Total
		1:25	1:50	
Tg-seropositive	59	1 (1.7%)	4 (6.8%)	5 (8.5%)
Tg-seronegative	104	3 (2.9%)	3 (2.9%)	6 (5.8%)
Total	163	4 (2.5%)	7 (4.3%)	11 (6.7%)

^a Serum samples tested by IHA, IFAT-Tg, ELISA and IB-Tg.

reactivity to the TgSAG1 antigen, the 163 dog sera were divided into two groups: 59 (36%) Tg-seropositive samples and 104 (64%) Tg-seronegative samples.

IFAT has been often used as a reference test for antibodies to *N. caninum*, and cut-off titers may present some variations among different studies, being often established at 1:50 for dogs (Bjorkman and Ugglä, 1999). In the present study, we evaluated two cut-off titers (1:25 and 1:50) in IFAT-Nc. As demonstrated in Table 1, antibodies to *N. caninum* were detected in 11 (6.7%) out of 163 dog sera. Thus, 4 (6.8%) samples of Tg-seropositive group were strongly reactive to *N. caninum* (>1:50), and 1 (1.7%) sample showed weak reactivity (1:25), totaling 5 (8.5%) samples reactive to both parasites (Tg+/Nc+). In the Tg-seronegative group, 3 (2.9%) were reactive to *N. caninum* at 1:50 dilution, and 3 (2.9%) at 1:25 dilution, computing a total of 6 (5.8%) samples reactive only to *Neospora* (Tg–/Nc+).

From the total of serum samples analyzed, 6 (3.7%) were reactive only to *N. caninum*, 54 (33%) reactive only to *T. gondii*, and 5 (3.1%) to both parasites. These results were slightly higher than seroprevalence data described by Lindsay et al. (1990) in dogs from Kansas. These authors found 2% only to *N. caninum*, 25% to *T. gondii* and 1% to both parasites. Such variations with our results might be due to regional differences as well as to the different serological assays, cut off titers and dog sampling analyzed.

Our seropositivity rates for *N. caninum* were not high, similarly to those reported in North America and Japan (6–7%) for canine populations (Cheadle et al., 1999; Sawada et al., 1998). In addition, seroprevalence values in dogs ranging from 0.2 to 29% were already reported by Dubey, 1999. However, such data may be underestimated and might not represent the current prevalence of the infection in canine populations, since that McAllister et al. (1998) and Lindsay et al. (1999) reported that some dogs can excrete oocysts in their feces and be non-reactive in IFAT.

In addition, a number of negative sera to *N. caninum* showed strong polar staining in IFAT-Nc, which was present in both Tg-seropositive and Tg-seronegative groups. Sole fluorescence of the apical part of the tachyzoites is regarded as a non-specific reaction caused by infection with cross-reactive apicomplexan species (Paré et al., 1995), due to common microneme and rhoptry antigens (Ganley and Comstock, 1980). Interestingly, it has been reported that the IFAT-Nc shows very little cross-reactivity with other coccidian parasites, particularly in relation to *T. gondii* (Dubey et al., 1996). Consequently, appears unlikely that these *Neospora*-negative sera showing polar staining could have cross-reacted with *T. gondii*.

In the present study, IB-Tg revealed various important antigens, particularly the 30 kDa band (TgSAG1), which was recognized by the five sera Tg+/Nc+ (Fig. 1A). No reactivity was observed to TgSAG1 in the six samples Tg–/Nc+, reinforcing the specificity of the assay, although other *T. gondii* antigens were weakly recognized. On the other hand, IP-Nc

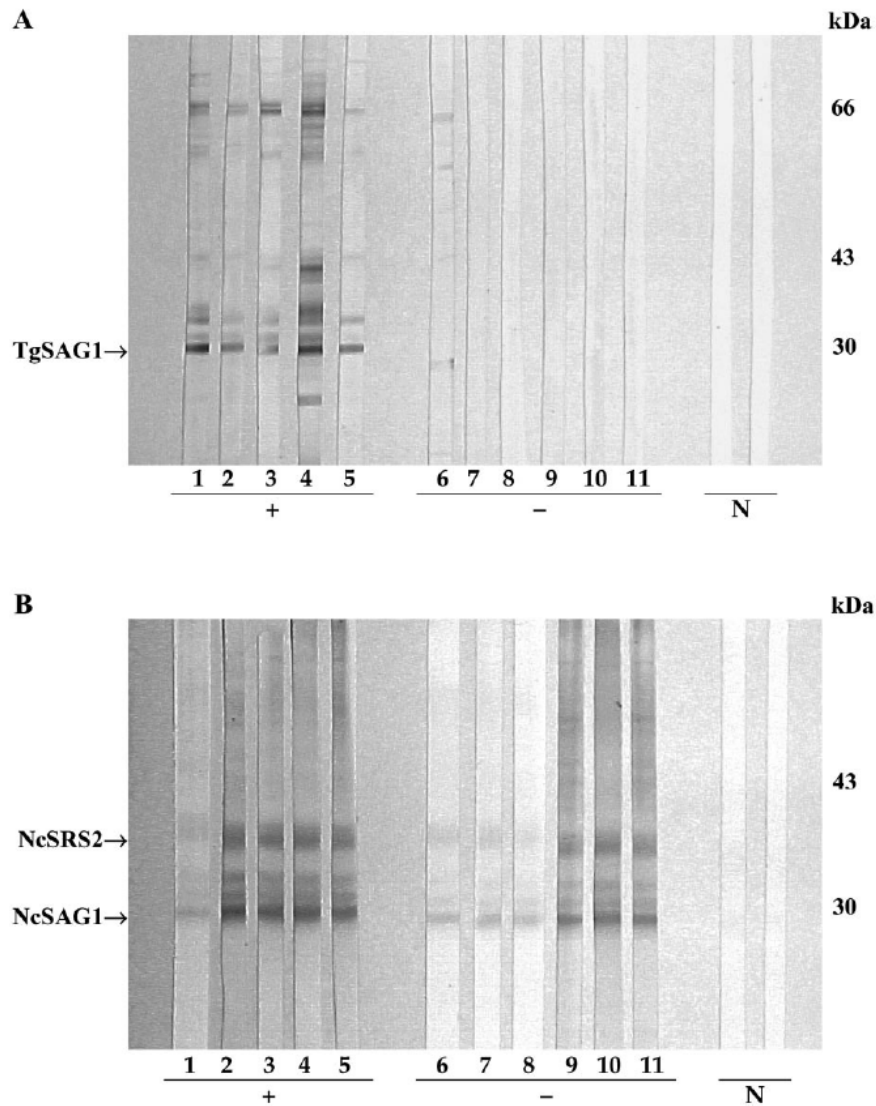


Fig. 1. Immunoblotting for *T. gondii* (A) and immunoprecipitation for *N. caninum* (B) of 11 *Neospora*-positive dog sera in IFAT-Nc; (+): five serum samples Tg+/Nc+ with IFAT-Nc titers of 25 (lane 1) and 50 (lanes 2–5); (–): six serum samples Tg–/Nc+ with IFAT-Nc titers of 25 (lanes 6–8) and 50 (lanes 9–11); (N): negative control sera. Molecular mass values (kDa) are shown on the right. Major surface antigens as TgSAG1 (p30) and NcSAG1 (p29)/NcSRS2 (p35) are shown on the left.

confirmed the specificity of the 11 *Neospora*-positive dog sera, where several *N. caninum* antigens were recognized, particularly two highly dominant 29 (NcSAG1) and 35 kDa (Nc-SRS2) proteins, although with weaker reactivity by sera with low antibody titers (1:25) (Fig. 1B). As reported by Howe and Sibley (1999), although NcSAG1 and NcSRS-2 are homologous to their *T. gondii* counterparts (TgSAG1 and TgSRS2), the percentage of sequence identity shared by the homologues is not sufficient to elicit antibody cross-reactivity. These proteins are highly immunogenic, being the predominantly recognized antigens by antisera from *Neospora*-infected animals.

Our results suggest that the infection by *N. caninum* can be concomitantly present in dogs from this area, although less common, and therefore should be considered in the differential clinical diagnosis with *T. gondii* in dogs presenting neuromuscular, respiratory and/or gastrointestinal disorders.

Acknowledgements

This work was financially supported by CNPq and FAPEMIG Brazilian Research Agencies.

References

- Ahmed, B.A., Gaafar, S.M., Weirich, W.E., Kanitz, C.L., 1983. Relationship of *Toxoplasma* infections to other diseases. *Vet. Parasitol.* 12, 199–203.
- Bjerkas, I., Mohn, S.F., Presthus, J., 1984. Unidentified cyst-forming sporozoan causing encephalomyelitis and myositis in dogs. *Z. Parasitenkd.* 70, 271–274.
- Bjorkman, C., Uggla, A., 1999. Serological diagnosis of *Neospora caninum* infection. *Int. J. Parasitol.* 29, 1497–1507.
- Brindley, P.J., Gazzinelli, R.T., Denkers, E.Y., Davis, S.W., Dubey, J.P., Belfort Jr., R., Martins, M.C., Silveira, C., Jamra, L., Waters, A.P., Sher, A., 1993. Differentiation of *Toxoplasma gondii* from closely related coccidia by riboprint analysis and a surface antigen gene polymerase chain reaction. *Am. J. Trop. Med. Hyg.* 48, 447–456.
- Cheadle, M.A., Lindsay, D.S., Blagburn, B.L., 1999. Prevalence of antibodies to *Neospora caninum* in dogs. *Vet. Parasitol.* 85, 325–330.
- Dubey, J.P., 1999. Recent advances in *Neospora* and neosporosis. *Vet. Parasitol.* 84, 349–367.
- Dubey, J.P., Lindsay, D.S., 1993. Neosporosis. *Parasitol Today* 9, 452–458.
- Dubey, J.P., Lindsay, D.S., Adams, D.S., Gay, J.M., Baszler, T.V., Blagburn, B.L., Thulliez, P., 1996. Serologic responses of cattle and other animals infected with *Neospora caninum*. *Am. J. Vet. Res.* 57, 329–336.
- Ganley, J.P., Comstock, G.W., 1980. Association of cats and toxoplasmosis. *Am. J. Epidemiol.* 111, 238–246.
- Hemphill, A., Gottstein, B., 1996. Identification of a major surface protein on *Neospora caninum* tachyzoites. *Parasitol. Res.* 82, 497–504.
- Howe, D.K., Sibley, L.D., 1999. Comparison of the major antigens of *Neospora caninum* and *Toxoplasma gondii*. *Int. J. Parasitol.* 29, 1489–1496.
- Howe, D.K., Crawford, A.C., Lindsay, D., Sibley, L.D., 1998. The p29 and p35 immunodominant antigens of *Neospora caninum* tachyzoites are homologous to the family of surface antigens of *Toxoplasma gondii*. *Infect. Immun.* 66, 5322–5328.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lindsay, D.S., Dubey, J.P., Upton, S.J., Ridley, R.K., 1990. Serological prevalence of *Neospora caninum* and *Toxoplasma gondii* in dogs from Kansas. *J. Helminthol. Soc. Wash.* 57, 86–88.

- Lindsay, D.S., Dubey, J.P., Duncan, R.B., 1999. Confirmation that the dog is a definitive host for *Neospora caninum*. *Vet. Parasitol.* 82, 327–333.
- Manger, I.D., Hehl, A.B., Boothroyd, J.C., 1998. The surface of *Toxoplasma* tachyzoites is dominated by a family of glycosylphosphatidylinositol-anchored antigens related to SAG-1. *Infect. Immun.* 66, 2237–2244.
- McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Willis, R.A., McGuire, A.M., 1998. Dogs are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* 28, 1473–1478.
- Paré, J., Hietala, S.K., Thurmond, M.C., 1995. Interpretation of an indirect fluorescent antibody test for diagnosis of *Neospora* sp. infection in cattle. *J. Vet. Diag. Invest.* 7, 273–275.
- Pearce, E.J., Sher, A., 1989. Three major surface antigens of *Schistosoma mansoni* are linked to the membrane by glycosylphosphatidylinositol. *J. Immunol.* 142, 979–984.
- Sawada, M., Park, C.H., Kondo, H., Morita, T., Shimada, A., Yamane, I., Umemura, T., 1998. Serological survey of antibody to *Neospora caninum* in Japanese dogs. *J. Vet. Med. Sci.* 60, 853–854.
- Silva, D.A.O., Cabral, D.D., Bernardina, B.L.D., Souza, M.A., Mineo, J.R., 1997. Detection of *Toxoplasma gondii*-specific antibodies in dogs. A comparative study of immunoenzymatic, immunofluorescent and haemagglutination titers. *Mem. Inst. Oswaldo Cruz* 92, 785–789.
- Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* 76, 4350–4354.

4.4 Estudo IV

Evaluation of serological tests for the diagnosis of *Neospora caninum* infection in dogs: optimization of cut off titers and inhibition studies of cross-reactivity with *Toxoplasma gondii*

Vários testes sorológicos têm sido desenvolvidos para o sorodiagnóstico de *N. caninum*. IFAT tem sido frequentemente utilizado como teste de referência para a detecção de anticorpos anti-*N. caninum* em cães e vários tipos de ELISA têm sido desenvolvidos e aplicados, principalmente em inquéritos soroepidemiológicos. Entretanto, os critérios para interpretação dos resultados, incluindo os títulos de *cut off*, são relativamente subjetivos e extremamente variáveis em diferentes estudos (LASRI et al., 2004). Além disso, reatividades inespecíficas ou cruzadas com parasitos relacionados têm sido relatadas em ELISA, dependendo da preparação do antígeno e dos antisoros policlonais utilizados (BJÖRKMAN; HOLMDAHL; UGGLA, 1997).

Diferentes diluições têm sido utilizadas em IFAT (1:16 a 1:200) e ELISA (1:50 a 1:100) para detecção de anticorpos anti-*N. caninum* em cães, não sendo possível fazer comparações válidas entre as soroprevalências de vários estudos. Além disso, não há um título de *cut off* estabelecido para cada teste que possa realmente ser considerado positivo para *N. caninum* e baixos títulos de anticorpos anti-*N. caninum* podem ser a expressão de reatividade cruzada com parasitos relacionados, particularmente *T. gondii*, devido a antígenos comuns de taquizoítas e bradizoítas (BJÖRKAS; JENKINS; DUBEY, 1994; McALLISTER et al., 1996).

No estudo IV, nós comparamos IFAT e ELISA para a detecção de anticorpos IgG anti-*N. caninum* em soros de cães através da determinação dos títulos ótimos de *cut off* usando a curva TG-ROC e considerando a reatividade aos antígenos imunodominantes de *N. caninum* em *immunoblotting* como marcador da infecção. Além disso, antígenos de reatividade cruzada com *T. gondii* foram também analisados por ELISA e *immunoblotting* de inibição.

Os resultados de IFAT e ELISA para a detecção de anticorpos IgG anti-*N. caninum* em 300 amostras de soros de cães revelaram 105 (35%) amostras positivas por ELISA, 32 (10,7%) por IFAT, sendo apenas 29 (9,7%) positivas em ambos testes (Tabela 1), resultando em um índice de concordância de 74% entre os testes, mas com um baixo coeficiente *kappa* ($\kappa = 0.30$), o que sugere uma pobre concordância. Entretanto, quando índices de concordância separados (positivos e negativos) foram analisados, uma alta associação de resultados negativos ($P_{\text{neg}} = 0,83$) em comparação aos positivos ($P_{\text{pos}} = 0,42$) foi encontrada, indicando que há uma boa concordância negativa, embora pobre concordância positiva entre IFAT e

ELISA para o sorodiagnóstico de *N. caninum* em cães, similarmente aos resultados obtidos por Lasri e colaboradores (2004).

Sabe-se que extratos antigênicos solúveis utilizados em ELISA contêm um grande número de antígenos, a maioria deles de origem intracelular, enquanto antígenos de membrana da superfície do parasito são preferencialmente reconhecidos em IFAT. Desta forma, IFAT tem sido considerado um teste mais específico, embora sua sensibilidade e especificidade podem variar dependendo dos valores de *cut off* estabelecidos (BJÖRKMAN; UGGLA, 1999). Portanto, resultados de ELISA para *N. caninum* devem ser interpretados com cautela e testes adicionais ou complementares, tais como *immunoblotting*, deveriam ser realizados para clarificar os resultados discordantes entre IFAT e ELISA.

Neste contexto, nós avaliamos antígenos de *N. caninum* que foram reconhecidos por soros de cães experimentalmente infectados com o parasito por *immunoblotting*, mostrando um forte reconhecimento de três antígenos imunodominantes (17, 29-32, 35-37 kDa) (Fig.2). A grande maioria (86%) dos soros com resultados concordantes positivos (IFAT+/ELISA+) reconheceram pelo menos dois destes antígenos, particularmente as bandas de 29-32 e 35-37 kDa, enquanto soros com resultados concordantes negativos (IFAT-/ELISA-) ou discordantes (IFAT+/ELISA- ou IFAT-/ELISA+) reconheceram apenas um destes três antígenos, especialmente as bandas de 35-37 kDa (Tabela 2).

Assim, os títulos ótimos de *cut off* em IFAT e ELISA para *N. caninum* foram determinados usando a curva TG-ROC, considerando a reatividade a pelo menos dois de seus três antígenos imunodominantes como marcador da infecção, obtendo-se um título de 50 para IFAT e ao redor de 200 para ELISA (Fig. 3). Para IFAT, a diluição de 1:50 tem sido recomendada e comumente usada para soros caninos em vários estudos (AZEVEDO et al., 2005; PINHEIRO et al., 2005; ANDREOTTI et al., 2006). Entretanto, os baixos valores de sensibilidade e especificidade equivalentes encontrados para ELISA sugerem que a eficiência do teste pode ser extremamente influenciada pelo tipo de extrato antigênico bem como características dos conjugados utilizados.

Quando a soropositividade a *N. caninum* foi avaliada em relação à sorologia para *T. gondii* (Fig. 1), amostras soropositivas para *T. gondii* mostraram significativa co-positividade com *N. caninum* em IFAT (18.2%) e, particularmente, em ELISA (55.4%) ($p < 0,001$). Assim, o grau de reatividade cruzada entre ambos parasitos foi investigado em ELISA de inibição, mostrando uma curva de inibição heteróloga parcial em ELISA-Nc, indicando que antígenos de *T. gondii* podem inibir a reatividade de anticorpos a *N. caninum* (Fig. 4). Por outro lado, curvas de inibição para ELISA-Tg mostraram uma clara inibição homóloga, mas

sem uma evidente inibição heteróloga, sugerindo que antígenos de *N. caninum* não conseguem inibir a reatividade de anticorpos a *T. gondii*. Uma provável explicação para estes achados pode estar relacionada aos maiores títulos de anticorpos anti-*T. gondii* que anti-*N. caninum* encontrados em soros de cães ou ainda possíveis diferenças na afinidade do anticorpo para estes antígenos.

Para identificar quais antígenos de reatividade cruzada entre *N. caninum* e *T. gondii* eram reconhecidos por soros de cães, *immunoblotting* de inibição foi realizado e a intensidade das bandas foi analisada em diferentes condições, com ou sem antígenos inibidores (Figs. 5 e 6). Uma alta inibição homóloga foi encontrada para as várias bandas antigênicas de ambos parasitos, mas inibição heteróloga parcial foi evidente para antígenos acima de 45-50 kDa para *N. caninum* e acima de 66 kDa para *T. gondii*, indicando que os antígenos de reatividade cruzada entre ambos parasitos são proteínas acima de 40 kDa. Neste contexto, Björkas, Jenkins e Dubey (1994) relataram que bandas antigênicas de 46, 88 e 97 kDa de *N. caninum* e de 45, 66 e 85 kDa de *T. gondii* foram reconhecidas por antisoros contra ambos parasitos. Recentemente, vários antígenos de reatividade cruzada entre *N. caninum* e *T. gondii* foram identificados e caracterizados por anticorpos monoclonais produzidos contra *N. caninum* (LIAO et al., 2005), entre eles, a proteína do choque térmico (HSP70).

No estudo IV, os antígenos imunodominantes de ambos parasitos, especialmente aqueles abaixo de 35 kDa, foram fracamente inibidos por antígenos heterólogos, indicando que eles são mais espécie-específicos. Os resultados deste estudo confirmaram que IFAT na diluição de 1:50 é a técnica mais apropriada para o sorodiagnóstico da infecção por *N. caninum* em cães e que ELISA deve ser empregado com cautela, particularmente quando preparações de antígenos totais são utilizadas, devido à possibilidade de reações cruzadas com outros parasitos como *T. gondii*. Além disso, nós sugerimos que antígenos imunodominantes de *N. caninum*, especialmente aqueles abaixo de 35 kDa, poderiam ser os candidatos selecionados para o aprimoramento de testes sorológicos para o diagnóstico da infecção por *N. caninum* em cães.

Evaluation of serological tests for the diagnosis of *Neospora caninum* infection in dogs: optimization of cut off titers and inhibition studies of cross-reactivity with *Toxoplasma gondii*

Deise A. O. Silva^a, Janaína Lobato^a, Tiago W. P. Mineo^b, José R. Mineo^{a,*}

^aLaboratory of Immunoparasitology, Federal University of Uberlândia, Uberlândia, MG, Brazil; ^bLaboratory of Immunoparasitology, FCAV/UNESP, Jaboticabal, SP, Brazil

Abstract

Diagnosis of *Neospora caninum* infection in dogs is based on serological assays such as the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA). This study evaluated two serological tests (IFAT and ELISA) for the detection of IgG antibodies to *N. caninum* in 300 serum samples of dogs through the optimization of cut off titers by using the two-graph receiver-operating characteristic (TG-ROC) curve. In addition, the major cross-reactive antigens with *Toxoplasma gondii* were analyzed by inhibition ELISA and immunoblotting (IB) assays. IFAT and ELISA results showed 74% agreement, with a good negative concordance ($P_{\text{neg}} = 0.83$) but a poor positive concordance ($P_{\text{pos}} = 0.42$). The great majority (86%) of sera with positive concordant results (IFAT+/ELISA+) recognized at least two out of three *N. caninum* immunodominant antigens, particularly the 29-32 and 35-37 kDa bands. Sera with negative concordant results (IFAT-/ELISA-) or discordant results (IFAT+/ELISA- ou IFAT-/ELISA+) recognized only the 35-37 kDa bands. Optimization of cut off titers in IFAT and ELISA was performed considering the reactivity to at least two out of three *N. caninum* immunodominant antigens as infection marker, obtaining a titer of 50 for IFAT and 200 for ELISA. Seropositivity to *N. caninum* was significantly associated with *T. gondii*-seropositive samples, particularly in ELISA (55.4%). Inhibition ELISA curves for *N. caninum* showed a partial heterologous inhibition, indicating some degree of cross-reactivity between *N. caninum* and *T. gondii* antigens. Inhibition IB assays showed a moderate heterologous inhibition for *N. caninum* antigens above 45-50 kDa. These results indicate that IFAT at 1:50 cut off dilution is the most appropriated test for *N. caninum* serology in dogs and ELISA, particularly when crude tachyzoite antigen preparations are employed, should be used critically due to possible cross-reactivity with other related parasites as *T. gondii*. Therefore, we suggest that *N. caninum* immunodominant antigens, specially the 17 and 29-32 kDa proteins, should be elected markers in order to develop serological diagnostic systems for *N. caninum* infection in dogs.

Keywords: *Neospora caninum*; *Toxoplasma gondii*; IFAT; ELISA; Immunoblotting; Dog

1. Introduction

Neospora caninum and *Toxoplasma gondii* are structurally, genetically, and immunologically related coccidian protozoan parasites that infect many warm-blooded vertebrates, although neosporosis and toxoplasmosis are biologically two distinct diseases (Dubey, 2003). *T. gondii* has been recognized as an important pathogen for humans and sheep, particularly during pregnancy and for immunocompromised patients, whereas *N. caninum* has been considered a major cause of neonatal mortality and/or abortion in cattle and neuromuscular disorders in dogs (Dubey, 2003). Thus, both neosporosis and toxoplasmosis have been recognized as economically important diseases that have considerable impact on the livestock industry (Liao et al., 2005).

N. caninum infection in dogs are epidemiologically relevant because dogs are the major definitive hosts of the protozoan (McAllister et al., 1998), by shedding oocysts into the environment and thus constituting an important risk factor for the horizontal transmission to other animals, particularly cattle (Paré et al., 1998). Clinical and subclinical infections with *N. caninum* in dogs have been extensively reported worldwide with seroprevalence rates ranging from 0.2 to 54% (Moore, 2005). Clinical canine neosporosis is characterized mainly by neuromuscular disorders, but may be generalized with the involvement of several organs, including the skin (Perlé et al., 2001), and it is more severe in young, congenitally infected pups (Barber and Trees, 1998).

A number of serological tests have been developed for the detection of *N. caninum* specific antibodies, but none of them are generally accepted and up to date there is not a standardized test available to be used. Indirect fluorescent antibody test (IFAT) has been often used as a reference test for the detection of *N. caninum* antibodies in dogs, showing very little cross-reactivity with related protozoan parasites (Dubey et al., 1996; Hemphill and Gottstein, 2000). In order to screen a large number of sera, commercially available or in-house enzyme-linked immunosorbent assays (ELISA) have been usually employed since they are cost effective and less time consuming. However, the criteria used for interpretation of the results, including the cut off titers, are relatively subjective and they are extremely varied in different seroepidemiological and clinical studies (Lasri et al., 2004). In addition, high background absorbance values and cross-reactivity with antibodies against related parasites have been reported depending on the antigen preparation and the polyclonal antisera used in ELISA (Björkman et al., 1997).

N. caninum antigens that are useful for the diagnosis of neosporosis have been identified and characterized in several domestic animals (Bjerkas et al., 1994). Thus, immunoblotting (IB) assays revealed that immune sera from a wide range of animal species exhibited a similar recognition pattern of four immunodominant (17-, 29-, 30- and 37-kDa) followed by several minor *N. caninum* antigens. Also, cross-reactive antigens between *N. caninum* and *T. gondii* have been identified by using monoclonal antibodies, such as the heat-shock protein 70 (HSP70), that could be useful to develop vaccines or drugs to control the diseases caused by both parasites (Liao et al., 2005). Inversely, the

identification of these cross-reactive antigens might contribute to identify parasite species-specific antigens to improve the serological diagnosis of both protozoan infections.

The aim of the present study was to evaluate comparatively two serological tests (IFAT and ELISA) for the diagnosis of *N. caninum* infection in dogs through the optimization of cut off titers by using the two-graph receiver-operating characteristic (TG-ROC) curve and the identification of major cross-reactive antigens with *T. gondii* through ELISA and IB inhibition assays.

2. Materials and methods

2.1. Serum samples

A total of 300 serum samples were obtained from dogs of both genders and different ages and breeds, acquired from the Veterinary Hospital of the Federal University of Uberlândia, private veterinary clinics of the city, and from the Zoonosis Control Center of Uberlândia, Brazil, between the years 2001 and 2004. Positive control sera were obtained from dogs experimentally infected with *N. caninum* (acquired from VMRD[®] Inc., Pullman, WA, USA) or *T. gondii* (Silva et al., 2002), and negative control sera were collected from healthy dogs with negative serology for both parasites by two serological tests (IFAT and ELISA).

2.2. Parasites and antigens

N. caninum tachyzoites (Nc-1 strain) were obtained by infections of bovine monocytes as host cells, cultured in RPMI 1640 medium supplemented with 3% heat-inactivated calf fetal serum in a 5% CO₂ atmosphere at 37°C and harvested by scraping off the cell monolayer after 2-3 days of infection (Speer et al., 1985). Tachyzoites were purified by forcible extrusion through a 27-gauge needle and centrifuged at 45 x g for 1 min at 4°C to remove host cell debris. The supernatant was collected and washed twice (720 x g, 10 min, 4°C) in phosphate-buffered saline (PBS, pH 7.2). *T. gondii* tachyzoites (RH strain) were maintained in Swiss mice by intraperitoneal serial passage at regular 48 h intervals (Mineo et al., 1980). Mouse peritoneal exudates were harvested and washed in PBS as described above.

N. caninum and *T. gondii* whole antigens were prepared according to Camargo (1964). Parasite suspensions were adjusted to 1 x 10⁶ tachyzoites/mL and treated with 1% formaldehyde for 30 min at room temperature. After washing in PBS, parasites were dry-fixed in microscopic slides and stored at -20°C until being used in IFAT.

N. caninum and *T. gondii* soluble antigens were prepared as described by Scott et al. (1987). Parasite suspensions were adjusted to 1 x 10⁸ tachyzoites/mL, treated with protease inhibitors (aprotinin, 10 µg/mL; leupeptin, 50 µg/mL; phenyl-methylsulfonyl fluoride [PMSF], 1.6 mM) and then lysed by five freeze-thaw (liquid nitrogen and water bath at 37°C) cycles and further by ultrasound (six 60 Hz cycles for 1 min each) on ice. After centrifugation (10,000 x g, 30 min, 4°C),

supernatants were collected, the protein concentration was determined (Lowry et al., 1951) and soluble antigen aliquots were stored at -20°C until being used in ELISA.

N. caninum and *T. gondii* tachyzoites (1×10^8 organisms/mL) were lysed in 4% sodium dodecyl sulfate (SDS) sample buffer and then submitted to electrophoresis in a 12% polyacrylamide gel (SDS-PAGE) under non-reducing conditions (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose membranes (Towbin et al., 1979) and stored at 4°C for further use in IB assays.

2.3. Serological assays

IFAT – Indirect fluorescent antibody tests were carried out to detect IgG antibodies to *N. caninum* (IFAT-Nc) and *T. gondii* (IFAT-Tg) as described by Mineo et al. (2001) and Camargo (1964), respectively. Antigen slides were incubated with dog sera diluted 1:25 (IFAT-Nc) or 1:16 (IFAT-Tg) in PBS and then with a fluorescein isothiocyanate-labeled rabbit anti-dog IgG (Fiocruz/Bio-Manguinhos, Rio de Janeiro, Brazil) diluted 1:50 in PBS plus 0.01% Evans blue. Positive and negative control sera were included in each slide. Slides were examined under fluorescence microscopy and only a bright, linear peripheral fluorescence of the tachyzoites was considered positive.

ELISA – Indirect ELISAs were carried out to detect IgG antibodies to *N. caninum* (ELISA-Nc) and *T. gondii* (ELISA-Tg), according to Silva et al. (2002a). Optimization of the reaction was established in preliminary experiments through block titration of the reagents. Microtiter plates were coated with *N. caninum* (20 $\mu\text{g/mL}$) or *T. gondii* (10 $\mu\text{g/mL}$) soluble antigens and then incubated with dog sera diluted 1:100 (ELISA-Nc) and 1:64 (ELISA-Tg) in PBS containing 0.05% Tween 20 (PBS-T) and 5% skim milk (PBS-TM). Subsequently, peroxidase-labeled rabbit anti-dog IgG (prepared according to Wilson and Nakane, 1978) diluted 1:8,000 in PBS-TM was added and the reaction was developed by adding the enzyme substrate consisting of 0.03% H_2O_2 and ortho-phenylenediamine (OPD) at 1 mg/mL in 0.1M citrate-phosphate buffer (pH 5.5). The optical density (OD) was read at 492 nm in a plate reader (Titertek Multiskan Plus spectrophotometer, Flow Laboratories, USA). Positive and negative control sera were included in each plate. The cut off of the reaction was determined as the mean OD for negative control sera plus three standard deviations. Antibody titers were expressed as ELISA index (EI) as described by Silva et al. (2002a) as follows: $\text{EI} = \text{OD}_{\text{sample}} / \text{OD}_{\text{cut off}}$ and values of $\text{EI} > 1.2$ were considered positive.

IB – Immunoblotting assays were performed to verify the reactivity to *N. caninum* (IB-Nc) and *T. gondii* (IB-Tg) immunodominant antigens as previously described (Mineo et al., 2001). *N. caninum* and *T. gondii* proteins blotted on nitrocellulose strips were blocked with 5% skim milk in PBS-T for 2 h at room temperature. Strips were incubated overnight at 4°C with dog sera diluted 1:50 in 1% skim milk in PBS-T, and then with peroxidase-rabbit anti-dog IgG diluted 1:6,000 for 2 h at room temperature. Alternatively, serum samples of mice experimentally infected with *N. caninum* or *T. gondii* were also tested and revealed with peroxidase-goat anti-mouse IgG (Sigma Chemical Co., MO, USA) diluted 1:1,000. The reaction was developed by adding enzyme substrate (Sigma FastTM 3,3'-

diaminobenzidine tablet sets; Sigma Chemical Co.). Bands were analyzed using the Kodak Digital Science 1D image analysis software and plotted against a standard molecular weight curve to determine their relative mobilities (R_f) and apparent molecular masses. Serum samples were considered positive when showing reactivity for at least two out of three (17, 29-32, 35-37 kDa) immunodominant antigens of *N. caninum* (Bjerkas et al., 1994; Howe et al., 1998) or to *T. gondii* SAG1 (p30) antigen (Silva et al., 2002b).

2.4. TG-ROC

In order to determine optimal cut off titers in IFAT-Nc and ELISA-Nc, sensitivity and specificity of the tests were evaluated considering IB-Nc as the reference test and the reactivity for at least two out of three (17, 29-32, 35-37 kDa) immunodominant antigens of *N. caninum* as infection markers. For this purpose, sensitivity and specificity were determined in different cut-off titers for each test by using the two-graph receiver-operating characteristic (TG-ROC) curve (Greiner et al., 1995).

2.5. ELISA and IB inhibition

To evaluate the degree of cross-reactivity between *N. caninum* and *T. gondii* antigens, inhibition assays were performed as previously described (Pereira et al., 2005). For ELISA inhibition, optimal serum dilutions were established at 1:50 for *N. caninum* and 1:200 for *T. gondii*, which were used as inhibitor antigens. Serum samples from seropositive dogs to both parasites were pre-adsorbed (v/v) overnight at 4°C with 10-fold serial concentrations (200 to 0.0002 µg/mL) of each inhibitor antigen. Sera incubated with diluent without inhibitors were used as positive controls. After adsorption, the inhibitor-serum mixtures were assayed in ELISA-Nc and ELISA-Tg as described above. The results were expressed as percentage of inhibition and calculated as follows: $(OD \text{ without inhibitor} - OD \text{ with inhibitor}) / OD \text{ without inhibitor} \times 100$ as previously described (Puerta et al., 2005).

For IB inhibition, dog sera with IgG antibodies to both parasites were diluted 1:50 and pre-adsorbed (v/v) with 200 µg/mL of each inhibitor antigen for 2 h at 37°C. As control, sera incubated with diluent only were used. Preadsorbed sera were centrifuged at 15,000 x g for 10 min at 4°C and supernatants were incubated with *N. caninum* and *T. gondii* proteins blotted on nitrocellulose strips, following the same protocol described for IB. Immunoblot profiles were analyzed using the Kodak Digital Science 1D image analysis software by calculating band intensity (*Band Int*) expressed in pixels. The percentage of inhibition was calculated as follows: $(Band \text{ Int without inhibitor} - Band \text{ Int with inhibitor}) / Band \text{ Int without inhibitor} \times 100$.

2.6. Statistical analysis

The agreement between IFAT-Nc and ELISA-Nc was assessed by calculating the proportion of observed agreement (P_0), the proportion of positive (P_{pos}) and negative (P_{neg}) agreement, and kappa (k) coefficient as previously reported (Cicchetti and Feinstein, 1990). Seropositivity percentages were compared using the chi-square (χ^2) test (GraphPad Prism 4.0, Graphpad Software, Inc). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1 Comparison between IFAT and ELISA for *N. caninum*

From 300 dog sera initially tested for IgG antibodies to *T. gondii*, 110 (36.7%) were positive in both IFAT-Tg and ELISA-Tg whereas 190 (63.3%) were negative for both tests. In the next step, the presence of IgG antibodies to *N. caninum* was investigated by IFAT-Nc and ELISA-Nc in both groups of *T. gondii* seropositive and seronegative dogs. From 300 serum samples tested, 105 (35%) were positive in ELISA-Nc, 32 (10.7%) in IFAT-Nc, being 29 (9.7%) positive in both tests (Table 1), resulting in a relatively good agreement ($P_o = 0.74$) between the tests, but with a low κ coefficient ($\kappa = 0.30$). When positive and negative agreement indices were separately analyzed, a poor concordance between IFAT-Nc and ELISA-Nc was found with regards to positive serological results ($P_{\text{pos}} = 0.42$), but a good concordance for negative results ($P_{\text{neg}} = 0.83$) was observed.

Seropositivity to *N. caninum* was then evaluated in relation to *T. gondii* serology (Fig. 1), showing significantly higher rates of seropositive samples for *N. caninum* in *T. gondii* seropositive than seronegative dogs in both IFAT-Nc (18.2%) and mostly in ELISA-Nc (55.4%) ($P < 0.001$).

3.2. Recognition of immunodominant antigens of *N. caninum* and *T. gondii*

Immunodominant antigens of *N. caninum* and *T. gondii* were evaluated using sera from both mouse and dog experimentally infected with either *N. caninum* or *T. gondii* probed in IB (Fig. 2). Both mouse and dog sera infected with *N. caninum* (Fig. 2, lanes 1 and 4, respectively) showed a similar antigenic profile, recognizing at least three immunodominant antigens (17, 29-32, and 35-37 kDa) of *N. caninum*, but *T. gondii* antigens with higher apparent molecular mass (80-85 kDa) were also recognized. Similarly, mouse and dog sera infected with *T. gondii* (Fig. 2, lanes 2 and 5, respectively) recognized at least three of its immunodominant antigens (22, 30 and 43 kDa), but also recognized *N. caninum* antigens above 45-50 kDa. Negative control sera (Fig. 2, lanes 3 and 6) showed weak or no reactivity to minor antigens of both parasites.

3.3. Comparison between IB, IFAT and ELISA for *N. caninum*

Immunoblotting for *N. caninum* (IB-Nc) was performed in a total of 200 dog sera selected on the following criteria: all serum samples with IFAT+/ELISA+ results (n=29), IFAT-/ELISA+ results (n=76), IFAT+/ ELISA- results (n=3) and 92 sera randomly selected among the 192 sera with IFAT-/ELISA- results (Table 2). From 29 sera with positive concordant results, 25 (86.2%) recognized at least two out of three *N. caninum* immunodominant antigens whereas only 2 (2.2%) from 92 negative concordant results and 3 (3.9%) from 76 discordant results were considered positive in IB-Nc.

Considering the reactivity for at least two out of three immunodominant antigens of *N. caninum* as a marker of infection, optimal cut off titers in IFAT-Nc and ELISA-Nc were determined by calculating the parameters of sensitivity and specificity of each test using the TG-ROC curve (Fig. 3). The intersection point of the two curves of IFAT (Fig. 3A) and ELISA (Fig. 3B) indicates the cut off point

at which equivalent sensitivity and specificity can be achieved. Thus, an optimization of cut off titers was obtained with a titer of 50 for IFAT-Nc and 200 for ELISA-Nc.

3.4. ELISA and IB inhibition

The degree of cross-reactivity between *N. caninum* and *T. gondii* antigens in dog sera was evaluated using ELISA inhibition (Fig. 4). ELISA-Nc inhibition curves showed a clear dose-dependent homologous (*N. caninum*) inhibition but also a partial heterologous (*T. gondii*) inhibition (Fig. 4A). In contrast, ELISA-Tg inhibition curves showed only homologous (*T. gondii*) dose-dependent inhibition with minor heterologous (*N. caninum*) inhibition (Fig. 4B). In addition, when positive sera to both *T. gondii* and *N. caninum* (Tg+Nc+) and positive to *N. caninum* only (Tg–Nc+) were individually assayed by ELISA-Nc inhibition assay, at maximal concentration (200 µg/mL) of inhibitor antigens, heterologous inhibition was significantly higher in Tg+Nc+ (25%) than Tg–Nc+ (5%) serum samples ($P < 0.05$), indicating some degree of cross-reactivity between *N. caninum* and *T. gondii* antigens (data not shown).

IB inhibition assays were performed to identify which cross-reactive antigens of *N. caninum* and *T. gondii* were recognized by serum samples containing IgG antibodies to both parasites (Figs. 5 and 6). A broad spectrum of *N. caninum* proteins ranging from 17 to 120 kDa were recognized by serum samples in the absence of inhibitor antigens (PBS) and showed a high percentage of inhibition with the homologous antigen, but only proteins of apparent molecular masses above 45–50 kDa showed some degree of *T. gondii* heterologous inhibition (Figs. 5A and 6A). In the same way, a high percentage of homologous inhibition was verified for the majority of proteins of *T. gondii* ranging from 22 to 116 kDa, and only antigens with apparent molecular masses above 43 kDa showed some degree of *N. caninum* heterologous inhibition (Figs. 5B and 6B). Immunodominant antigens from both parasites (below 35 kDa) showed very low levels of heterologous inhibition, indicating they were species-specific antigens.

4. Discussion

Diagnosis of *N. caninum* infection in dogs is mainly based on serological assays that have been used as important tools for both clinical and seroepidemiological investigations. However, the criteria used for serum samples to be considered positive are quite variable and depend on the investigator and on the kind of the assays performed. Different dilutions have been considered as positive in different tests, with IFAT titers ranging from 1:16 to 1:100 (Lasri et al., 2004; Pinheiro et al., 2005; Capelli et al., 2005) and ELISA titers ranging from 1:50 to 1:100 (Lasri et al., 2004; Pinheiro et al., 2005). So far, it has not been possible to make suitable comparisons among prevalence of antibody to *N. caninum* in dogs from these various studies because of the differences in sensitivity and specificity of the employed serological tests and the different cut offs established for each test (Björkman and Uggla, 1999). Therefore, no conclusive data have been presented to demonstrate which titers might

really be considered positive to *N. caninum* among various assays. In addition, low titers of antibodies to *N. caninum* can be the expression of cross-reactivity to related parasites, particularly *T. gondii*, due to common tachyzoite and bradizoyte antigens (Bjerkas et al. 1994; McAllister et al., 1996).

In the present study, we initially compared IFAT and ELISA results for *N. caninum* in dog sera, showing 74% agreement index between the tests, but with a low ($\kappa = 0.30$) kappa coefficient, indicating a poor concordance between these tests for *N. caninum* serology. However, when separate indices of agreement were analyzed, a good association was found regarding the negative agreement index ($P_{\text{neg}} = 0.83$) in contrast to the positive agreement ($P_{\text{pos}} = 0.42$) index. These results were similar to those reported by Lasri et al. (2004) who found a poor positive but good negative agreement between IFAT and ELISA for the serodiagnosis of *N. caninum* in dogs.

It is widely known that soluble antigen extracts used in ELISA contain a large number of antigens, particularly those from intracellular origin, whereas parasite surface membrane antigens are preferentially recognized in IFAT. Thus, IFAT has been considered a more species-specific test, although its sensitivity and specificity can be altered based on the established cut off values (Bjorkman and Ugglä, 1999). In canine neosporosis, the first described ELISA was an iscom ELISA (Bjorkman et al., 1994) that showed a high concordance with IFAT as the reference test, presenting 98% sensitivity and 96% specificity. Since then, several ELISAs using crude tachyzoite antigens have been developed and tested for *N. caninum* serology in dogs (Lasri et al., 2004; Pinheiro et al., 2005; Capelli et al., 2005), including a commercially available competitive ELISA (cELISA) (VMRD® Inc., Pullman, WA, USA), which was recently validated for canine neosporosis using IFAT as the reference test at cut off dilution of 1:16 (Capelli et al., 2005). Therefore, the great variation in cut off titers used for IFAT in different studies could be influencing on the performance of ELISA. For that reason, ELISA results for *N. caninum* should be interpreted with carefulness and, thus, additional or complementary tests, such as immunoblotting, should be performed in order to clarify the discordant results found by IFAT-Nc and ELISA-Nc.

In this context, we evaluated antigens of *N. caninum* that were recognized by sera from experimentally infected dogs with the parasite by immunoblotting, showing a strong recognition of three immunodominant antigens (17, 29-32, and 35-57 kDa). Interestingly, the great majority (86%) of sera with positive concordant (IFAT+/ELISA+) results recognized at least two out of these three antigens, particularly the 29-32 and 35-37 kDa bands, whereas sera with negative concordant (IFAT–/ELISA–) or discordant (IFAT–/ELISA+ or IFAT+/ELISA–) results recognized just one out of these three antigens, especially the 35-37 kDa bands. Recently, Pinheiro et al. (2005) reported that the 35-37 kDa bands of *N. caninum* were recognized by both positive and negative serum samples, indicating that other nonspecific antigens might be present in both groups of animals. Thus, optimization of cut off titers in IFAT-Nc and ELISA-Nc were determined by TG-ROC curve, considering the reactivity to at least two out of three immunodominant antigens of *N. caninum* as marker of infection, obtaining a titer of 50 for IFAT-Nc and 200 for ELISA-Nc. For IFAT, dilution of 1:50 has been recommended and

commonly used for canine sera in several studies (Azevedo et al., 2005; Pinheiro et al., 2005; Andreotti et al., 2005). However, the equivalent sensitivity and specificity values found for ELISA-Nc were lower than those obtained in IFAT-Nc, suggesting that the diversity of crude antigen preparations used in ELISA as well as the affinity characteristics of the secondary antibody used as conjugates might be influencing the performance of the test. A recent study comparing different methods for the solubilisation of *N. caninum* antigens showed that extraction with SDS and sulphobetaines gave higher yields and the best signal versus noise ratio when used in ELISA as compared to the standard sonication method (Zintl et al., 2005). Therefore, the optimal extraction method for parasite proteins depends on the intended application. In this context, Capelli et al. (2005) recently validated cELISA for canine neosporosis, suggesting its use to confirm clinical suspicion of the disease due to its good specificity (96%), but not indicated for seroepidemiological surveys because of the low sensitivity (47%). Thus, the use of this cELISA in canine population requires some techniques for adjustment of misclassification in prevalence and risk-factor studies (Capelli et al., 2005).

When seropositivity to *N. caninum* was evaluated in relation to *T. gondii* serology, a significantly higher number of *N. caninum*-seropositive dogs were found in *T. gondii*-seropositive dogs in both ELISA-Nc and IFAT-Nc tests, particularly for ELISA-Nc. Thus, the degree of cross-reactivity between both parasites was investigated by using inhibition assays, showing a partial heterologous inhibition curve in ELISA-Nc, indicating that antibodies to *N. caninum* were partially adsorbed with *T. gondii* antigens, reflecting in lower reactivity and consequently higher inhibition rates in ELISA-Nc. Such fact was particularly evident in *T. gondii*-seropositive samples, indicating that antibodies to *T. gondii* were also adsorbed with their specific antigens and as a result, lower reactivity and higher inhibition rates also occurred. These findings indicate that the ELISA-Nc here developed showed some degree of cross-reactivity between *N. caninum* and *T. gondii* antigens. Interestingly, inhibition curves for ELISA-Tg showed a clear homologous inhibition, but a minor heterologous inhibition, suggesting that antibodies to *T. gondii* were less adsorbed with *N. caninum* antigens, resulting in higher reactivity and lower inhibition rates in ELISA-Tg. A probable explanation for these findings could be the differential titers of antibodies to *N. caninum* and *T. gondii* in dog sera, with higher titers being found for the former and lower titers for the latter, or possible differences in the antibody affinity for the different antigens.

To identify which cross-reactive antigens of *N. caninum* and *T. gondii* were recognized by dog sera, immunoblot inhibition assays were performed by determining the band intensity in different conditions, with or without inhibitor antigens. A high homologous inhibition was seen for the large spectrum of antigenic bands of both parasites, but heterologous inhibition was more evident for proteins of higher molecular masses, above 45-50 kDa for *N. caninum* and 66 kDa for *T. gondii*. Accordingly, cross-reaction of *N. caninum* and *T. gondii* antigens has been previously reported in hyperimmune rabbit sera (Bjerkas et al., 1994), showing that *N. caninum* protein bands of 46, 88 and 97 kDa were labeled by both anti-*Neospora* and anti-*Toxoplasma* sera and likewise, *T. gondii* proteins

of 45, 66 and 85 kDa were recognized by both types of sera. A recent study has reported that several proteins with apparent molecular masses around 50-60 kDa were recognized by *N. caninum*- and *T. gondii*-negative dog sera, suggesting nonspecific recognition to unrelated organisms (Pinheiro et al., 2005). Also, cross-reactive antigens between *N. caninum* and *T. gondii* were recently identified and characterized by monoclonal antibodies produced against *N. caninum* (Liao et al., 2005), and consisted of proteins located on the surface of parasites (molecular masses from 28 to 76 kDa), on interior organelles (molecular masses of 35, 50 and 64 kDa) and on the apical end of parasites (unknown molecular masses). Three these proteins could be identified as the heat shock protein 70 (HSP70), the protein disulfide isomerase (PDI), and ribosomal protein 1 (RP1) (Liao et al., 2005). Proteomic analyses in two-dimensional electrophoresis (2-DE) immunoblotting using rabbit antiserum against *N. caninum* have also identified a number of homologue proteins, ranging from 41 to 76 kDa, between *N. caninum* and *T. gondii* tachyzoites, such as HSP70, PDI, tubulin α - and β -chains, actin and enolase, which were believed as common antigens between both parasites (Lee et al., 2005).

On the other hand, in our study, immunodominant antigens from both parasites, especially those below 35 kDa, were slightly inhibited by heterologous antigens, indicating that they are more species-specific antigens. Accordingly, Bjerkas et al. (1994) found that anti-*Toxoplasma* rabbit sera exhibited faint and inconsistent binding to the immunodominant (17, 29, and 35-37 kDa) *N. caninum* antigens and that proteins with apparent molecular masses between 17 and 37 kDa were recognized by sera from infected animals of several species, including dogs. On the 2-DE maps, proteins ranging from 26-33 kDa (NcGRA2) and from 31-40 kDa (NcGRA7), two proteins of *N. caninum* dense granules, exhibited low homology with the respective *T. gondii* dense granule proteins, suggesting as possible species-specific proteins of *N. caninum* (Lee et al., 2005).

Altogether, the results of our study showed that IFAT at 1:50 cut off dilution is the most appropriated technique for the serodiagnosis of *N. caninum* infection in dogs. Also, ELISA with different designs should be used critically, particularly when crude tachyzoite antigen preparations are employed, due to possible cross-reactivity with other related parasites as *T. gondii*. Therefore, we suggest that the *N. caninum* immunodominant antigens, especially the 17 and 29-32 kDa proteins, should be the selected candidates to develop serological diagnostic systems for *N. caninum* infection in dogs.

Acknowledgments

We thank Dr Solange M. Gennari for kindly providing the NC-1 strain of *N. caninum* and also Dr Mônica Camargo Sopenete and MSc Leandro Hideki Ynoue for helping with image analysis software. This study was supported by grants from the Brazilian funding agencies (CNPq, FAPEMIG and CAPES).

References

- Andreotti, R., Oliveira, J.M., Silva, E.A, Oshiro, L.M., Matos, M.F.C., 2006. Occurrence of *Neospora caninum* in dogs and its correlation with visceral leishmaniasis in the urban area of Campo Grande, Mato Grosso do Sul, Brazil. *Vet. Parasitol.* 135, 375-379.
- Azevedo, S.S., Batista, C.S.A., Vasconcellos, S.A., Aguiar, D.M., Ragozo, A.M.A., Rodrigues, A.A.R., Alves, C.J., Gennari, S.M., 2005. Seroepidemiology of *Toxoplasma gondii* and *Neospora caninum* in dogs from the state of Paraíba, Northeast region of Brazil. *Res. Vet. Sci.* 79, 51-56.
- Barber, J.S., Trees, A.J., 1998. Naturally occurring vertical transmission of *Neospora caninum* in dogs. *Int. J. Parasitol.* 28, 57-64.
- Bjerkas, I., Jenkins, M.C., Dubey, J.P., 1994. Identification and characterization of *Neospora caninum* tachyzoite antigens useful for diagnosis of neosporosis. *Clin. Diagn. Lab. Immunol.* 1, 214-221.
- Björkman, C., Holmdahl, O.J.M., Uggla, A., 1997. An indirect enzyme-linked immunoassay (ELISA) for demonstration of antibodies to *Neospora caninum* in serum and milk of cattle. *Vet. Parasitol.* 68, 251-260.
- Björkman, C., Lundén, A., Holmdahl, J., Barber, J., Trees, A.J., Uggla, A., 1994. *Neospora caninum* in dogs: detection of antibodies by ELISA using an iscom antigen. *Parasite Immunol.* 16, 643-648.
- Björkman, C., Uggla, A., 1999. Serological diagnosis of *Neospora caninum* infection. *Int. J. Parasitol.* 29, 1497-1507.
- Camargo, M.E., 1964. Improved technique of indirect immunofluorescence for serological diagnosis of toxoplasmosis. *Rev. Inst. Med. Trop. São Paulo* 12, 117-118.
- Capelli, G., Natale, A., Nardelli, S., Regalbono, A.F., Pietrobelli, M., 2006. Validation of a commercially available cELISA test for canine neosporosis against an indirect fluorescent antibody test (IFAT). *Prev. Vet. Med.* 73, 315-320.
- Cicchetti, D.V., Feinstein, A.R., 1990. High agreement but low kappa: II. Resolving the paradoxes. *J. Clin. Epidemiol.* 43, 551-558.
- Dubey, J.P., 2003. Review of *Neospora caninum* and neosporosis in animals. *Korean J. Parasitol.* 41, 1-16.
- Dubey, J.P., Lindsay, D.S., Adams, D.S., Gay, J.M., Baszler, T.V., Blagburn, B.L., Thulliez, P., 1996. Serological responses of cattle and other animals infected with *Neospora caninum*. *Am. J. Vet. Res.* 57, 329-336.
- Greiner, M., Sohr, D., Göbel, P., 1995. A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. *J. Immunol. Methods* 185, 123-132.
- Hemphill, A., Gottstein, B., 2000. A European perspective on *Neospora caninum*. *Int. J. Parasitol.* 30, 877-924.
- Howe, D.K., Crawford, A.C., Lindsay, D.S., Sibley, L.D., 1998. The p29 and p35 immunodominant antigens of *Neospora caninum* tachyzoites are homologous to the family of surface antigens of *Toxoplasma gondii*. *Infect. Immun.* 66, 5322-5328.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
- Lasri, S., De Meerschman, F., Rettigner, C., Focant, C., Losson, B., 2004. Comparison of three techniques for the serological diagnosis of *Neospora caninum* in the dog and their use for epidemiological studies. *Vet. Parasitol.* 123, 25-32.
- Lee, E.G., Kim, J.H., Shin, Y.S., Shin, G.W., Kim, Y.R., Palaksha, K.J., Kim, D.Y., Yamane, I. Kim, Y.H., Kim, G.S., Suh, M.D., Jung, T.S., 2005. Application of proteomics for comparison of proteome of *Neospora caninum* and *Toxoplasma gondii* tachyzoites. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 815, 305-314.
- Liao, M., Xuan, X., Huang, X., Shirafugi, H., Fukumoto, S., Hirata, H., Suzuki, H. Fujisaki, K., 2005. Identification and characterization of cross-reactive antigens from *Neospora caninum* and *Toxoplasma gondii*. *Parasitology* 130, 481-488.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275.
- McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., McGuire, A.M., 1998. Dogs are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* 28, 1473-1478.

- McAllister, M.M., Parmley, S.F., Weiss, L.M., Welch, V.J., McGuire, A.M., 1996. An immunohistochemical method for detecting bradyzoite antigen (BAG5) in *Toxoplasma gondii*-infected tissues cross-reacts with a *Neospora caninum* bradizoyte antigen. *J. Parasitol.* 82, 354-355.
- Mineo, J.R., Camargo, M.E., Ferreira, A.W., 1980. Enzyme-linked immunosorbent assay for antibodies to *Toxoplasma gondii* polysaccharides in human toxoplasmosis. *Infect. Immun.* 27, 283-287.
- Mineo, T.W.P., Silva, D.A.O., Costa, G.H.N., von Ancken, A.C.B., Kasper, L.H., Souza, M.A., Cabral, D.D., Costa, A.J., Mineo, J.R., 2001. Detection of IgG antibodies to *Neospora caninum* and *Toxoplasma gondii* in dogs examined in a veterinary hospital from Brazil. *Vet. Parasitol.* 98, 239-245.
- Moore, D.P., 2005. Neosporosis in South America. *Vet. Parasitol.* 127, 87-97.
- Paré, J., Fecteau, G., Fortin, M., Marsolais, G., 1998. Seroepidemiologic study of *Neospora caninum* in dairy herds. *J. Am. Vet. Med. Assoc.* 213, 1595-1598.
- Pereira, E.A.L., Silva, D.A.O., Cunha-Junior, J.P., Almeida, K.C., Alves, R., Sung, S.J., Taketomi, E.A., 2005. IgE, IgG1, and IgG4 antibody responses to *Blomia tropicalis* in atopic patients. *Allergy* 60, 401-406.
- Perlé, K.M.D., Del Piero, F., Carr, R.F., Harris, C., Stromberg, P.C., 2001. Cutaneous neosporosis in two adult dogs on chronic immunosuppressive therapy. *J. Vet. Diagn. Invest.* 13, 252-255.
- Pinheiro, A.M., Costa, M.F., Paule, B., Vale, V., Ribeiro, M., Nascimento, I., Schaer, R.E., Almeida, M.A.O., Meyer, R., Freire, S.M., 2005. Serologic immunoreactivity to *Neospora caninum* antigens in dogs determined by indirect immunofluorescence, western blotting and dot-ELISA. *Vet. Parasitol.* 130, 73-79.
- Puerta, L., Lagares, A., Mercado, D., Fernández-Caldas, E., Caraballo, L., 2005. Allergenic composition of the mite *Suidasia medanensis* and cross-reactivity with *Blomia tropicalis*. *Allergy* 60, 41-47.
- Scott, P., Pearce, E., Natovitz, P., Sher, A., 1987. Vaccination against cutaneous leishmaniasis in murine model. I. Induction of protective immunity with a soluble extract of promastigotes. *J. Immunol.* 139, 221-227.
- Silva, D.A.O., Silva, N.M., Mineo, T.W.P., Pajuaba Neto, A.A., Ferro, E.A., Mineo, J.R., 2002a. Heterologous antibodies to evaluate the kinetics of the humoral immune response in dogs experimentally infected with *Toxoplasma gondii* RH strain. *Vet. Parasitol.* 107, 181-195.
- Silva, N.M., Lourenço, E.V., Silva, D.A.O., Mineo, J.R., 2002b. Optimisation of cut-off titres in *Toxoplasma gondii* specific ELISA and IFAT in dog sera using immunoreactivity to SAG-1 antigen as a molecular marker of infection. *Vet. J.* 163, 94-98.
- Speer, C.A., Reduker, D.W., Burgess, D.E., Whitmire, W.M., Splitter, G.A., 1985. Lymphokine-induced inhibition of growth of *Eimeria bovis* and *Eimeria papillata* (Apicomplexa) in cultured bovine monocytes. *Infect. Immun.* 50, 566-571.
- Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci.* 76, 4350-4356.
- Wilson M.B., Nakane P.K., 1978. Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In: Knapp W., Holubar K., Wick G. (Eds.), *Immunofluorescence and related staining techniques*. Elsevier North Holland Biomedical Press, Amsterdam, pp. 215-224.
- Zintl, A., Pennington, S.R., Mulcahy, G., 2006. Comparison of different methods for the solubilisation of *Neospora caninum* (Phylum Apicomplexa) antigen. *Vet. Parasitol.* 135, 205-213.

Table 1. Comparison between IFAT and ELISA results for the detection of IgG antibodies to *N. caninum* in dog sera.

<i>ELISA</i>	<i>IFAT</i>		<i>Total</i> n (%)
	<i>Positive</i> n (%)	<i>Negative</i> n (%)	
<i>Positive</i>	29 (9.7)	76 (25.3)	105 (35)
<i>Negative</i>	3 (1.0)	192 (64.0)	195 (65)
<i>Total</i>	32 (10.7)	268 (89.3)	300 (100)

Proportion of observed agreement (P_o) = 0.74; Proportion of positive agreement (P_{pos}) = 0.42; Proportion of negative agreement (P_{neg}) = 0.83; *Kappa* (κ) coefficient = 0.30.

Table 2. Reactivity to *Neospora caninum* immunodominant antigens in immunoblotting (IB) of dog sera with concordant or discordant results in IFAT and ELISA for IgG antibodies to *N. caninum*.

<i>Results</i>	No. of serum samples	No. (%) of positive sera in IB ^a	Reactivity to <i>N. caninum</i> immunodominant antigens ^b		
			1	2	3
IFAT+ / ELISA+	29	25 (86.2%)	4	10	15
IFAT– / ELISA–	92	2 (2.2%)	10	2	0
IFAT– / ELISA+	76	3 (3.9%)	29	3	0
IFAT+ / ELISA–	3	0 (0%)	3	0	0
Total	200	30 (15%)	46	15	15

^aSera were considered positive in IB when at least 2 from 3 immunodominant antigens were recognized.

^bNumber of sera reactive to at least 1, 2 or 3 immunodominant antigens (17, 29-32, and 35-37 kDa) of *N. caninum*.

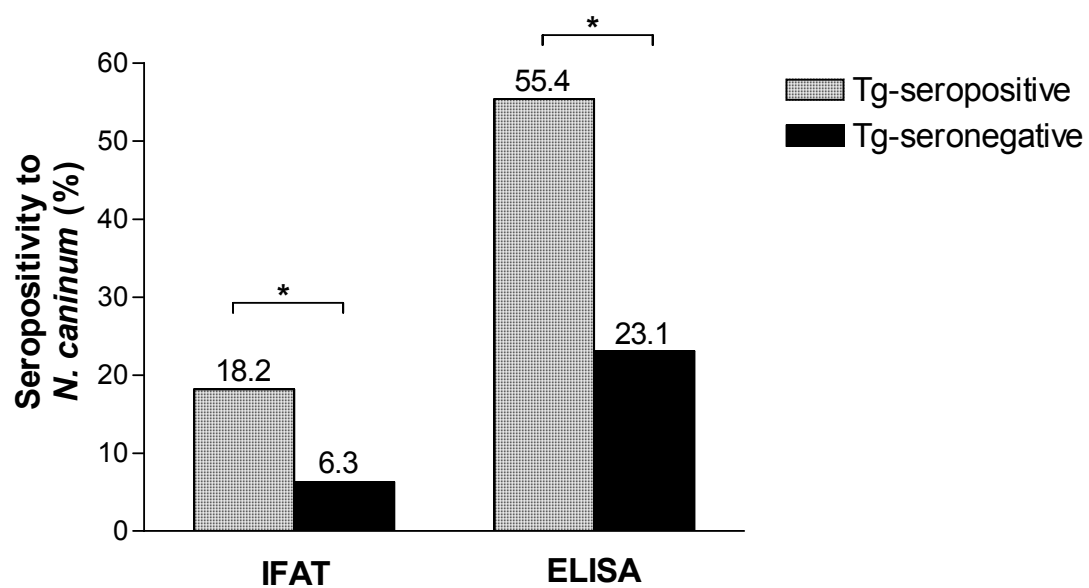


Fig. 1. Serological results obtained by IFAT and ELISA for the detection of IgG antibodies to *Neospora caninum* in *Toxoplasma gondii* seropositive and seronegative dogs (* $P < 0.05$).

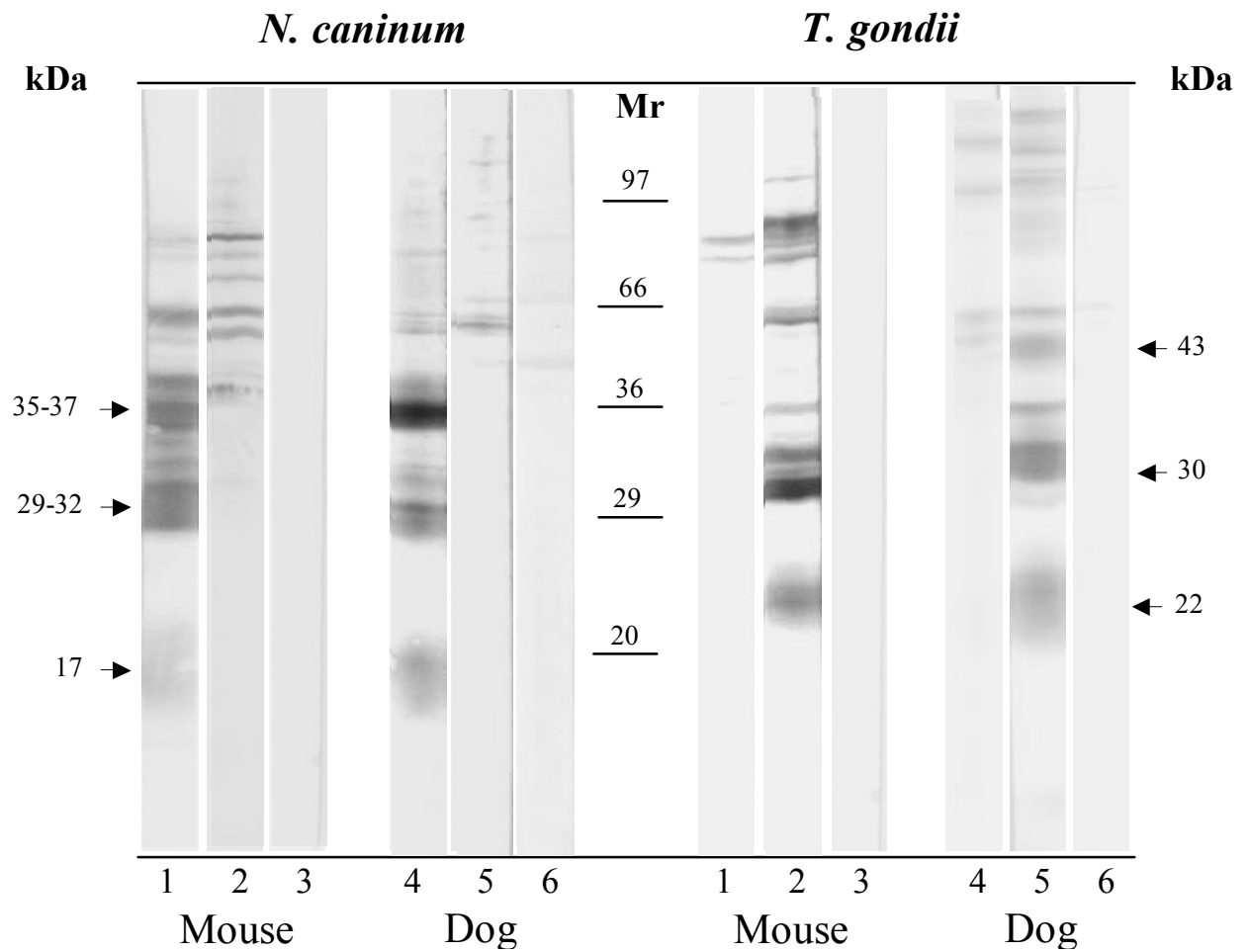


Fig. 2. Immunoblotting of *Neospora caninum* and *Toxoplasma gondii* proteins separated by 12% SDS-PAGE under nonreducing conditions probed with sera from a mouse and a dog experimentally infected with *N. caninum* (lanes 1 and 4) or *T. gondii* (lanes 2 and 5) and respective mouse and dog negative control sera (lanes 3 and 6). Molecular mass markers (Mr) and immunodominant antigens (arrows) of *N. caninum* and *T. gondii* are indicated in kilodaltons.

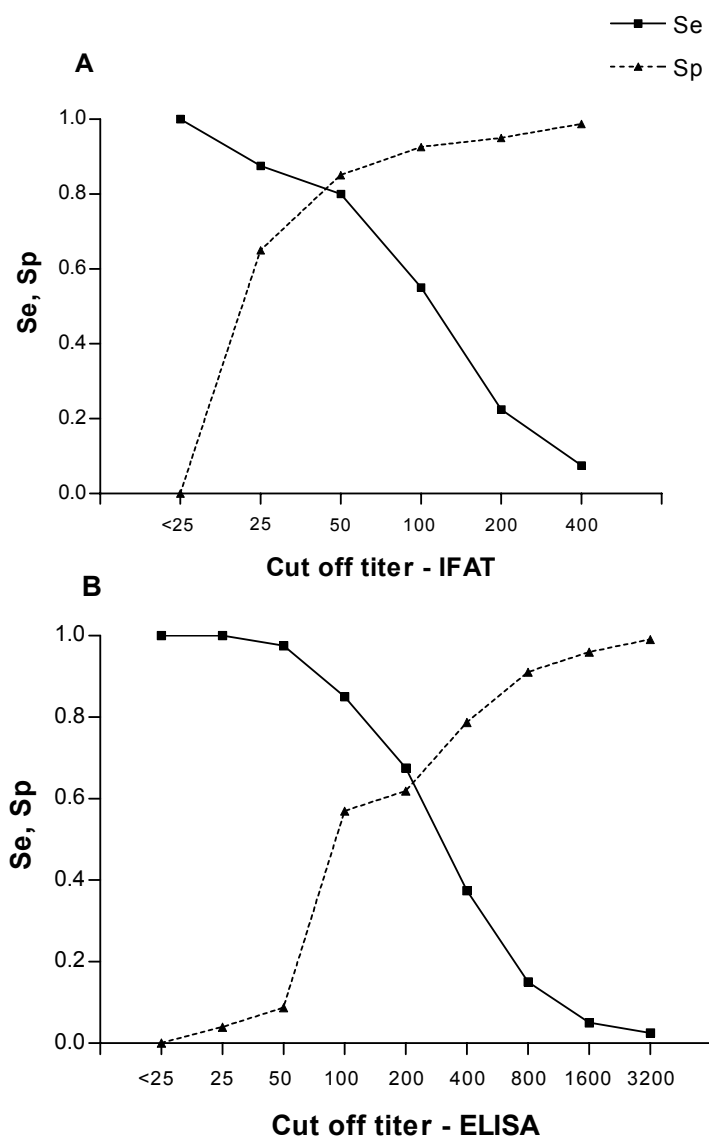


Fig. 3. TG-ROC curve of IFAT (**A**) and ELISA (**B**) to detect IgG antibodies to *Neospora caninum* in dog sera. Sensitivity (Se) and Specificity (Sp) of IFAT and ELISA were determined for each cut off titer, considering the reactivity to at least two out of three (17, 29-32 and 35-37 kDa) immunodominant antigens of *N. caninum* as marker of infection. The intersection point of the two curves (Se, Sp) of each test indicates the cut off point at which equivalent sensitivity and specificity can be achieved.

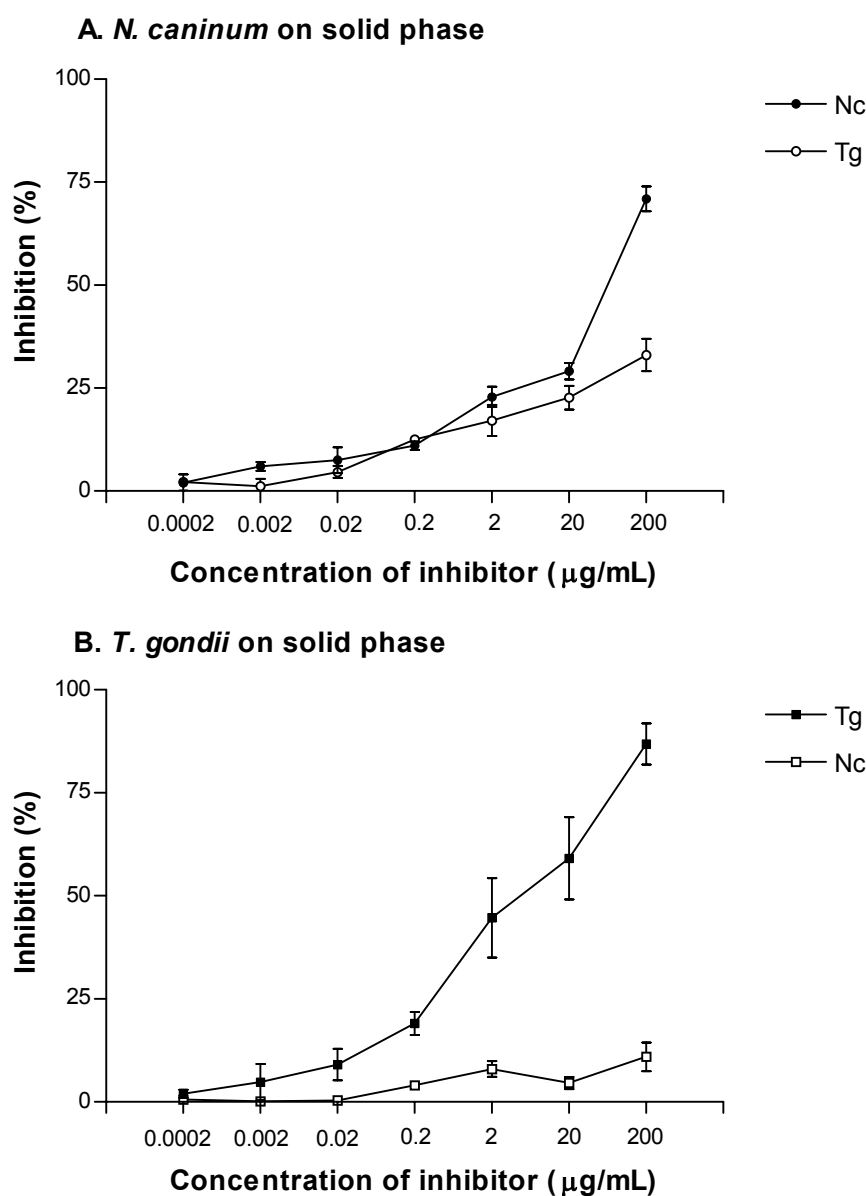


Fig. 4. ELISA inhibition curves with *Neospora caninum* (A) and *Toxoplasma gondii* (B) on solid phase. Dog sera with IgG antibodies to both parasites were pre-adsorbed with different concentrations of *N. caninum* (Nc) or *T. gondii* (Tg) inhibitor antigens. Data represent the mean plus 95% confidence interval of six dog sera with IgG antibodies to both parasites.

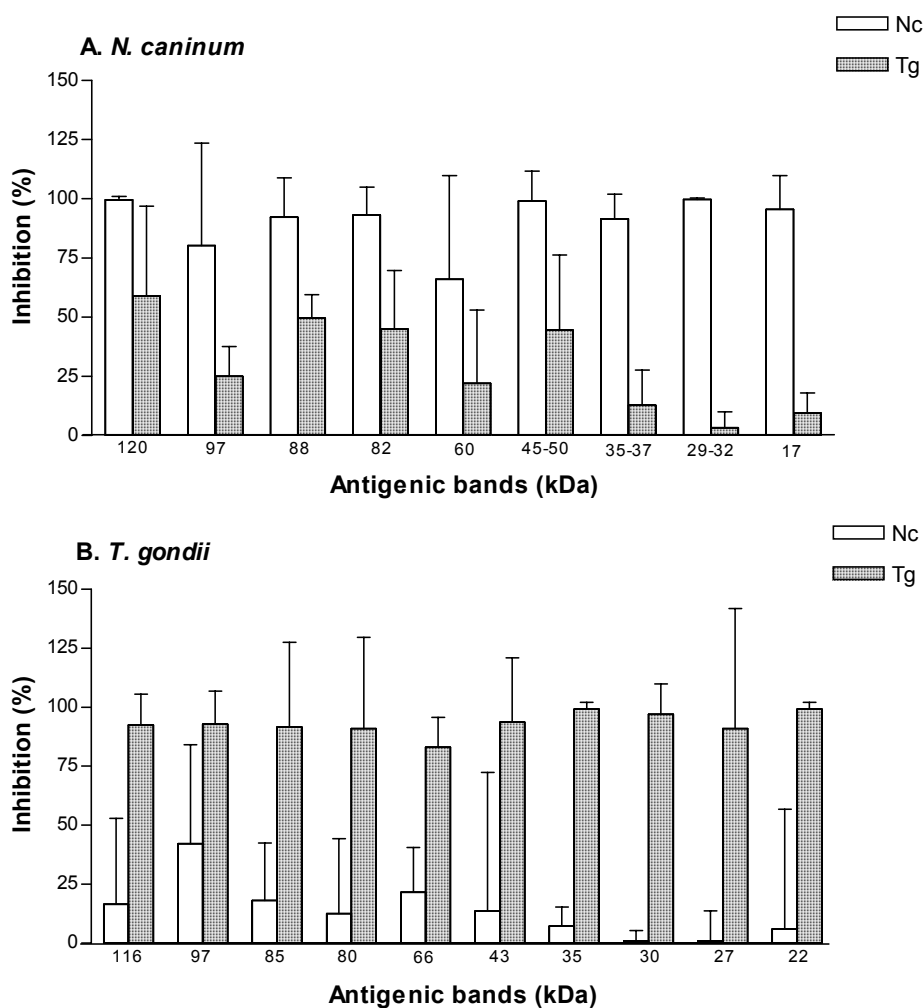


Fig. 5. Inhibition (%) of antigenic bands of *Neospora caninum* (A) and *Toxoplasma gondii* (B) recognized by dog sera when pretreated with *N. caninum* (Nc) or *T. gondii* (Tg) inhibitor antigens at maximal concentration of 200 µg/mL. Data represent the mean plus 95% confidence interval of six dog sera with IgG antibodies to both parasites.

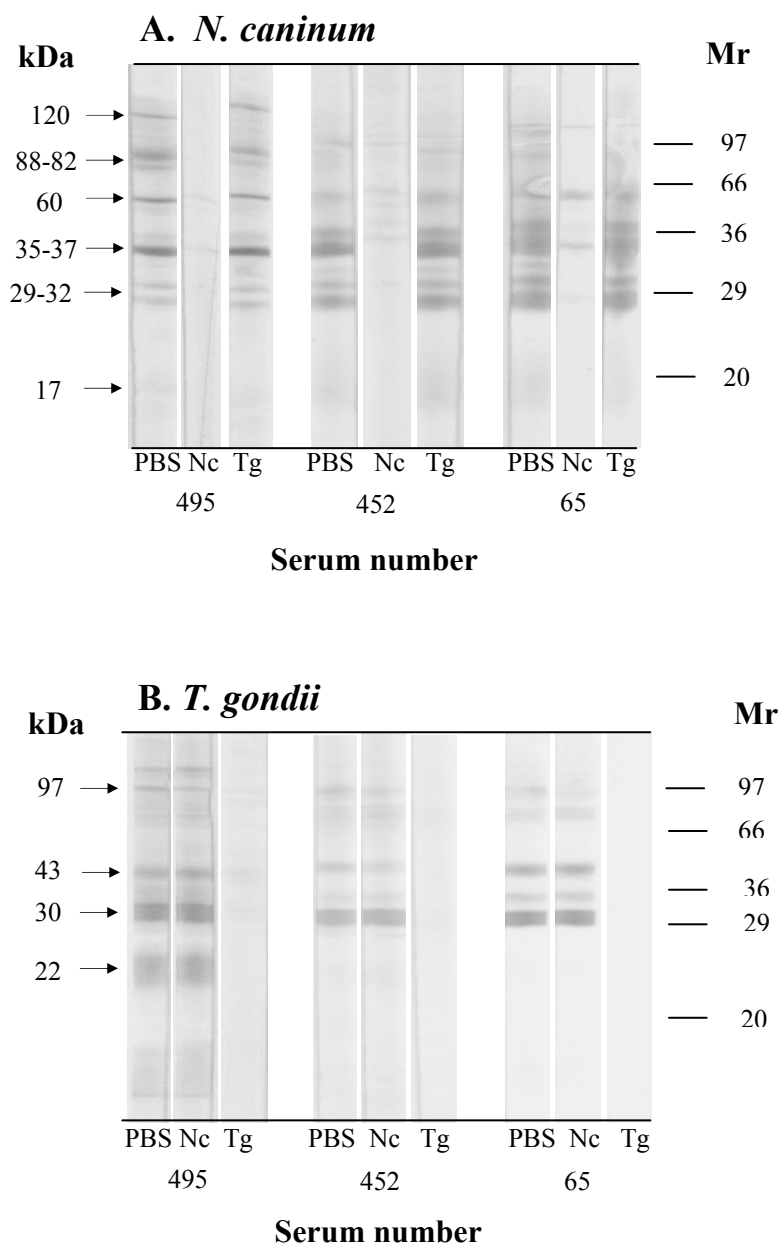


Fig. 6. Immunoblot inhibition of *Neospora caninum* (A) and *Toxoplasma gondii* (B) antigens recognized by three representative sera from dogs with IgG antibodies to both parasites. Sera were pretreated with *N. caninum* (Nc) or *T. gondii* (Tg) inhibitor antigens or with diluent only (PBS). Molecular mass markers (Mr) and immunodominant antigens (arrows) of *N. caninum* and *T. gondii* are indicated in kilodaltons.

4.5 Estudo V

Evaluation of homologous, heterologous and affinity conjugates for the serodiagnosis of *Toxoplasma gondii* and *Neospora caninum* in maned wolves (*Chrysocyon brachyurus*)

Um crescente número de estudos tem sido realizado sobre a prevalência da infecção por *T. gondii* e *N. caninum* em animais silvestres, especialmente após a descoberta que coiotes são também hospedeiros definitivos para *N. caninum* e a existência de um ciclo silvestre para este parasito (GONDIM et al., 2004 a,c).

O lobo-guará (*Chrysocyon brachyurus*) é considerado o maior canídeo silvestre da América do Sul e encontra-se em extinção em seu habitat natural devido a vários fatores, incluindo predação e aquisição de doenças infecciosas. Como dados sobre a prevalência da infecção por *T. gondii* e *N. caninum* nesta espécie não eram conhecidos, em um estudo prévio (VITALIANO et al., 2004), nós investigamos a ocorrência de anticorpos anti-*T. gondii* e anti-*N. caninum* em amostras de soros de 59 lobos-guará procedentes de 6 zoológicos e uma reserva ecológica das regiões sudeste e centro-oeste do Brasil. Soros foram submetidos à sorologia para *T. gondii* (ELISA) e *N. caninum* (IFAT) e, em ambos testes, um conjugado heterólogo anti-IgG de cão foi utilizado, marcado com peroxidase (ELISA) ou isotiocianato de fluoresceína (FITC). Os resultados mostraram uma maior soropositividade (74,6%) para *T. gondii* que para *N. caninum* (8,5%), confirmando os relatos sobre a maior soroprevalência de *T. gondii* em relação a *N. caninum* em várias espécies de canídeos silvestres (JAKUBEK et al., 2001; LINDSAY; WESTON; LITTLE, 2001; WOLFE et al., 2001). Não houve diferença significativa na soropositividade entre os diferentes zoológicos nem em relação ao sexo, para ambos parasitos. Entretanto, a soropositividade para *T. gondii* foi fortemente relacionada com a idade, com taxas significativamente maiores entre os lobos adultos (91,7%) quando comparado a animais jovens (33,3%). Embora a soropositividade para *N. caninum* foi detectada somente em lobos adultos (12,5%), nenhuma relação significativa com a idade pôde ser demonstrada.

Uma das principais limitações no diagnóstico sorológico de doenças infecciosas em animais silvestres é a complexidade para a obtenção de reagentes espécie-específicos de qualidade, tornando difícil fazer estimativas e avaliações de soroprevalências para *T. gondii* e *N. caninum* em animais silvestres. A utilização de anticorpos heterólogos ou conjugados de afinidade tem mostrado ser de grande valor para o sorodiagnóstico de *T. gondii* (SILVA et al., 1997; MINEO et al., 2001; SILVA et al., 2002) e *N. caninum* (BARBER et al., 1997a; MINEO et al., 2001) tanto em animais domésticos como silvestres.

No estudo V, nós avaliamos três conjugados: (1) homólogo (anti-IgG de lobo), (2) heterólogo (anti-IgG de cão) e de afinidade (proteína A) marcados com peroxidase (ELISA) ou isotiocianato de fluoresceína (IFAT) para o sorodiagnóstico de *T. gondii* e *N. caninum* em lobos-guará. Os resultados mostraram que a soropositividade para *T. gondii* foi comparável entre os conjugados homólogo (69,5%), heterólogo (74,6%) e de afinidade (71,2%) em ELISA (Tabela 1), com uma alta e significativa correlação positiva entre os níveis de anticorpos detectados pelos três testes (Fig. 2), indicando que os três conjugados enzimáticos são úteis na detecção de anticorpos IgG anti-*T. gondii* em soros de lobo-guará. Entretanto, os mais altos níveis médios de anticorpos (EI = 4,5) foram encontrados para o conjugado de afinidade proteína A-peroxidase (Fig. 1), provavelmente devido aos menores valores de reatividade inespecífica detectada em soros controles negativos quando comparado às reatividades inespecíficas produzidas pelos conjugados homólogo e heterólogo. Como resultado, menores valores de *cut off* são obtidos para o conjugado de afinidade e, conseqüentemente, maiores valores de índice ELISA (IE) são obtidos como determinado pela divisão da $DO_{amostra}$ pela $DO_{cut\ off}$. Nossos estudos anteriores também têm destacado o uso com sucesso de proteína A-peroxidase em ELISA para a detecção de IgG anti-*T. gondii* em soros de cães (SILVA et al., 1997; MINEO et al., 2001; SILVA et al., 2002).

Analizando os três conjugados fluorescentes em IFAT para detecção de anticorpos IgG anti-*N. caninum* em soros de lobo-guará, a mesma soropositividade foi obtida com os conjugados homólogo e heterólogo (8,5%), com baixos títulos de anticorpos (25 a 100), mostrando variações no título em somente uma diluição entre os conjugados. Estes resultados reforçam o uso efetivo de anticorpos heterólogos em IFAT para inquéritos soroepidemiológicos de *N. caninum* em diferentes populações de canídeos (BARBER et al., 1997a). Surpreendentemente, o conjugado de afinidade proteína A-FITC não foi capaz de detectar ou confirmar as amostras de soros consideradas positivas pelos conjugados homólogo e heterólogo, mesmo na mais baixa diluição (1:10) do conjugado de afinidade. Assim, proteína A-FITC não mostrou ser uma ferramenta útil para o sorodiagnóstico de *N. caninum* em lobos-guará, em contraste ao seu uso com sucesso para detectar IgG anti-*T. gondii* nesta espécie (dados não mostrados). Estes achados podem ser explicados pelos mais baixos títulos de anticorpos que freqüentemente são encontrados na infecção por *N. caninum* que para *T. gondii* em canídeos silvestres (LINDSAY; WESTON; LITTLE, 2001; VITALIANO et al., 2004) como também por características particulares de afinidade desta matriz (proteína A de *Staphylococcus*) para diferentes isotipos de imunoglobulinas, especialmente, as subclasses de IgG.

Os resultados do estudo V demonstraram que conjugados homólogos, heterólogos e de afinidade podem ser usados como métodos sensíveis e específicos para determinar a soroprevalência de *T. gondii* em canídeos silvestres, enquanto para *N. caninum*, somente os conjugados homólogos e heterólogos foram úteis para este propósito, contribuindo assim para aprimorar procedimentos de manejo e preservação do lobo-guará em cativeiro. Além disso, a presença de anticorpos anti-*N. caninum* foi demonstrada, pela primeira vez, em canídeos silvestres da América do Sul (VITALIANO et al., 2004), em contraste aos achados de Melo e colaboradores (2002) que também investigaram a ocorrência de anticorpos anti-*N. caninum* em lobos-guará da mesma região, mas nenhuma amostra soropositiva foi detectada. Os resultados destes estudos refletem a grande exposição desta espécie a *T. gondii* e, em menor grau, a *N. caninum* e apontam para a importância destes canídeos silvestres na epidemiologia da neosporose, quer como hospedeiros intermediários naturais ou como potenciais hospedeiros definitivos de *N. caninum*.

EVALUATION OF HOMOLOGOUS, HETEROLOGOUS, AND AFFINITY CONJUGATES FOR THE SERODIAGNOSIS OF *TOXOPLASMA GONDII* AND *NEOSPORA CANINUM* IN MANED WOLVES (*CHRYSOCYON BRACHYURUS*)

D. A. O. Silva, S. N. Vitaliano, T. W. P. Mineo*, R. A. Ferreira†, E. Bevilacqua‡, and J. R. Mineo#

Laboratory of Immunoparasitology, Universidade Federal de Uberlândia, Av. Pará 1720, Campus Umuarama, CEP 38400-902, Uberlândia, MG, Brasil. e-mail: jrmineo@ufu.br

ABSTRACT: Use of serological tests in the diagnosis of infectious diseases in wild animals has several limitations, primarily the difficulty of obtaining species-specific reagents. Wild canids, such as maned wolves (*Chrysocyon brachyurus*), are highly predisposed to infection by *Toxoplasma gondii* and, to a lesser extent, to *Neospora caninum*. The aim of the present study was to evaluate homologous, heterologous, and affinity conjugates in enzyme-linked immunosorbent assays (ELISAs) and indirect fluorescent antibody tests (IFATs) for detecting immunoglobulin (Ig) G antibodies against *T. gondii* and *N. caninum* in maned wolves. Serum samples were obtained from 59 captive animals in Brazil and tested by ELISA for *T. gondii* serology and IFAT for *N. caninum* serology using 3 different enzymatic and fluorescent conjugates: homologous (guinea pig anti-maned wolf IgG–peroxidase and –fluorescein isothiocyanate [FITC]), heterologous (rabbit anti-dog IgG–peroxidase and –FITC), and affinity (protein A–peroxidase and –FITC). Seropositivity to *T. gondii* was comparable among the homologous (69.5%), heterologous (74.6%), and affinity (71.2%) enzymatic conjugates. A significant positive correlation was found between the antibody levels determined by the 3 enzymatic conjugates. The highest mean antibody levels (ELISA index = 4.5) were observed with the protein A–peroxidase conjugate. The same seropositivity to *N. caninum* (8.5%) was found with the homologous and heterologous fluorescent conjugates, but protein A–FITC was not able to detect or confirm any positive samples with homologous or heterologous conjugates. Our results demonstrate that homologous, heterologous, and affinity conjugates might be used in ELISA for serological assays of *T. gondii* in wild canids, whereas for *N. caninum* infection, only the homologous or heterologous fluorescent conjugates have been shown to be useful.

Toxoplasma gondii and *Neospora caninum* are apicomplexan parasites with worldwide distribution, using felids and some canids (domestic dogs and coyotes) as definitive hosts, respectively. A wide range of domestic and wild animal species may act as intermediate hosts for these parasites (Dubey and Beattie, 1988; Buxton et al., 1997; Dubey et al., 1999).

Wild canids are highly predisposed to infection by these parasites not only because of their carnivorous habits, which facilitate ingestion of cyst-positive tissues, but also because of close contact with soil contaminated by sporulated oocysts (Zarnke et al., 2000; Lindsay et al., 2001; Wolfe et al., 2001; Almería et al., 2002). Although maned wolves (*Chrysocyon brachyurus*), the largest wild canid in South America, are omnivores, the high seroprevalence of these animals to *T. gondii* and, in lower extension, to *N. caninum* (Vitaliano et al., 2004) have been reported recently.

Maned wolves are an endangered species in their natural habitat, the Brazilian savannah, because of various factors: predation, loss of natural habitat, and introduced infectious diseases. Thus, a worldwide effort to preserve this species in zoos or ecological reserve areas is meaningful. However, inappropriate nutrition and management conditions associated with other stress conditions in captivity may be critical to the acquisition of infectious diseases. In this context, several studies have shown that *T. gondii* may cause not only infection but also clinical disease in wildlife species, such as foxes (Dubey et al., 1990; Dubey and Liu, 1994), which also are natural interme-

diate hosts for *N. caninum* (Almería et al., 2002). Therefore, wild canids could be involved in a sylvatic cycle of these parasites and play an important role in the epizootiology of these coccidiosis in wild mammal populations. Accordingly, Gondim et al. (2004) demonstrated recently that coyotes are a second definitive host of *N. caninum*, after dogs, and represent a potential risk in transmission to ruminant livestock.

Use of serological tests for the immunodiagnosis of infectious diseases in wild animals has several limitations. One is the difficulty in obtaining species-specific reagents. Because of the lack of data and reagents, it is difficult to obtain and compare prevalence of *T. gondii* and *N. caninum* infections in wildlife. The use of heterologous and affinity conjugates in enzyme-linked immunosorbent assays (ELISAs) or indirect fluorescent antibody tests (IFATs) may represent a new tool to help in understanding the sylvatic cycles of these parasites.

The aim of the present study was to evaluate homologous, heterologous, and affinity conjugates in ELISA and IFAT for the detection of immunoglobulin (Ig) G antibodies to *T. gondii* and *N. caninum* in maned wolves from Brazil.

MATERIALS AND METHODS

Animals

In total, 59 serum samples were studied from captive maned wolves (*C. brachyurus*) collected between 1992 and 2002 from 6 zoos and 1 ecological reserve of the southeastern and midwestern regions of Brazil. The epidemiological data from these samples have been presented previously (Vitaliano et al., 2004). Blood samples were obtained by cephalic or jugular venipuncture. Sera were separated after centrifugation at 300 g for 10 min and stored at –20 °C until analysis.

Parasites and antigens

Toxoplasma gondii tachyzoites (RH strain) were maintained by serial passage in Swiss mice every 48–72 hr as described previously (Mineo et al., 1980). Briefly, parasites were obtained from mouse peritoneal exudates, washed 3 times by centrifugation at 1,000 g for 10 min at 4 °C with phosphate-buffered saline (PBS), and then submitted to 3 freeze–thaw cycles and 6 sonication cycles. After centrifugation at 10,000 g

Received 24 November 2005; revised 14 January 2005; accepted 1 February 2005.

* Laboratory of Immunoparasitology, Faculdade de Ciências Agrárias e Veterinárias, (FCAV/UNESP), Campus de Jaboticabal, CEP 13884-900, Jaboticabal, SP, Brasil.

† Laboratory of Parasitology, Universidade Federal de Minas Gerais, CEP 31270-901, Belo Horizonte, MG, Brasil.

‡ Secretaria do Meio Ambiente, Prefeitura de Uberlândia, CEP 38408-900, Uberlândia, MG, Brasil.

TABLE I. Seropositivity of *Toxoplasma gondii* in 59 captive maned wolves (*Chrysocyon brachyurus*) from 7 different locales (zoos or an ecological reserve) of the southeastern and midwestern regions of Brazil using homologous, heterologous, or affinity conjugates in enzyme-linked immunosorbent assays.

Origin*	No. of animals	Seropositivity (%)†		
		Homologous (anti-wolf IgG-HRPO)	Heterologous (anti-dog IgG-HRPO)	Affinity (protein A-HRPO)
Brasília-DF	7	7 (100%)	7 (100%)	7 (100%)
São Paulo-SP	6	6 (100%)	6 (100%)	6 (100%)
Bauru-SP	4	2 (50%)	3 (75%)	2 (50%)
Uberaba-MG	3	3 (100%)	3 (100%)	3 (100%)
Uberlândia-MG	8	4 (50%)	4 (50%)	4 (50%)
Belo Horizonte-MG	24	19 (79.2%)	21 (87.5%)	20 (83.4%)
Araxá-MG	7	0 (0%)	0 (0%)	0 (0%)
Total	59	41 (69.5%)	44 (74.6%)	42 (71.2%)

* Captive maned wolves originated from zoos except for those from Araxá-MG (ecological reserve).

†: Homologous, heterologous, and affinity conjugates were labeled with horseradish peroxidase (HRPO). Ig, immunoglobulin.

for 30 min at 4 °C, the supernatant was collected, and the protein concentration was determined (Lowry et al., 1951). Antigen aliquots were stored at -20 °C until used as soluble antigen in ELISA.

Neospora caninum tachyzoites (NC-1 strain) were cultured in bovine monocytes and harvested by scraping off the cell monolayer 2–3 days after infection. Antigen slides were prepared according to the method described by Camargo (1964). In brief, parasites were washed 3 times by centrifugation at 1,000 g at 4 °C for 10 min with PBS and then incubated with gentle rotation in 1% formaldehyde for 30 min at room temperature. After a new washing cycle, parasites were homogenized in distilled water up to an adjusted concentration of approximately 20 tachyzoites/field at ×40 magnification. Antigen slides were wrapped and stored at -20 °C until IFAT testing.

Enzymatic or fluorescent conjugates

Three different conjugates (homologous, heterologous, and affinity) were analyzed using ELISA and IFAT. The homologous (guinea pig anti-maned wolf IgG) and heterologous (rabbit anti-dog IgG) antisera were labeled with horseradish peroxidase (HRPO) as described by Wilson and Nakane (1978) or with fluorescein isothiocyanate (FITC) as described by Clark and Shepard (1963). The affinity conjugate consisted of protein A labeled with HRPO and FITC for ELISA and IFAT, respectively (Sigma Chemical Co., St Louis, Missouri).

Serological assays

Enzyme-linked immunosorbent assay: Indirect ELISA was carried out with minor modifications for the detection of IgG antibodies to *T. gondii* as described by Silva et al. (1997). Optimization of the reaction was determined in preliminary experiments through block titration of the reagents (antigen, sera, and conjugates). Briefly, polystyrene microtiter plates were coated overnight at 4 °C with *T. gondii* soluble antigen (10 µg/ml) diluted in 0.06 M carbonate buffer (pH 9.6). After washing with PBS containing 0.05% Tween 20 (PBS-T), plates were incubated with maned wolf serum samples diluted 1:64 in PBS-T plus 5% nonfat milk (PBS-TM) for 45 min at 37 °C. After a new washing cycle, plates were incubated with the 3 different enzymatic conjugates: (1) homologous (i.e., guinea pig anti-maned wolf IgG-HRPO diluted 1:50 in PBS-TM), (2) heterologous (i.e., rabbit anti-dog IgG-HRPO diluted 1:3,000 in PBS-TM), and (3) affinity (i.e., protein A-HRPO diluted 1:25,000 in PBS-TM). After incubation for 45 min at 37 °C, plates were again washed and incubated with enzyme substrate consisting of 0.03% H₂O₂ (Sigma) and ortho-phenylenediamine (Merck, Darmstadt, Germany) in 0.1 M citrate-phosphate buffer (pH 5.0) for 15 min at room temperature. The reaction was stopped by adding 2 N H₂SO₄; the optical density (OD) was read at 492 nm using a plate reader (Titertek Multiskan Plus; Flow Laboratories, Helsinki, Finland). Positive- and negative-control sera were obtained from dogs with previously determined serology and included in each assay. Antibody levels were arbitrarily expressed as the ELISA reactivity index (EI) according to the following formula: EI

= OD sample/OD cutoff, where the cutoff was calculated as the mean OD values from negative controls plus 3 standard deviations. Samples were considered seropositive when EI > 1.0.

Indirect fluorescent antibody tests: Serology for *N. caninum* was carried out by IFAT, with some modifications, as described previously (Minea et al., 2001). Antigen slides were incubated with maned wolf serum samples (1:25 and 1:50) and then with the 3 different fluorescent conjugates: (1) homologous (i.e., guinea pig anti-maned wolf IgG-FITC diluted 1:100 in PBS), (2) heterologous (i.e., rabbit anti-dog IgG-FITC diluted 1:50 in PBS), and (3) affinity (i.e., protein A-FITC diluted 1:10 in PBS). Positive- and negative-control sera were obtained from dogs with previously determined serology as in the ELISA assays. Cutoff titers were established at 1:25 dilution, and positive results were considered only when a bright, linear, peripheral fluorescence of the tachyzoites was observed.

Statistical analysis

Statistical analysis consisted of determinations of means with the 95% confidence interval (CI) of antibody levels (expressed in EI) in serum samples, and the differences between the means were examined by analysis of variance using Tukey's multiple comparison (GraphPad Prism 3.0; GraphPad Software, Inc., San Diego, California) as a post-hoc test. Percentages of seropositivity found for each assay were compared by the chi-square test with the Yates correction. The correlation coefficients between the antibody levels obtained by the different enzymatic conjugates were determined using the Pearson correlation test. Values of *P* < 0.05 were considered to be statistically significant.

RESULTS

Immunoglobulin G antibodies to *T. gondii* in maned wolves were detected by ELISA using different HRPO-labeled conjugates (Table I). From a total of 59 maned wolves, 41 (69.5%) were seropositive with the homologous conjugate, 44 (74.6%) with the heterologous conjugate, and 42 (71.2%) with the affinity conjugate. Similar results for the 3 conjugates were found in 5 of 7 wolves. Only 2 zoos (Bauru/SP and Belo Horizonte/MG) showed variations in seropositivity among the different conjugates, but these differences were not statistically significant (*P* > 0.05).

As illustrated in Figure 1, mean levels of IgG antibodies to *T. gondii* obtained with the homologous conjugate (EI = 2.0, 95% CI = 1.7–2.2) were not significantly different from those obtained with the heterologous conjugate (EI = 2.5, 95% CI = 2.1–2.8). In contrast, mean levels of IgG anti-*T. gondii* determined with the affinity conjugate (EI = 4.5, 95% CI = 3.7–

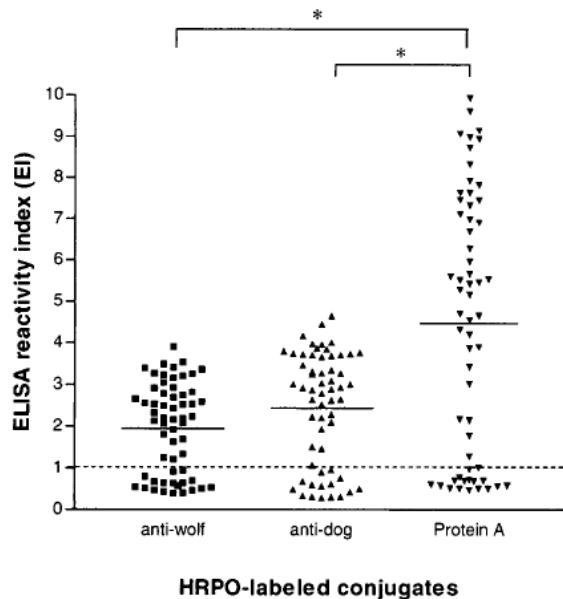


FIGURE 1. Levels of immunoglobulin (Ig) G antibodies to *Toxoplasma gondii* expressed as the enzyme-linked immunosorbent assay reactivity index (EI) in 59 serum samples from captive maned wolves (*Chrysocyon brachyurus*) using horseradish peroxidase (HRPO)-labeled homologous (anti-wolf IgG), heterologous (anti-dog IgG), or affinity (protein A) conjugates. The dashed line indicates the cutoff (EI > 1.0), and horizontal bars represent the mean EI values for each assay. * $P < 0.001$ as determined by analysis of variance and Tukey's multiple-comparison test.

5.3) were significantly higher than those obtained with the homologous and the heterologous conjugates ($P < 0.001$). In addition, a high and significant, positive correlation was found between the EIs obtained by the 3 conjugates (Fig. 2): homologous versus affinity ($r = 0.9706$, $P < 0.0001$) (Fig. 2A), homologous versus heterologous ($r = 0.9672$, $P < 0.0001$) (Fig. 2B), and heterologous versus affinity ($r = 0.9511$, $P < 0.0001$) (Fig. 2C).

Seropositivity to *N. caninum* in maned wolves was evaluated by IFAT using 3 FITC-labeled conjugates (Table II). From 59 serum samples, only 5 (8.5%) were positive with both homologous and heterologous conjugates, and concordant results were found in all serum samples analyzed from different locales of origin. However, when using the affinity conjugate (protein A-FITC), no reactivity was found in any serum sample, even at

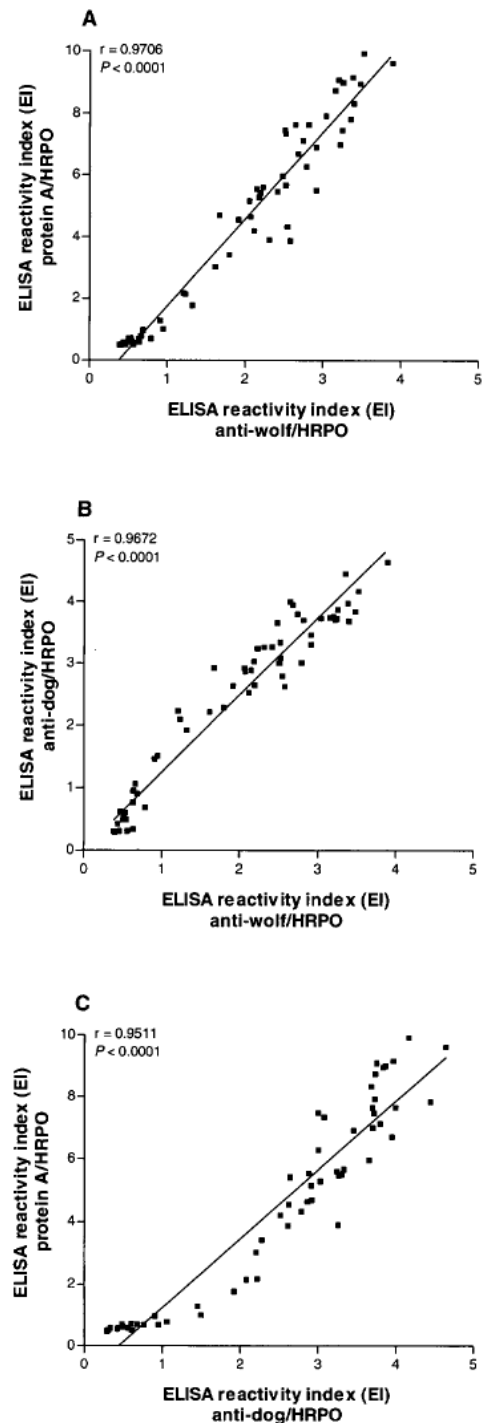


FIGURE 2. Correlation between the levels of immunoglobulin (Ig) G antibodies to *Toxoplasma gondii* expressed as the enzyme-linked immunosorbent assay reactivity index (EI) using different horseradish peroxidase (HRPO)-labeled conjugates in 59 serum samples from captive maned wolves (*Chrysocyon brachyurus*). A. Homologous (anti-wolf IgG) versus affinity (protein A) conjugates. B. Homologous (anti-wolf IgG) versus heterologous (anti-dog IgG) conjugates. C. Heterologous (anti-dog IgG) versus affinity (protein A) conjugates. Coefficients of correlation were determined by Pearson's correlation test.

TABLE II. Seropositivity to *Neospora caninum* in 59 captive maned wolves (*Chrysocyon brachyurus*) from 7 different locales (zoos or an ecological reserve) of the southeastern and midwestern regions of Brazil using homologous, heterologous, or affinity conjugates in indirect fluorescent antibody tests.

Origin*	No. of animals	Seropositivity (%)†		
		Homologous (anti-wolf IgG-FITC)	Heterologous (anti-dog IgG-FITC)	Affinity (protein A-FITC)
Brasília-DF	7	0 (0%)	0 (0%)	0 (0%)
São Paulo-SP	6	2 (33.3%)	2 (33.3%)	0 (0%)
Bauru-SP	4	2 (50%)	2 (50%)	0 (0%)
Uberaba-MG	3	1 (33.3%)	1 (33.3%)	0 (0%)
Uberlândia-MG	8	0 (0%)	0 (0%)	0 (0%)
Belo Horizonte-MG	24	0 (0%)	0 (0%)	0 (0%)
Araxá-MG	7	0 (0%)	0 (0%)	0 (0%)
Total	59	5 (8.5%)	5 (8.5%)	0 (0%)

* Captive maned wolves were originated from zoos except for those from Araxá-MG (ecological reserve).

† Homologous, heterologous, and affinity conjugates were labeled with fluorescein isothiocyanate (FITC). Ig, immunoglobulin.

the initial dilution (1:25). The distribution of IgG antibody titers to *N. caninum* showed that the 5 positive sera had low titers, from 1:25 to 1:50 with the homologous conjugate and from 1:50 to 1:100 when the heterologous conjugate was used.

DISCUSSION

A number of studies have shown the great exposure of wild-life species, particularly wild canids, to *T. gondii* when compared to infection with *N. caninum* (Lindsay et al., 1996; Dubey et al., 1999; Jakubek et al., 2001; Wolfe et al., 2001; Vitaliano et al., 2004). Some seroepizootiological studies have shown that *N. caninum* elicits an antibody response in wild canids (Buxton et al., 1997; Simpson et al., 1997; Lindsay et al., 2001) and that some species, such as the red foxes, are natural intermediate hosts for the parasite (Almería et al., 2002), but its role in the sylvatic cycle of these parasites needs further investigation. Moreover, *N. caninum* definitive hosts seem to take longer to seroconvert, because IgG-specific antibodies against the parasite are detected in dogs only after at least 25 days of experimental infection (Gondim et al., 2002), making the serological diagnosis of clinical neosporosis difficult to establish.

Recently, Gondim et al. (2004) reported that coyotes also are definitive hosts for *N. caninum*, and the expanding population and range of these wild canids increases the probability of contact with domestic animals or locales where feed is stored, thereby increasing the risk of transmission of *N. caninum* to intensively farmed cattle. In our previous studies (Vitaliano et al., 2004), we demonstrated that maned wolves are highly susceptible to *T. gondii*.

In recent years, the use of heterologous antibodies or affinity conjugates has shown to be of great value for the serodiagnosis of *T. gondii* (Silva et al., 1997; Mineo et al., 2001; Silva et al., 2002) and *N. caninum* (Barber et al., 1997; Mineo et al., 2001) in both domestic and wild animals because of the great difficulty in obtaining species-specific reagents. In the present study, we evaluated 3 enzymatic and fluorescent conjugates (homologous, heterologous, or affinity) by ELISA and IFAT for the detection of IgG antibodies to *T. gondii* and *N. caninum* in captive Brazilian maned wolves.

Seropositivity to *T. gondii* was comparable among the homologous (69.5%), heterologous (74.6%), and affinity (71.2%)

conjugates with ELISA, indicating that the 3 enzymatic conjugates were suitable to detect IgG antibodies to *T. gondii* in maned wolves. In addition, high and significant, positive correlation coefficients were found between the antibody levels determined by the 3 enzymatic conjugates. However, the highest mean antibody levels (EI = 4.5) were observed with the protein A-HRPO conjugate. This observation likely results from lower nonspecific reactivity detected in negative-control sera when using protein A-HRPO as compared to a higher background for negative controls with the homologous (anti-wolf IgG-HRPO) or heterologous (anti-dog IgG-HRPO) conjugates. As a result, high antibody levels, expressed as EI, are obtained when dividing the mean OD of samples by lower cut-off values (affinity conjugate) in contrast to higher cutoff values (homologous or heterologous conjugates). In this context, our previous studies already have shown the successful use of protein A-HRPO in ELISA for the serodiagnosis of canine toxoplasmosis in dogs (Silva et al., 1997; Mineo et al., 2001; Silva et al., 2002).

When evaluating the 3 fluorescent conjugates in IFAT to detect IgG antibodies to *N. caninum* in maned wolves, the same seropositivity (8.5%) was obtained with the homologous (anti-wolf IgG-FITC) and heterologous (anti-dog IgG-FITC) conjugates, and no divergent result was observed among the different wolves. Also, similar low antibody titers (1:25 to 1:100) were determined by both conjugates, with titer variations in only a single dilution. These results support the effective use of heterologous antibodies in IFAT-IgG for seroepizootiological surveys of *N. caninum* in different canid populations (Barber et al., 1997). Surprisingly, the protein A-FITC conjugate was not able to detect or confirm any serum samples that were positive when homologous or heterologous conjugates were used, even when the lowest dilution (1:10) of the affinity conjugate was tested. Thus, protein A-FITC was not shown to be suitable for the serodiagnosis of *N. caninum* in maned wolves, in contrast of its successful use to detect IgG antibodies to *T. gondii* in this wildlife species (data not shown). Interestingly, in our recent study on the occurrence of IgG antibodies to *N. caninum* in dogs presenting neuromuscular, respiratory, and/or gastrointestinal disorders, protein A-FITC was effectively used in IFAT at optimal dilution of 1:100 (Mineo et al., 2001).

These unexpected findings may be explained by the low antibody titers that often are seen in *N. caninum* infection as compared to *T. gondii* infection, not only in wild canids (Jakubek et al., 2001; Lindsay et al., 2001; Vitaliano et al., 2004) but also in other possible intermediate hosts, including humans (Tranas et al., 1999). Another probable explanation that needs to be investigated further refers to particular and differential affinities of this affinity matrix (staphylococcal protein A) for various immunoglobulin isotypes and, particularly, IgG sub-isotypes (Lindmark et al., 1983). In this context, little is known about the IgG sub-isotypes in *T. gondii* and *N. caninum* infections, particularly in wild canids.

Altogether, our results demonstrate that homologous, heterologous, and affinity conjugates can be used as sensitive and specific tests for determining the seroprevalence of *T. gondii* in wild canids, whereas for *N. caninum* infection, only the homologous or heterologous conjugates are useful for this purpose, thus contributing to adequate management procedures and preservation of the maned wolf. In addition, evaluation of more available or purified reagents becomes indispensable in determining their use in serological assays, particularly those directed to seroepizootiological surveys, because the majority of infected animals are usually asymptomatic.

ACKNOWLEDGMENTS

The present work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), FAPEMIG, and CAPES.

LITERATURE CITED

- ALMERIA, S., D. FERRER, M. PABÓN, J. CASTELLÀ, AND S. MAÑAS. 2002. Red foxes (*Vulpes vulpes*) are a natural intermediate host of *Neospora caninum*. *Veterinary Parasitology* **107**: 287–294.
- BARBER, J. S., R. B. GASSER, J. ELLIS, M. P. REICHEL, D. McMILLAN, AND A. J. TREES. 1997. Prevalence of antibodies to *Neospora caninum* in different canid populations. *Journal of Parasitology* **83**: 1056–1058.
- BUXTON, D., S. W. MALEY, P.-P. PASTORET, B. BROCHIER, AND E. A. INNES. 1997. Examination of red foxes (*Vulpes vulpes*) from Belgium for antibody to *Neospora caninum* and *Toxoplasma gondii*. *Veterinary Records* **141**: 308–309.
- CAMARGO, M. E. 1964. Improved technique of indirect immunofluorescence for serological diagnosis of toxoplasmosis. *Revista do Instituto de Medicina Tropical de São Paulo* **6**: 117–118.
- CLARK, H. F., AND C. C. SHEPARD. 1963. A dialysis technique for preparing fluorescent antibody. *Virology* **20**: 642–644.
- DUBEY, J. P., AND C. P. BEATTIE. 1988. *Toxoplasmosis of animals and man*. CRC Press, Boca Raton, Florida, 220 p.
- , A. N. HAMIR, AND C. E. RUPPRECHT. 1990. Acute disseminated toxoplasmosis in a red fox (*Vulpes vulpes*). *Journal of Wildlife Diseases* **26**: 286–290.
- , AND T. L. LIU. 1994. Acute toxoplasmosis in a gray fox (*Urocyon cinereoargenteus*). *Veterinary Parasitology* **51**: 321–325.
- , S. T. STORANDT, O. C. H. KWOK, P. THULLIEZ, AND K. R. KAZACOS. 1999. *Toxoplasma gondii* antibodies in naturally exposed wild coyotes, red foxes, and gray foxes and serologic diagnosis of toxoplasmosis in red foxes fed *T. gondii* oocysts and tissue cysts. *Journal of Parasitology* **85**: 240–243.
- GONDIM, L. F. P., L. GAO, AND M. M. MCALLISTER. 2002. Improved production of *Neospora caninum* oocysts, cyclical oral transmission between dogs and cattle, and in vitro isolation from oocysts. *Journal of Parasitology* **88**: 1159–1163.
- , M. M. MCALLISTER, W. C. PITT, AND D. E. ZEMLICKA. 2004. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. *International Journal for Parasitology* **34**: 159–161.
- JAKUBEK, E.-B., C. BRÖJER, C. REGNERSEN, A. UGGLA, G. SCHARS, AND C. BJÖRKMAN. 2001. Seroprevalences of *Toxoplasma gondii* and *Neospora caninum* in Swedish red foxes (*Vulpes vulpes*). *Veterinary Parasitology* **102**: 167–172.
- LINDMARK, R., K. THOREN-TOLLING, AND J. SJÖQUIST. 1983. Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera. *Journal of Immunological Methods* **62**: 1–13.
- LINDSAY, D. S., E. J. KELLY, R. D. MCKOWN, F. J. STEIN, J. PLOZER, J. HERMAN, B. L. BLAGBURN, AND J. P. DUBEY. 1996. Prevalence of *Neospora caninum* and *Toxoplasma gondii* antibodies in coyotes (*Canis latrans*) and experimental infections of coyotes with *Neospora caninum*. *Journal of Parasitology* **82**: 657–659.
- , J. L. WESTON, AND S. E. LITTLE. 2001. Prevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in gray foxes (*Urocyon cinereoargenteus*) from South Carolina. *Veterinary Parasitology* **97**: 159–164.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with folin phenol reagent. *Journal of Biological Chemistry* **193**: 265–275.
- MINEO, J. R., M. E. CAMARGO, AND A. W. FERREIRA. 1980. Enzyme-linked immunosorbent assay for antibodies to *Toxoplasma gondii* polysaccharides in human toxoplasmosis. *Infection and Immunity* **27**: 283–287.
- MINEO, T. W. P., D. A. O. SILVA, G. H. N. COSTA, A. C. B. VON ANCKEN, L. H. KASPER, M. A. SOUZA, D. D. CABRAL, A. J. COSTA, AND J. R. MINEO. 2001. Detection of IgG antibodies to *Neospora caninum* and *Toxoplasma gondii* in dogs examined in a veterinary hospital from Brazil. *Veterinary Parasitology* **98**: 239–245.
- SILVA, D. A. O., D. D. CABRAL, B. L. D. BERNARDINA, M. A. SOUZA, AND J. R. MINEO. 1997. Detection of *Toxoplasma gondii*-specific antibodies in dogs. A comparative study of immunoenzymatic, immunofluorescent, and hemagglutination titers. *Memórias do Instituto Oswaldo Cruz* **92**: 785–789.
- , N. M. SILVA, T. W. P. MINEO, A. A. PAJUBA NETO, E. A. V. FERRO, AND J. R. MINEO. 2002. Heterologous antibodies to evaluate the kinetics of the humoral immune response in dogs experimentally infected with *Toxoplasma gondii* RH strain. *Veterinary Parasitology* **107**: 181–195.
- SIMPSON, V. R., R. J. MONIES, P. RILEY, AND D. S. CROMEY. 1997. Foxes and neosporosis. *Veterinary Record* **141**: 503.
- TRANAS, J., R. A. HEINZEN, L. M. WEISS, AND M. M. MCALLISTER. 1999. Serological evidence of human infection with the protozoan *Neospora caninum*. *Clinical and Diagnostic Laboratory Immunology* **6**: 765–767.
- VITALIANO, S. N., D. A. O. SILVA, T. W. P. MINEO, R. A. FERREIRA, E. BEVILACQUA, AND J. R. MINEO. 2004. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in captive maned wolves (*Chrysocyon brachyurus*) from southeastern and midwestern regions of Brazil. *Veterinary Parasitology* **122**: 253–260.
- WILSON, M. B., AND P. K. NAKANE. 1978. Antibody conjugated to horseradish peroxidase. In *Immunofluorescence and related staining technique*, W. Knapp, K. Holubar, and G. Wick (eds.). Elsevier North Holland Biomedical, Amsterdam, Netherlands, p. 215–224.
- WOLFE, A., S. HOGAN, D. MACGUIRE, C. FITZPATRICK, L. VAUGHAN, D. WALL, T. J. HAYDEN, AND G. MULCAHY. 2001. Red foxes (*Vulpes vulpes*) in Ireland as hosts for parasites of potential zoonotic and veterinary significance. *Veterinary Records* **149**: 759–763.
- ZARNKE, R. L., J. P. DUBEY, O. C. KWOK, AND J. M. VER HOEF. 2000. Serologic survey for *Toxoplasma gondii* in selected wildlife species from Alaska. *Journal of Wildlife Diseases* **36**: 219–224.

5. CONCLUSÕES

Os resultados da análise de todos os estudos permitiram concluir que:

- A reatividade ao antígeno de superfície de *T. gondii* (SAG1) em *immunoblotting* mostrou ser uma poderosa ferramenta para determinar os títulos ótimos de *cut off* em IFAT (título 16) e ELISA (título 64) e reforçam a combinação de testes sorológicos com ênfase na determinação dos títulos de anticorpos para o diagnóstico da infecção por *T. gondii* em cães (estudo I).
- ELISA de captura IgM (McELISA) mostrou ser um método relevante para o diagnóstico da toxoplasmose aguda em cães, particularmente utilizando anticorpos heterólogos (anti-IgM humana) que são reagentes comercialmente disponíveis e mais acessíveis com alto grau de purificação e qualidade. A infecção aguda por *T. gondii* em cães foi caracterizada por um perfil transitório de IgM que pode constituir em importante marcador da infecção ativa (estudo II).
- A cinética da resposta de anticorpos IgG anti-*T. gondii* em cães experimentalmente infectados, avaliada por IFAT e ELISA, revelou uma resposta imune precoce (7 a 10 dias pós-inoculação) com títulos máximos entre 20 e 40 dias pós-inoculação e de longa duração. Parasitos foram isolados por bioensaio em camundongos e detectados por imunohistoquímica somente dos tecidos do cão que apresentou toxoplasmose fatal (estudo II).
- A infecção por *N. caninum* parece estar presente em cães de Uberlândia, MG, embora com menores taxas de soropositividade (6,7%) quando comparado a *T. gondii* (36%), incluindo casos de co-infecção (3,1%) e infecção somente por *N. caninum* (3,7%) e, portanto, deve ser considerada no diagnóstico clínico diferencial com *T. gondii* em cães apresentando sintomas neuromusculares, respiratórios e/ou gastrointestinais (estudo III).
- IFAT com o título ótimo de *cut off* estabelecido na diluição de 1:50 mostrou ser a técnica mais apropriada para o sorodiagnóstico da infecção por *N. caninum* em cães, enquanto ELISA deve ser empregado com cautela, particularmente quando preparações de antígenos totais são utilizadas, devido à possibilidade de reações cruzadas com outros parasitos, incluindo *T. gondii* (estudo IV).

- Os antígenos imunodominantes de *N. caninum*, especialmente aqueles abaixo de 35 kDa, foram fracamente inibidos por antígenos de *T. gondii*, indicando que eles são mais espécie-específicos e poderiam ser os candidatos selecionados para o aprimoramento de testes para o sorodiagnóstico da infecção por *N. caninum* em cães (estudo IV).
- Anticorpos anti-*N. caninum* foram detectados, pela primeira vez, em um canídeo silvestre da América do Sul, o lobo-guará (*Chrysocyon brachyurus*), embora em menor taxa de soropositividade (8,5%) quando comparado a *T. gondii* (74,6%), refletindo a grande exposição desta espécie de canídeo silvestre a *T. gondii* e, em menor extensão, a *N. caninum* (estudo V).
- Conjugados homólogos (anti-IgG lobo), heterólogos (anti-IgG de cão) ou de afinidade (Proteína A) marcados com peroxidase ou isotiocianato de fluoresceína, mostraram ser úteis em métodos ELISA ou IFAT para determinar a prevalência da infecção por *T. gondii* em lobos-guará (*Chrysocyon brachyurus*), enquanto para *N. caninum*, somente os conjugados homólogos e heterólogos mostraram ser efetivos para este propósito (estudo V).

REFERÊNCIAS

- ALI, C. N.; HARRIS, J. A.; WATKINS, J. D.; ADESIYUN, A. A. Seroepidemiology of *Toxoplasma gondii* in dogs in Trinidad and Tobago. **Veterinary Parasitology**, Amsterdam, v. 113, n. 3-4, p. 179-187, 2003.
- ANDREOTTI, R.; OLIVEIRA, J. M.; SILVA, E. A.; OSHIRO, L. M.; MATOS, M. F. C. Occurrence of *Neospora caninum* in dogs and its correlation with visceral leishmaniasis in the urban area of Campo Grande, Mato Grosso do Sul, Brazil. **Veterinary Parasitology**, Amsterdam, v. 135, n. 3-4, p. 375-379, 2006.
- ANTONY, A.; WILLIAMSON, N. B. Prevalence of antibodies to *Neospora caninum* in dogs of rural or urban origin in central New Zealand. **New Zealand Veterinary Journal**, Wellington, v. 51, n. 5, p. 232-237, 2003.
- ASLANTAS, O.; OZDEMIR, V.; KILIC, S.; BABUR, C. Seroepidemiology of leptospirosis, toxoplasmosis, and leishmaniosis among dogs in Ankara, Turkey. **Veterinary Parasitology**, Amsterdam, v. 129, n. 3-4, p. 187-191, 2005.
- AZEVEDO, S. S.; BATISTA, C. S.; VASCONCELLOS, S. A.; AGUIAR, D. M.; RAGOZO, A. M.; RODRIGUES, A. A.; ALVES, C. J.; GENNARI, S. M. Seroepidemiology of *Toxoplasma gondii* and *Neospora caninum* in dogs from the state of Paraíba, Northeast region of Brazil. **Research in Veterinary Science**, London, v. 79, n. 1, p. 51-56, 2005.
- BARBER, J. S.; GASSER, R. B.; ELLIS, J.; REICHEL, M. P.; McMILLAN, D.; TREES, A. J. Prevalence of antibodies to *Neospora caninum* in different canid populations. **Journal of Parasitology**, Lawrence, v. 83, n. 6, p. 1056-1058, 1997a.
- BARBER, J. S.; HOLMDAHL, O. J. M.; OWEN, M. R.; GUY, F.; UGLLA, A.; TREES, A. J. Characterization of the first European isolate of *Neospora caninum* (Dubey, Carpenter, Speer, Topper and Uggla). **Parasitology**, London, v. 111, n. 5, p. 563-568, 1995.
- BARBER, J. S.; TREES, A. J. Clinical aspects of 27 cases of neosporosis in dogs. **The Veterinary Record**, London, v. 139, n. 18, p. 439-443, 1996.
- BARBER, J. S.; TREES, A. J. Naturally occurring vertical transmission of *Neospora caninum* in dogs. **International Journal for Parasitology**, New York, v. 28, n. 1, p. 57-64, 1998.
- BARBER, J. S.; VAN HAM, L.; POLIS, I.; TREES, A. J. Seroprevalence of antibodies to *Neospora caninum* in Belgian dogs. **The Journal of Small Animal Practice**, Oxford, v. 38, n. 1, p. 15-16, 1997b.
- BARLING, K. S.; McNEILL, J. W.; THOMPSON, J. A.; PASCHAL, J. C.; MCCOLLUM, F. T. 3rd; CRAIG, T. M.; ADAMS, L. G. Association of serologic status for *Neospora caninum* with postweaning weight gain and carcass measurements in beef calves. **Journal of the American Veterinary Medical Association**, Chicago, v. 217, n. 9, p. 1356-1360, 2000.
- BASSO, W.; VENTURINI, L.; VENTURINI, M. C.; HILL, D. E.; KWOK, O. C.; SHEN, S. K. First isolation of *Neospora caninum* from the feces of a naturally infected dog. **Journal of Parasitology**, Lawrence, v. 87, n. 3, p. 612-618, 2001a.

BASSO, W.; VENTURINI, L.; VENTURINI, M. C.; MOORE, P.; RAMBEAU, M.; UNZAGA, J. M.; CAMPERO, C.; BACIGALUPE, D.; DUBEY J. P. Prevalence of *Neospora caninum* infection in dogs from beef-cattle farms, dairy farms, and from urban areas of Argentina. **Journal of Parasitology**, Lawrence, v. 87, n. 4, p. 906-907, 2001b.

BASSO, W.; VENTURINI, M. C.; BACIGALUPE, D.; KIENAST, M.; UNZAGA, J. M.; LARSEN, A.; MACHUCA, M.; VENTURINI, L. Confirmed clinical *Neospora caninum* infection in a boxer puppy from Argentina. **Veterinary Parasitology**, Amsterdam, v. 131, n. 3-4, p. 299-303, 2005.

BASZLER, T. V.; KNOWLES, D. P.; DUBEY, J. P.; GAY, J. M.; MATHISON, B. A.; McELWAIN, T. F. Serological diagnosis of bovine neosporosis by *Neospora caninum* monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. **Journal of Clinical Microbiology**, Washington DC, v. 34, n. 6, p. 1423-1428, 1996.

BEVERLEY, J. K. A. Congenital transmission of toxoplasmosis through successive generations of mice. **Nature**, London, v. 183, n. 4671, p. 1348-1349, 1959.

BJÆRKAS, I.; DUBEY, J. P. Evidence that *Neospora caninum* is identical to the *Toxoplasma*-like parasite of Norwegian dogs. **Acta Veterinaria Scandinavica**, Copenhagen, v. 32, n. 3, p. 407-410, 1991.

BJÆRKAS, I.; MOHN, S. F.; PRESTHUS, J. Unidentified cyst-forming sporozoan causing encephalomyelitis and myositis in dogs. **Zeitschrift für Parasitenkunde**, Berlin, v. 70, n. 2, p. 271-274, 1984.

BJÆRKAS, I.; JENKINS, M. C.; DUBEY, J. P. Identification and characterization of *Neospora caninum* tachyzoite antigens useful for diagnosis of neosporosis. **Clinical and Diagnostic Laboratory Immunology**, Washington DC, v. 1, n. 2, p. 214-221, 1994.

BJÖRKMAN, C.; HEMPHILL, A. Characterization of *Neospora caninum* iscom antigens using monoclonal antibodies. **Parasite Immunology**, Oxford, v. 20, n. 2, p. 73-80, 1998.

BJÖRKMAN, C.; HOLMDAHL, O. J. M.; UGGLA, A. An indirect enzyme-linked immunoassay (ELISA) for demonstration of antibodies to *Neospora caninum* in serum and milk of cattle. **Veterinary Parasitology**, Amsterdam, v. 68, n. 3, p. 251-260, 1997.

BJÖRKMAN, C., LUNDÉN, A., HOLMDAHL, J., BARBER, J., TREES, A. J., UGGLA, A. *Neospora caninum* in dogs: detection of antibodies by ELISA using an iscom antigen. **Parasite Immunology**, Oxford, v. 16, n. 12, p. 643-648, 1994.

BJÖRKMAN, C.; UGGLA, A. Serological diagnosis of *Neospora caninum* infection. **International Journal for Parasitology**, New York, v. 29, n. 10, p. 1497-1507, 1999.

BLIXENKRONE-MÖLLER, M.; PEDERSEN, I. R.; APPEL, M. J.; GRIOT, C. Detection of IgM antibodies against canine distemper virus in dog and mink sera employing enzyme-linked immunosorbent assay (ELISA). **Journal of Veterinary Diagnostic Investigation**, Columbia, v. 3, n. 1, p. 3-9, 1991.

BRITO, A. F.; SOUZA, L. C.; SILVA, A. V.; LANGONI, H. Epidemiological and serological aspects in canine toxoplasmosis in animals with nervous symptoms. **Memórias do Instituto Oswaldo Cruz**, Rio de Janeiro, v. 97, n. 1, p. 31-35, 2002.

BURG, J. L.; GROVER, C. M.; POULETTY, P.; BOOTHROPYD, J. C. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. **Journal of Clinical Microbiology**, Washington DC, v. 27, n. 8, p. 1787-1792, 1989.

BUXTON, D.; MALEY, S. W.; PASTORET, P. P.; BROCHIER, B.; INNES, E. A. Examination of red foxes (*Vulpes vulpes*) from Belgium for antibody to *Neospora caninum* and *Toxoplasma gondii*. **Veterinary Record**, London, v.141, n.12, p.308-309, 1997.

BUXTON, D.; McALLISTER, M. M.; DUBEY, J. P. The comparative pathogenesis of neosporosis. **Trends in Parasitology**, Oxford, v. 18, n. 12, p. 546-552, 2002.

CABRAL, D. D.; SILVA, D. A. O.; MINEO, J. R.; FERREIRA, F. A.; DURAN, F. P. Frequency of anti-*Toxoplasma gondii* antibodies in apparently healthy dogs of the city of Uberlândia – MG. **Revista Brasileira de Parasitologia Veterinária**, São Paulo, v. 7, n. 2, p. 87-90, 1998a.

CABRAL, D. D.; SILVA, D. A. O.; MIRANDA, E. O.; CUNHA, L.; FUKUSSIMA, A. C.; STUTZ, W.; BASTOS, J. E. D.; FERREIRA, F. A. Detecção de anticorpos anti-*Leishmania (Viannia) braziliensis* e *L. donovani*, anti-*Trypanosoma cruzi* e anti-*Toxoplasma gondii* em cães da área rural do município de Uberlândia, MG, Brasil. **Veterinária Notícias**, Uberlândia, v. 4, n. 1, p. 15-19, 1998b.

CAMARGO, M. E. Improved technique of indirect immunofluorescence for serological diagnosis of toxoplasmosis. **Revista do Instituto de Medicina Tropical de São Paulo**, São Paulo, v. 12, p. 117-118, 1964.

CAMARGO, M. E.; FERREIRA, A. W.; MINEO, J. R.; TAKIGUTI, C. K.; NAKAHARA, O. S. Immunoglobulin G and immunoglobulin M enzyme-linked immunosorbent assays and defined toxoplasmosis serological patterns. **Infection and Immunity**, Washington DC, v. 21, n. 1, p. 55-58, 1978.

CAMARGO, M. E.; LESER, P. G.; ROCCA, A. Detection of IgM anti-*Toxoplasma* antibodies in acute acquired and congenital toxoplasmosis after protein A treatment of serum. **Revista do Instituto de Medicina Tropical de São Paulo**, São Paulo, v. 25, n. 5, p. 201-206, 1983.

CAMARGO, M. E.; MOURA, M. E. G.; LESER, P. G. Toxoplasmosis serology: An efficient hemagglutination procedure to detect IgG and IgM antibodies. **Revista do Instituto de Medicina Tropical de São Paulo**, São Paulo, v. 31, n. 4, p. 279-285, 1989.

CAMARGO, M. E.; SILVA, S. M.; LESER, P. G.; GRANATO, C. H. Avidéz de anticorpos IgG específicos como marcadores de infecção primária recente pelo *Toxoplasma gondii*. **Revista do Instituto de Medicina Tropical de São Paulo**, São Paulo, v. 33, n. 3, p. 213-218, 1991.

CAÑÓN-FRANCO, W. A.; BERGAMASCHI, D. P.; LABRUNA, M. B.; CAMARGO, L. M.; SILVA, J. C.; PINTER, A.; GENNARI, S. M. Occurrence of anti-*Toxoplasma gondii* antibodies in dogs in the urban area of Monte Negro, Rondonia, Brazil. **Veterinary Research Communications**, Dordrecht, v. 28, n. 2, p. 113-118, 2004a.

CAÑÓN-FRANCO, W. A.; BERGAMASCHI, D. P.; LABRUNA, M. B.; CAMARGO, L. M.; SOUZA, S. L.; SILVA, J. C.; PINTER, A.; DUBEY, J. P.; GENNARI, S. M. Prevalence of antibodies to *Neospora caninum* in dogs from Amazon, Brazil. **Veterinary Parasitology**, Amsterdam, v. 115, n. 1, p. 71-74, 2003.

CAÑÓN-FRANCO, W. A.; YAI, L. E.; SOUZA, S. L.; SANTOS, L. C.; FARIAS, N. A.; RUAS, J.; ROSSI, F. W.; GOMES, A. A.; DUBEY, J. P.; GENNARI, S. M. Detection of antibodies to *Neospora caninum* in two species of wild canids, *Lycalopex gymnocercus* and *Cerdocyon thous* from Brazil. **Veterinary Parasitology**, Amsterdam, v. 123, n. 3-4, p. 275-277, 2004b.

CAPELLI, G.; NARDELLI, S.; di REGALBONO, A. F.; SCALA, A.; PIETROBELLI, M. Sero-epidemiological survey of *Neospora caninum* infection in dogs in north-eastern Italy. **Veterinary Parasitology**, Amsterdam, v. 123, n. 3-4, p. 143-148, 2004.

CESBRON-DELAUW, M. F.; TOMAVO, S.; BEAUCHAMPS, P.; FOURMAUX, M. F.; CAMUS, D.; CAPRON, A.; DUBREMETZ, J. F. Similarities between the primary structures of two distinct major surface protein of *Toxoplasma gondii*. **The Journal of Biological Chemistry**, Baltimore, v. 269, n. 23, p. 16217-16222, 1994.

CLARK, H. F.; SHEPARD, C. C. A dialysis technique for preparing fluorescent antibody. **Virology**, New York, v. 20, p. 642-644, 1963.

COBEA. **Princípios Éticos na Experimentação Animal** – Colégio Brasileiro de Experimentação Animal (COBEA), 1991.

COSKUN, S. Z.; AYDYN, L. BAUER, C. Seroprevalence of *Neospora caninum* infection in domestic dogs in Turkey. **Veterinary Record**, London, v. 146, n. 22, p. 649, 2000.

CRINGOLI, G.; RINALDI, L.; CAPUANO, F.; BALDI, L.; VENEZIANO, V.; CAPELLI, G. Serological survey of *Neospora caninum* and *Leishmania infantum* co-infection in dogs. **Veterinary Parasitology**, Amsterdam, v. 106, n. 4, p. 307-313, 2002.

CUDDON, P.; LIN, D. S.; BOWMAN, D. D.; LINDSAY, D. S.; MILLER, T. K.; DUNCAN, I. D.; deLAHUNTA, A.; CUMMINGS, J.; SUTER, M.; COOPER, B. *Neospora caninum* infection in English springer spnaiel littermatesÇ diagnostic evaluation and organism isolation. **Journal of Veterinary Internal Medicine**, Philadelphia, v. 6, n. 6, p. 325-332, 1992.

da SILVA, A. V.; PEZERICO, S. B.; de LIMA, V. Y.; d'ARC MORETTI, L.; PINHEIRO, J. P.; TANAKA, E. M.; RIBEIRO, M. G.; LANGONI, H. Genotyping of *Toxoplasma gondii* strains isolated from dogs with neurological signs. **Veterinary Parasitology**, Amsterdam, v. 127, n. 1, p. 27-27, 2005.

DECOSTER, A.; DARCY, F.; CAPRON, A. Recognition of *Toxoplasma gondii* excreted and secreted antigens by human sera from acquired and congenital toxoplasmosis: identification of markers of acute and chronic infection. **Clinical and Experimental Immunology**, London, v. 73, n. 3, p. 376-382, 1988.

de SOUZA, S. L.; GENNARI, S. M.; YAI, L. E. O.; D'ÁURIA, S. R. N.; CARDOSO, S. M. S.; GUIMARÃES JUNIOR, J. S.; DUBEY, J. P. Occurrence of *Toxoplasma gondii* antibodies in sera from dogs of the urban and rural areas from Brazil. **Revista Brasileira de Parasitologia Veterinária**, São Paulo, v. 12, p. 1-3, 2003.

de SOUZA, S. L.; GUIMARAES, J. S. Jr; FERREIRA, F.; DUBEY, J. P.; GENNARI, S. M. Prevalence of *Neospora caninum* antibodies in dogs from dairy cattle farms in Parana, Brazil. **Journal of Parasitology**, Lawrence, v. 88, n. 2, p. 408-409, 2002.

DESMONTS, G.; REMINGTON, J. S. Direct agglutination test for diagnosis of *Toxoplasma* infection: method for increasing sensitivity and specificity. **Journal of Clinical Microbiology**, Washington DC, v. 11, n. 6, p. 562-568, 1980.

DIJKSTRA, T.; EYSKER, M.; SCHARES, G.; CONRATHS, F. J.; WOUDE, W.; BARKEMA, H. W. Dogs shed *Neospora caninum* oocysts after ingestion of naturally infected bovine placenta but not after ingestion of colostrum spiked with *Neospora caninum* tachyzoites. **International Journal for Parasitology**, New York, v. 31, n. 8, p. 747-752, 2001.

DOMINGUES, L. M.; MACHADO, R. Z.; TINUCCI COSTA, M.; CARVALHO, C. S.; COSTA, A. J.; MALHEIROS, E. B. Canine toxoplasmosis: a comparative evaluation of the detection of anti-*Toxoplasma gondii* antibodies by the indirect immunoenzymatic assay (ELISA) and the indirect immunofluorescence reaction (IIF). **Revista Brasileira de Parasitologia Veterinária**, São Paulo, v.7, n. 2, p. 79-85, 1998.

DUBEY, J. P. Toxoplasmosis in dogs. **Canine Practice**, Montreal, v. 12, n. 6, p. 7-20, 25-28, 1985.

DUBEY, J. P. Toxoplasmosis. **Veterinary Clinics of North America - Small Animal Practice**, Philadelphia, v. 17, n. 6, p.1389-1404, 1987.

DUBEY, J. P. Recent advances in *Neospora* and neosporosis. **Veterinary Parasitology**, Amsterdam, v. 84, n. 3-4, p. 349-367, 1999.

DUBEY, J. P. Review of *Neospora caninum* and neosporosis in animals. **The Korean Journal of Parasitology**, Seoul, v. 41, n. 1, p. 1-16, 2003.

DUBEY, J. P.; BARR, B. C.; BARTA, J. R.; BJERKAS, I.; BJÖRKMAN, C.; BLAGBURN, B. L.; BOWMAN, D. D.; BUXTON, D.; ELLIS, J. T.; GOTTSTEIN, B.; HEMPHILL, A.; HILL, D. E.; HOWE, D. K.; JENKINS, M. C.; KOBAYASHI, Y.; KOUDELA, B.; MARSH, A. E.; MATTSSON, J. G.; McALLISTER, M. M.; MODRÝ, D.; OMATA, Y.; SIBLEY, L. D.; SPEER, C. A.; TREES, A. J.; UGGLA, A.; UPTON, S. J.; WILLIAMS, D. J. L.; LINDSAY, D. S. Redescription of *Neospora caninum* and its differentiation from related coccidian. **International Journal for Parasitology**, New York, v. 32, n. 8, p. 929-946, 2002a.

DUBEY, J. P.; BHAIYAT, M. I.; de ALLIE, C.; MACPHERSON, C. N.; SHARMA, R. N.; SREEKUMAR, C.; VIANNA, M. C.; SHEN, S. K.; KWOK, O. C.; MISKA, K. B.; KILL, D. E.; LEHMANN, T. Isolation, tissue distribution, and molecular characterization of *Toxoplasma gondii* from chickens in Grenada, West Indies. **Journal of Parasitology**, Lawrence, v. 91, n. 3, p. 557-560, 2005b.

DUBEY, J. P.; CARPENTER, J. L.; SPEER, C. A.; TOPPER, M. J.; UGGLA, A. Newly recognized fatal protozoan disease of dogs. **Journal of the American Veterinary Medical Association**, Chicago, v. 192, n. 9, p. 1269-1285, 1988a.

DUBEY, J. P.; CARPENTER, J. L.; TOPPER, M. J.; UGGLA, A. Fatal toxoplasmosis in dogs. **Journal of the American Animal Hospital Association**, v. 25, p. 659-665, 1989.

DUBEY, J. P.; DOROUGH, K. R.; JENKINS, M. C.; LIDDELL, S.; SPEER, C. A.; KWOK, O. C.; SHEN, S. K. Canine neosporosis: clinical signs, diagnosis, treatment and isolation of *Neospora caninum* in mice and cell culture. **International Journal for Parasitology**, New York, v. 28, n. 8, p. 1293-1304, 1998.

DUBEY, J. P.; GOMEZ-MARIN, J. E.; BEDOYA, A.; LORA, F.; VIANNA, M. C.; HILL, D.; KWOK, O. C.; SHEN, S. K.; MARCET, P. L.; LEHMANN, T. Genetic and biologic characteristics of *Toxoplasma gondii* isolates in free-range chickens from Colombia, South America. **Veterinary Parasitology**, Amsterdam, v. 134, n. 1-2, p. 67-72, 2005a.

DUBEY, J. P.; GRAHAM, D. H.; BLACKSTON, C. R.; LEHMANN, T.; GENNARI, S. M.; RAGOZO, A. M.; NISHI, S. M.; SHEN, S. K.; KWOK, O. C.; HILL, D. E.; THULLIEZ, P. Biological and genetic characterisation of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*) from Sao Paulo, Brazil: unexpected findings. **International Journal for Parasitology**, New York, v. 32, n. 1, p. 99-105, 2002b.

DUBEY, J. P.; GRAHAM, D. H.; da SILVA, D. S.; LEHMANN, T.; BAHIA-OLIVEIRA, L. M. *Toxoplasma gondii* isolates of free-ranging chickens from Rio de Janeiro, Brazil: mouse mortality, genotype, and oocyst shedding by cats. **Journal of Parasitology**, Lawrence, v. 89, n. 4, p. 851-853, 2003a.

DUBEY, J. P.; HATTEL, A. L.; LINDSAY, D. S.; TOPPER, M. J. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and the experimental transmission. **Journal of the American Veterinary Medical Association**, Chicago, v. 193, n. 10, p. 1259-1263, 1988b.

DUBEY, J. P.; KARHEMERE, S.; DAHL, E.; SREEKUMAR, C.; DIABATÉ, A.; DABIRÉ, K. R.; VIANNA, M. C. B.; KWOK, O. C. H.; LEHMANN, T. First biologic and genetic characterization of *Toxoplasma gondii* isolates from chickens from Africa (Democratic Republic of Congo, Mali, Burkina Faso, and Kenya). **Journal of Parasitology**, Lawrence, v. 91, n. 1, p. 69-72, 2005c.

DUBEY, J. P.; LINDSAY, D. S. A review of *Neospora caninum* and neosporosis. **Veterinary Parasitology**, Amsterdam, v. 67, n. 1-2, p. 1-59, 1996.

DUBEY, J. P.; LINDSAY, D. S.; SPEER, C. A. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites and sporozoites and biology and development of tissue cysts. **Clinical Microbiology Reviews**, Washington DC, v. 11, n. 2, p.267-299, 1998.

DUBEY, J. P.; LIN, T. L. Acute toxoplasmosis in a gray fox (*Urocyon cinereoargenteus*). **Veterinary Parasitology**, Amsterdam, v. 51, n. 3-4, p. 321-325, 1994.

DUBEY, J. P.; NAVARRO, I. T.; GRAHAM, D. H.; DAHL, E.; FREIRE, R. L.; PRUDENCIO, L. B.; SREEKUMAR, C.; VIANNA, M. C.; LEHMANN, T. Characterization of *Toxoplasma gondii* isolates from free range chickens from Parana, Brazil. **Veterinary Parasitology**, Amsterdam, v. 117, n. 3, p. 229-234, 2003b.

DUBEY, J. P.; NAVARRO, I. T.; SREEKUMAR, C.; DAHL, E.; FREIRE, R. L.; KAWABATA, H. H.; VIANNA, M. C.; KWOK, O. C.; SHEN, S. K.; THULLIEZ, P.; LEHMANN, T. *Toxoplasma gondii* infections in cats from Parana, Brazil: seroprevalence, tissue distribution, and biologic and genetic characterization of isolates. **Journal of Parasitology**, Lawrence, v. 90, n. 4, p. 721-726, 2004b.

DUBEY, J. P.; SREEKUMAR, C.; KNICKMAN, E.; MISKA K. B.; VIANNA M. C.; KWOK O. C.; HILL, D. E.; JENKINS, M. C.; LINDSAY, D. S.; GREENE, C. E. Biologic, morphologic, and molecular characterisation of *Neospora caninum* isolates from littermate dogs. **International Journal for Parasitology**, New York, v. 34, n. 10, p. 1157-1167, 2004a.

DUBEY, J. P.; STORANDT, S. T.; KWOK, O. C.; THULLIEZ, P.; KAZACOS, K. R. *Toxoplasma gondii* antibodies in naturally exposed wild coyotes, red foxes, and gray foxes and serologic diagnosis of *Toxoplasmosis* in red foxes fed *T. gondii* oocysts and tissue cysts. **Journal of Parasitology**, Lawrence, v. 85, n. 2, p. 240-243, 1999.

DUBEY, J. P.; THULLIEZ, P. Prevalence of antibodies to *Neospora caninum* in wild animals. **Journal of Parasitology**, Lawrence, v. 91, n. 5, p. 1217-1218, 2005.

DUBEY, J. P.; VENTURINI M. C.; VENTURINI, L.; PISCOPO, M.; GRAHAM, D. H.; DAHL, E.; SREEKUMAR, C.; VIANNA, M. C.; LEHMANN, T. Isolation and genotyping of *Toxoplasma gondii* from free-ranging chickens from Argentina. **Journal of Parasitology**, Lawrence, v. 89, n. 5, p. 1063-1064, 2003c.

FERNANDES, B. C.; GENNARI, S. M.; SOUZA, S. L.; CARVALHO, J. M.; OLIVEIRA, W. G.; CURY, M. C. Prevalence of anti-*Neospora caninum* antibodies in dogs from urban, periurban and rural areas of the city of Uberlandia, Minas Gerais--Brazil. **Veterinary Parasitology**, Amsterdam, v. 123, n. 1-2, p. 33-40, 2004.

FRENKEL, J. K.; LINDSAY, D. S.; PARKER, B. B.; DOBESH, M. Dogs as possible mechanical carriers of *Toxoplasma*, and their fur as a source of infection of young children. **International Journal of Infectious Diseases**, Hamilton, v. 7, n. 4, p. 292-293, 2003.

FRENKEL, J. K.; PARKER, B. B. An apparent role of dogs in the transmission of *Toxoplasma gondii*. The probable importance of xenosmophilia. **Annals of the New York Academy of Science**, New York, v. 791, p. 402-407, 1996.

FULTON, J. D.; TURK, J. L. Direct agglutination tests for *Toxoplasma gondii*. **Lancet**, London, v. 2, p.1068-1069, 1959.

GALLEGO, C.; SAAVEDRA-MATIZ, C.; GOMEZ-MARIN, J. E. Direct genotyping of animal and human isolates of *Toxoplasma gondii* from Colombia (South America). **Acta Tropica**, Basel, v. 97, n. 2, p. 161-167, 2006.

GANLEY, J. P.; COMSTOCK, G. W. Association of cats and toxoplasmosis. **American journal of Epidemiology**, Baltimore, v. 111, n. 2, p. 238-246, 1980.

GAZZINELLI, R. T.; WYSOCKA, M.; HIENY, S.; SCHARTON-KERSTEN, T.; CHEEVER, A.; KUHN, R.; MULLER, W.; TRINCHIERI, G.; SHER, A. In absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4⁺ cells and accompanied by over production of IL-12, IFN- γ and TNF- α . **Journal of Immunology**, Baltimore, v. 157, n. 2, p. 798-805, 1996.

GENNARI, S. M.; CAÑÓN-FRANCO, W. A.; YAI, L. E.; de SOUZA, S. L.; SANTOS, L. C.; FARIAS, N. A.; RUAS, J.; ROSSI, F. W.; GOMES, A. A. Seroprevalence of *Toxoplasma gondii* antibodies from wild canids from Brazil. **Veterinary Parasitology**, Amsterdam, v. 121, n. 3-4, p. 337-340, 2004.

GENNARI, S. M.; YAI, L. E.; D'AURIA S. N.; CARDOSO, S. M.; KWOK, O. C.; JENKINS, M. C.; DUBEY, J. P. Occurrence of *Neospora caninum* antibodies in sera from dogs of the city of Sao Paulo, Brazil. **Veterinary Parasitology**, Amsterdam, v. 106, n. 2, p. 177-179, 2002.

GONDIM, L. F.; GAO, L.; McALLISTER, M. M. Improved production of *Neospora caninum* oocysts, cyclical oral transmission between dogs and cattle, and in vitro isolation from oocysts. **Journal of Parasitology**, Lawrence, v. 88, n. 6, p. 1159-1163, 2002.

GONDIM, L. F.; LASKI, P.; GAO, L.; McALLISTER, M. M. Variation of the internal transcribed spacer 1 sequence within individual strains and among different strains of *Neospora caninum*. **Journal of Parasitology**, Lawrence, v. 90, n. 1, p. 119-122, 2004b.

GONDIM, L. F.; McALLISTER, M. M.; GAO, L. Effects of host maturity and prior exposure history on the production of *Neospora caninum* oocysts by dogs. **Veterinary Parasitology**, Amsterdam, v. 134, n. 1-2, p. 33-39, 2005.

GONDIM, L. F.; McALLISTER, M. M.; MATEUS-PINILLA, N. E.; PITT, W. C.; MECH, L. D.; NELSON, M. E. Transmission of *Neospora caninum* between wild and domestic animals. **Journal of Parasitology**, Lawrence, v. 90, n. 6, p. 1361-1365, 2004c.

GONDIM, L. F.; McALLISTER, M. M.; PITT, W. C.; ZEMPLICKA, D. E. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. **International Journal for Parasitology**, New York, v. 34, n. 2, p. 159-161, 2004a.

GONDIM, L. F.; PINHEIRO, A. M.; SANTOS, P. O.; JESUS, E. E.; RIBEIRO, M. B.; FERNANDES, H. S.; ALMEIDA, M. A.; FREIRE, S. M.; MEYER, R.; McALLISTER, M. M. Isolation of *Neospora caninum* from the brain of a naturally infected dog, and production of encysted bradyzoites in gerbils. **Veterinary Parasitology**, Amsterdam, v. 101, n. 1, p. 1-7, 2001.

GRAHAM, D. A.; CALVERT, V.; WHYTE, M.; MARKS, J. Absence of serological evidence for human *Neospora caninum* infection. **The Veterinary Record**, London, v. 144, n. 24, p. 672-673, 1999.

GREINER, M.; SOHR, D.; GÖBEL, P. A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. **Journal of Immunological Methods**, Amsterdam, v. 185, n. 1, p. 123-132, 1995.

GUIMARÃES, A. M.; RIBEIRO, M. F. B.; LIMA, J. D.; CURY, M. C.; SPIEWAK, G. Frequência de anticorpos anti-*Toxoplasma gondii* em cães de Belo Horizonte, Minas Gerais. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, Belo Horizonte, v. 44, p. 67-68, 1992.

HAU, J.; NILSSON, M.; SKOVGAARD-JENSEN, H. J.; SOUZA, A.; ERIKSEN, E.; WANDALL, L. T. Analysis of animal serum proteins using antisera against human analogous proteins. **Scandinavia Journal of Laboratory and Animal Science**, Copenhagen, v. 17, p. 3-7, 1990.

HAY, W. H.; SHELL, L. G.; LINDSAY, D. S.; DUBEY, J. P. Diagnosis and treatment of *Neospora caninum* infection in a dog. **Journal of the American Veterinary Medical Association**, Chicago, v. 197, n. 1, p. 87-89, 1990.

HEGAB, S. M.; AL-MUTAWA, S. A. Immunopathogenesis of toxoplasmosis. **Clinical and Experimental Medicine**, Milano, v. 3, n. 2, p. 84-105, 2003.

HEMPHILL, A.; FUCHS, N.; SONDA, S.; HEHL, A. The antigenic composition of *Neospora caninum*. **International Journal for Parasitology**, New York, v. 29, n. 8, p. 1175-1188, 1999.

HOLLIMAN, R. E.; RAYMOND, R.; RENTON, N.; JOHNSON, J. D. The diagnosis of toxoplasmosis using IgG avidity. **Epidemiology and Infection**, Cambridge, v. 112, n. 2, p. 399-408, 1994.

HOWE, D. K.; CRAWFORD, A. C.; LINDSAY, D.; SIBLEY, L. D. The p29 and p35 immunodominant antigens of *Neospora caninum* tachyzoites are homologous to the family of surface antigens of *Toxoplasma gondii*. **Infection and Immunity**, Washington DC, v. 66, n. 11, p. 5322-5328, 1998.

HOWE, D. K.; SIBLEY, L. D. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. **The Journal of Infectious Diseases**, Chicago, v. 172, n. 6, p. 1561-1566, 1995.

HUTCHISON, W. M. Experimental transmission of *Toxoplasma gondii*. **Nature**, London, v. 206, n. 987, p. 961-962, 1965.

INNES, E. A.; ANDRIANARIVO, A. G.; BJÖRKMAN, C.; WILLIAMS, D. J.; CONRAD, P. A. Immune responses to *Neospora caninum* and prospects for vaccination. **Trends in Parasitology**, Oxford, v. 18, n. 11, p. 497-504, 2002.

JACOBS, L.; LUNDE, M. N. A hemagglutination test for toxoplasmosis. **Journal for Parasitology**, New York, v. 43, n. 3, p. 308-314, 1957.

JACOBS, L.; REMINGTON, J. S.; MELTON, M. L. A survey of meat samples from swine, cattle, and sheep for the presence of encysted *Toxoplasma*. **Journal for Parasitology**, New York, v. 46, p. 23-28, 1960.

JAKUBEK, E. B.; BRÖJER, C.; REGNERSEN, C.; UGGLA, A.; SCHARES, G.; BJÖRKMAN, C. Seroprevalences of *Toxoplasma gondii* and *Neospora caninum* in Swedish red foxes (*Vulpes vulpes*). **Veterinary Parasitology**, Amsterdam, v. 102, n. 1-2, p. 167-172, 2001.

JUNG, C.; LEE, C. Y. F.; GRIGG, M. E. The SRS superfamily of *Toxoplasma* surface proteins. **International Journal for Parasitology**, New York, v. 34, n. 3, p. 285-296, 2004.

KASPER, L. H.; CRABB, J. H.; PFEFFERKORN, E. R. Purification of a major membrane protein of *Toxoplasma gondii* by immunoabsorption with a monoclonal antibody. **Journal of Immunology**, Baltimore, v. 130, n. 5, p. 2407-2412, 1983.

KIMBITA, E. N.; XUAN, X.; HUANG, X.; MIYAZAWA, T.; FUKUMOTO, S.; MISHIMA, M.; SUZUKI, H.; SUGIMOTO, C.; NAGASAWA, H.; FUJISAKI, K.; SUZUKI, N.; MIKAMI, T.; IGARASHI, I. Serodiagnosis of *Toxoplasma gondii* infection in cats by enzyme-linked immunosorbent assay using recombinant SAG1. **Veterinary Parasitology**, Amsterdam, v. 102, n. 1-2, p. 35-44, 2001.

LAEMMLI, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature**, London, v. 227, n. 5259, p. 680-685, 1970.

LALLY, N. C.; JENKINS, M.; LIDELL, S.; DUBEY, J. P. A dense granule protein (NCDG1) gene from *Neospora caninum*. **Molecular and Biochemical Parasitology**, Amsterdam, v. 87, n. 2, p. 239-243, 1997.

LASRI, S.; De MEERSCHMAN, F.; RETTIGNER, C.; FOCANT, C.; LOSSON, B. Comparison of three techniques for the serological diagnosis of *Neospora caninum* in the dog and their use for epidemiological studies. **Veterinary Parasitology**, Amsterdam, v. 123, n. 1-2, p. 25-32, 2004.

LEMBERGER, K. Y.; GONDIM, L. F. P.; PESSIER, A. P.; McALLISTER, M. M.; KINSEL, M. J. *Neospora caninum* infection in a free-ranging raccoon (*Procyon lotor*) with concurrent canine distemper virus infection. **Journal of Parasitology**, Lawrence, v. 91, n. 4, p. 960-961, 2005.

LIAO, M.; XUAN, X.; HUANG, X.; SHIRAFUGI, H.; FUKUMOTO, S.; HIRATA, H.; SUZUKI, H.; FUJISAKI, K. Identification and characterization of cross-reactive antigens from *Neospora caninum* and *Toxoplasma gondii*. **Parasitology**, London, v. 130, n. 5, p. 481-488, 2005.

LIN, D. S. Seroprevalence to *Toxoplasma gondii* in privately-owned dogs in Taiwan. **Preventive Veterinary Medicine**, Amsterdam, v. 35, n. 1, p. 21-27, 1998.

LINDSAY, D. S.; DUBEY, J. P.; BUTLER, J. M.; BLAGBURN, B. L. Mechanical transmission of *Toxoplasma gondii* oocysts by dogs. **Veterinary Parasitology**, Amsterdam, v. 73, n. 1-2, p. 27-33, 1997.

LINDSAY, D. S.; DUBEY, J. P.; DUNCAN, R. B. Confirmation that the dog is a definitive host for *Neospora caninum*. **Veterinary Parasitology**, Amsterdam, v. 82, n. 4, p. 327-333, 1999.

LINDSAY, D. S.; DUBEY, J. P.; UPTON, S. J.; RIDLEY, R. K. Serological prevalence of *Neospora caninum* and *Toxoplasma gondii* in dogs from Kansas. **Journal of the Helminthological Society of Washington**, Washington DC, v. 57, p. 86-88, 1990.

LINDSAY, D. S.; KELLY, E. J.; McKOWN, R. S.; STEIN, F. J.; PLOZER, J.; HERMAN, J.; BLAGBURN, B. L.; DUBEY, J. P. Prevalence of *Neospora caninum* and *Toxoplasma gondii* antibodies in coyotes (*Canis latrans*) and experimental infections of coyotes with *Neospora caninum*. **Journal of Parasitology**, Lawrence, v. 82, n. 4, p. 657-659, 1996.

LINDSAY, D. S.; WESTON, J. L.; LITTLE, S. E. Prevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in gray foxes (*Urocyon cinereoargenteus*) from South Carolina. **Veterinary Parasitology**, Amsterdam, v. 97, n. 2, p. 159-164, 2001.

LOBATO, J.; SILVA, D. A.; MINEO, T. W.; AMARAL, J. D.; SEGUNDO, G. R.; COSTA-CRUZ, J. M.; FERREIRA, M. S.; BORGES, A. S.; MINEO, J. R. Detection of immunoglobulin G antibodies to *Neospora caninum* in humans: high seropositivity rates in patients who are infected by human immunodeficiency virus or have neurological disorders. **Clinical and Vaccine Immunology**, Washington DC, v. 13, n.1, p. 84-89, 2006.

LOWRY, O. H.; ROSEBROUGH, N. J.; FARR, A. L.; RANDALL, R. J. Protein measurement with folin phenol reagent. **Journal of Biological Chemistry**, Baltimore, v. 193, n. 1, p. 265-275, 1951.

MARCOLINO, P. T.; SILVA, D. A. O.; LESER, P. G.; CAMARGO, M. E.; MINEO, J. R. Molecular markers in acute and chronic phases of human toxoplasmosis: determination of immunoglobulin G avidity by Western blotting. **Clinical and Diagnostic Laboratory Immunology**, Washington DC, v.7, n.3, p.384-389, 2000.

MARSH, A. E.; BARR, B. C.; PACKHAM, A. E.; CONRAD, P. A. Description of a new *Neospora* species (Protozoa: Apicomplexa: Sarcocystidae). **Journal of Parasitology**, Lawrence, v. 84, n. 5, p. 983-991, 1998.

McALLISTER, M. M.; DUBEY, J. P.; LINDSAY, D. S.; JOLLEY, W. R.; WILLIS, R. A.; McGUIRE, A. M. Dogs are definitive hosts of *Neospora caninum*. **International Journal for Parasitology**, New York, v. 28, n. 9, p. 1473-1478, 1998.

- McALLISTER, M. M.; PARMLEY, S. F.; WEISS, L. M.; WELCH, V. J.; MCGUIRE, A. M. An immunohistochemical method for detecting bradyzoite antigen (BAG5) in *Toxoplasma gondii*-infected tissues cross-reacts with a *Neospora caninum* bradizoyte antigen. **Journal of Parasitology**, Lawrence, v. 82, n. 2, p. 354-355, 1996.
- McGARRY, J. W.; STOCKTON, C. M.; WILLIAMS, D. J. L.; TREES, A. J. Protracted shedding of oocysts of *Neospora caninum* by a naturally infected foxhound. **Journal of Parasitology**, Lawrence, v. 89, n. 3, p. 628-630, 2003.
- MEIRELES, L. R.; GALISTEO, A. J. JR.; POMPEU, E.; ANDRADE, H. F. JR. *Toxoplasma gondii* spreading in an urban area evaluated by seroprevalence in free-living cats and dogs. **Tropical Medicine and International Health**, Oxford, v. 9, n. 8, p. 876-881, 2004.
- MELO, C. B.; LEITE, R. C.; LEITE, F. S. C.; LEITE, R. C. Serological surveillance on South American wild canids for *Neospora caninum*. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, Belo Horizonte, v. 54, n. 4, p. 444-447, 2002.
- MINEO, J. R.; CAMARGO, M. E.; FERREIRA, A. W. Enzyme-linked immunosorbent assay for antibodies to *Toxoplasma gondii* polysaccharides in human toxoplasmosis. **Infection and Immunity**, Washington DC, v. 27, n. 2, p. 283-287, 1980.
- MINEO, J. R.; CAMARGO, M. E.; FERREIRA, A. W.; ALMEIDA, G. Research on IgM anti-*Toxoplasma gondii* antibodies by using a reverse immunoenzymatic technique. **Revista do Instituto de Medicina Tropical de São Paulo**, São Paulo, v. 28, p. 6-11, 1986.
- MINEO, T. W. P.; SILVA, D. A. O.; COSTA, G. H. N.; VON ANCKEN, A. C. B.; KASPER, L. H.; SOUZA, M. A.; CABRAL, D. D.; COSTA, A. J.; MINEO, J. R. Detection of IgG antibodies to *Neospora caninum* and *Toxoplasma gondii* in dogs examined at a veterinary hospital from Brazil. **Veterinary Parasitology**, Amsterdam, v. 98, n. 4, p. 239-245, 2001.
- MINEO, T. W. P.; SILVA, D. A. O.; NÄSLUND, K.; BJÖRKMAN, C.; UGGLA, A.; MINEO, J. R. *Toxoplasma gondii* and *Neospora caninum* serological status of different canine populations from Uberlândia, Minas Gerais. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, Belo Horizonte, v. 56, n. 3, p. 414-417, 2004.
- MONTOYA, J. G.; LIESENFELD, O. Toxoplasmosis. **Lancet**, London, v. 363, n. 9425, p. 1965-1976, 2004.
- MOORE, D.P. Neosporosis in South America. **Veterinary Parasitology**, Amsterdam, v. 127, n. 2, p. 87-97, 2005.
- MUGRIDGE, N. B.; MORRISON, D. A.; HECKEROTH, A. R.; JOHNSON, A. M.; TENTER, A. M. Phylogenetic analysis based on full-length large subunit ribosomal RNA gene sequence comparison reveals that *Neospora caninum* is more closely related to *Hammondia heydorni* than to *Toxoplasma gondii*. **International Journal for Parasitology**, New York, v. 29, n. 10, p. 1545-1556, 1999.

- MÜLLER, N.; ZIMMERMANN, V.; HENTRICH, B.; GOTTSTEIN, B. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. **Journal of Clinical Microbiology**, Washington DC, v. 34, n. 11, p. 2850-2852, 1996.
- NAM, H.; KANG, S.; CHOI, W. Antibody reaction of human anti-*Toxoplasma gondii* positive and negative sera with *Neospora caninum* antigens. **The Korean Journal of Parasitology**, Sedul, v. 36, n. 4, p. 269-275, 1998.
- NEVES, D. P. **Parasitologia Humana**. 10 ed. São Paulo: Atheneu, 2000, 428p.
- NICOLLE, C.; MANCEAUX, L. Sur une infection à corps de Leishman (ou organismes voisins) du gondi. **C. R. Hebd Séances Academy Sciences**, v. 147, p. 763-766, 1908.
- ORDEIX, L.; LLORET, A.; FONDEVILA, D.; DUBEY, J. P.; FERRER, L.; FONDATI, A. Cutaneous neosporosis during treatment of pemphigus foliaceus in a dog. **Journal of the American Animal Hospital Association**, South Bend In, v. 38, n. 5, p. 415-419, 2002.
- PARÉ, J.; FECTEAU, G.; FORTIN, M.; MARSOLAIS, G. Seroepidemiologic study of *Neospora caninum* in dairy herds. **Journal of the American Veterinary Medical Association**, Chicago, v. 213, n. 11, p. 1595-1598, 1998.
- PATITUCCI, A. N.; PHIL, M.; PÉREZ, M. J.; ROZAS, M. A.; ISRAEL, K. F. Neosporosis canina: presencia de anticuerpos séricos en poblaciones caninas rurales y urbanas de Chile. **Journal of Veterinary Diagnostic Investigation**, Columbia, v. 13, p. 252-255, 2001.
- PEARCE, E. J.; SHER, A. Three major surface antigens of *Schistosoma mansoni* are linked to the membrane by glycosylphosphatidylinositol. **Journal of Immunology**, Baltimore, v. 142, n. 3, p. 979-984, 1989.
- PENA, H. F.; SOARES, R. M.; AMAKU, M.; DUBEY, J. P.; GENNARI, S. M. *Toxoplasma gondii* infection in cats from Sao Paulo state, Brazil: Seroprevalence, oocyst shedding, isolation in mice, and biologic and molecular characterization. **Research in Veterinary Science**, London, v. 81, n. 1, p. 58-67, 2006.
- PEREIRA, E. A. L, SILVA, D. A. O., CUNHA-JUNIOR, J. P., ALMEIDA, K. C., ALVES, R., SUNG, S. J., TAKETOMI, E. A. IgE, IgG1, and IgG4 antibody responses to *Blomia tropicalis* in atopic patients. **Allergy**, Copenhagen, v. 60, n. 3, p. 401-406, 2005.
- PERLÉ, K. D. M.; DEL PIERO, F.; CARR, R. F.; HARRIS, C.; STROMBERG, P. C. Cutaneous neosporosis in two adult dogs on chronic immunosuppressive therapy. **Journal of Veterinary Diagnostic Investigation**, Columbia, v. 13, p. 252-255, 2001.
- PETERS, M.; WAGNER, F., SCHARES, G. Canine neosporosis: clinical and pathological findings and first isolation of *Neospora caninum* in Germany. **Parasitology Research**, Berlin, v. 86, n. 1, p.1-7, 2000.

PETERS, M.; LUTKEFELS, E.; HECKEROTH, A. R.; SCHARES, G. Immunohistochemical and ultrastructural evidence for *Neospora caninum* tissue cysts in skeletal muscles of naturally infected dogs and cattle. **International Journal for Parasitology**, New York, v. 31, n. 10, p. 1144-1148, 2001.

PETERSEN, E.; LEBECH, M.; JENSEN, L.; LIND, P.; RASK, M.; BAGGER, P.; BJORKMAN, C.; UGGLA, A. *Neospora caninum* infection and repeated abortions in humans. **Emerging Infectious Diseases**, Atlanta, v. 5, n. 2, p. 278-280, 1999.

PINHEIRO, A. M.; COSTA, M. F.; PAULE, B.; VALE, V.; RIBEIRO, M.; NASCIMENTO, I.; SCHAEER, R. E.; ALMEIDA, M. A. O.; MEYER, R.; FREIRE, S. M. Serologic immunoreactivity to *Neospora caninum* antigens in dogs determined by indirect immunofluorescence, western blotting and dot-ELISA. **Veterinary Parasitology**, v. 130, n. 1-2, p. 73-79, 2005.

PUERTA, L.; LAGARES, A.; MERCADO, D.; FERNÁNDEZ-CALDAS, E.; CARABALLO, L. Allergenic composition of the mite *Suidasia medanensis* and cross-reactivity with *Blomia tropicalis*. **Allergy**, Copenhagen, v. 60, n. 1, p. 41-47, 2005.

RHYAN, J.; DUBEY, J. P. Toxoplasmosis in an adult dog with hepatic necrosis and associated tissue cysts and tachyzoites. **Canine Practice**, Montreal, v. 17, p. 6-10, 1992.

RODRIGUEZ, C.; AFCHAIN, D.; CAPRON, A.; DISSOUS, C.; SANTORO, F. Major surface protein of *Toxoplasma gondii* (p30) contains an immunodominant region with repetitive epitopes. **European Journal of Immunology**, Weinheim, v. 15, n. 7, p. 747-749, 1985.

ROMAND, S.; THULLIEZ, P.; DUBEY, J. P. Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. **Parasitology Research**, Berlin, v. 84, n. 1, p. 50-53, 1998.

ROSYPAL, A. C.; LINDSAY, D. S. The sylvatic cycle of *Neospora caninum*: where do we go from here? **Trends in Parasitology**, Oxford, v. 21, n. 10, p. 439-440, 2005.

SABIN, A.; FELDMAN, H. A. Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoan parasitic (*Toxoplasma*). **Science**, Washington DC, v. 108, p. 660-663, 1948.

SÁNCHEZ, G. F.; MORALES, S. E.; MARTÍNEZ, M. J.; TRIGO, J. F. Determination and correlation of anti-*Neospora caninum* antibodies in dogs and cattle from Mexico. **The Canadian Journal of Veterinary Research**, Ottawa, v. 67, n. 2, p. 142-145, 2003.

SCHARS, G.; HEYDORN, A. O.; CÜPPERS, A.; CONRATHS, F. J.; MEHLHORN, H. Cyclic transmission of *Neospora caninum*: serological findings in dogs shedding oocysts. **Parasitology Research**, Berlin, v. 87, n. 10, p. 873-877, 2001a.

SCHARS, G.; HEYDORN, A. O.; CÜPPERS, A.; CONRATHS, F. J.; MEHLHORN, H. Hammondia heydorni-like oocysts shed by a naturally infected dog and *Neospora caninum* NC-1 cannot be distinguished. **Parasitology Research**, Berlin, v. 87, n. 10, p. 808-816, 2001b.

SCHARES, G.; PANTCHEV, N.; BARUTZKI, D.; HEYDORN, A. O.; BAUER, C.; CONRATHS, F. J. Oocysts of *Neospora caninum*, *Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. **International Journal for Parasitology**, New York, v. 35, n. 14, p. 1525-1537, 2005.

SCHATZBERG, S. J.; HALEY, N. J.; BARR, S. C.; deLAHUNTA, A.; OLBY, N.; MUNANA, K.; SHARP, N. J. Use of a multiplex polymerase chain reaction assay in the antemortem diagnosis of toxoplasmosis and neosporosis in the central nervous system of cats and dogs. **American Journal of Veterinary Research**, Chicago, v. 64, n. 12, p. 1507-1513, 2003.

SCHOCK, A.; INNES, E. A.; YAMANE, I.; LATHAM, S. M.; WASTLING, J. M. Genetic and biological diversity among isolates of *Neospora caninum*. **Parasitology**, London, v. 123, n. 1, p. 13-23, 2001.

SCOTT, P.; PEARCE, E.; NATOVITZ, P.; SHER, A. Vaccination against cutaneous leishmaniasis in murine model. Induction of protective immunity with a soluble extract of promastigotes. **Journal of Immunology**, Baltimore, v.139, n. 1, p. 221-227, 1987.

SEDLAK, K.; BARTOVA, E. Seroprevalences of antibodies to *Neospora caninum* and *Toxoplasma gondii* in zoo animals. **Veterinary Parasitology**, Amsterdam, v. 136, n. 3-4, p. 223-231, 2006.

SILVA, D. A. O.; CABRAL, D. D.; BERNARDINA, B. L. D.; SOUZA, M. A.; MINEO, J. R. Detection of *Toxoplasma gondii*-specific antibodies in dogs. A comparative study of immunoenzymatic, immunofluorescent and haemagglutination titers. **Memórias do Instituto Oswaldo Cruz**, Rio de Janeiro, v. 92, n. 6, p.785-789, 1997.

SILVA, D. A. O.; SILVA, N. M.; MINEO, T. W. P.; PAJUABA NETO, A. A.; FERRO, E. A. V.; MINEO, J. R. Heterologous antibodies to evaluate the kinetics of the humoral immune response in dogs experimentally infected with *Toxoplasma gondii* RH strain. **Veterinary Parasitology**, Amsterdam, v. 107, n. 3, p. 181-195, 2002.

SLAPETA, J. R.; MODRÝ, D.; KYSELOVÁ, I.; HOREJS, R.; LUKES, J.; KOUDELA, B. Dog shedding oocysts of *Neospora caninum*: PCR diagnosis and molecular phylogenetic approach. **Veterinary Parasitology**, Amsterdam, v. 109, n. 3-4, p. 157-167, 2002.

SPEER, C. A.; DUBEY, J. P.; McALLISTER, M. M.; BLIXT, J. A. Comparative ultrastructure of tachyzoites, bradyzoites, and tissue cysts of *Neospora caninum* and *Toxoplasma gondii*. **International Journal for Parasitology**, New York, v. 29, n. 10, p. 1509-1519, 1999.

SPEER, C. A.; REDUKER, D. W.; BURGESS, D. E.; WHITMIRE, W. M.; SPLITTER, G. A. Lymphokine-induced inhibition of growth of *Eimeria bovis* and *Eimeria papillata* (Apicomplexa) in cultures bovine monocytes. **Infection and Immunity**, Washington DC, v.50, n. 2, p. 566-571, 1985.

SPLENDRE, A. Un nuovo protozoa parassita dei conigli. Incontrato nelle lesioni Anatomiche d'una malattia che Ricorda in molti punti il kala-azar dell'uomo. **Revista da Sociedade de Ciências de São Paulo**, São Paulo, v. 3, p. 109-112, 1908.

STEINMAN, A.; SHPIGEL, N. Y.; MAZAR, S.; KING, R.; BANETH, G.; SAVITSKY, I.; SHKAP, V. Low seroprevalence of antibodies to *Neospora caninum* in wild canids in Israel. **Veterinary Parasitology**, Amsterdam, v. 137, n. 1-2, p. 155-158, 2006.

SU, C.; EVANS, D.; COLE, R. H.; KISSINGER, J. C.; AJIOKA, J. W.; SIBLEY, L. D. Recent expansion of *Toxoplasma* through enhanced oral transmission. **Science**, Washington DC, v. 299, n. 5605, p. 414-416, 2003.

SWITAJ, K.; MASTER, A.; SKRZYPCZAK, M.; ZABOROWSKI, P. Recent trends in molecular diagnostics for *Toxoplasma gondii* infections. **Clinical Microbiology and Infection**, Oxford, v. 11, n. 3, p. 170-176, 2005.

TARANTINO, C.; ROSSI, G.; KRAMER, L. H.; PERRUCCI, S.; CRINGOLI, G.; MACCHIONI, G. *Leishmania infantum* and *Neospora caninum* simultaneous skin infection in a young dog in Italy. **Veterinary Parasitology**, Amsterdam, v. 102, n. 1-2, p. 77-83, 2001.

TENTER, A. M.; HECKEROTH, A. R.; WEISS, L. M. *Toxoplasma gondii*: from animals to humans. **International Journal for Parasitology**, New York, v. 30, n. 12-13, p. 1217-1258, 2000.

TOWBIN, H.; STAEHELIN, T.; GORDON, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. **Proceedings of the National Academy of Sciences of the United States of America**, Washington DC, v. 76, n. 9, p. 4350-4356, 1979.

TRANAS, J.; HEINZEN, R. A.; WEISS, L. M.; McALLISTER, M. M. Serological evidence of human infection with the protozoan *Neospora caninum*. **Clinical and Diagnostic Laboratory Immunology**, Washington DC, v. 6, n. 5, p. 765-767, 1999.

TREES, A. J.; WILLIAMS, D. J. L. Endogenous and exogenous transplacental infection in *Neospora caninum* and *Toxoplasma gondii*. **Trends in Parasitology**, Oxford, v. 21, n. 12, p. 558-561, 2005.

TREES, A. J.; WILLIAMS, D. J. L. Neosporosis in the United Kingdom. **International Journal for Parasitology**, New York, v. 30, p. 891-893, 2000.

VITALIANO, S. N.; SILVA, D. A. O.; MINEO, T. W. P.; FERREIRA, R. A.; BEVILACQUA, E.; MINEO, J. R. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in captive maned wolves (*Chrysocyon brachyurus*) from southeastern and midwestern regions of Brazil. **Veterinary Parasitology**, Amsterdam, v. 122, n. 4, p. 253-260, 2004.

VOLLER, A.; BIDWELL, D. E.; BARTLETT, A.; FLECK, D. G.; PERKINS, M.; OLADEHIN, B. A microplate enzyme-immunoassay for toxoplasma antibody. **Journal of Clinical Pathology**, London, v. 29, n. 2, p. 150-153, 1976.

WANHA, K.; EDELHOFER, R.; GABLER-EDUARDO, C.; PROSL, H. Prevalence of antibodies against *Neospora caninum* and *Toxoplasma gondii* in dogs and foxes in Austria. **Veterinary Parasitology**, Amsterdam, v. 128, n. 3-4, p. 189-193, 2005.

WEBB, J. A.; KELLER, S. L.; SOUTHERN, E. P.; ARMSTRONG, J.; ALLEN, D. G.; PEREGRINE, A. S.; DUBEY, J. P. Cutaneous manifestations of disseminated toxoplasmosis in an immunosuppressed dog. **Journal of the American Animal Hospital Association**, South Bend In, 41, n. 3, p. 198-202, 2005.

WILSON, M. B.; NAKANE, P. K. Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In: Knapp, W.; Holubar, K.; Wick, G. (Eds.), **Immunofluorescence and related staining techniques**. Amsterdam: Elsevier North Holland Biomedical Press, pp. 215-224, 1978.

WOLFE, A.; HOGAN, S.; MAGUIRE, D.; FITZPATRICK, C.; VAUGHAN, L.; WALL, D.; HAYDEN, T. J.; MULCAHY, G. Red foxes (*Vulpes vulpes*) in Ireland as hosts for parasites of potential zoonotic and veterinary significance. **The Veterinary Record**, London, v. 149, n. 25, p. 759-763, 2001.

WOUDA, W.; DIJKSTRA, T.; KRAMER, A. M. H.; van MAANEN, C.; BRINKHOF, J. M. A. Seroepidemiological evidence for a relationship between *Neospora caninum* infections in dogs and cattle. **International Journal for Parasitology**, New York, v. 29, n. 10, p. 1677-1682, 1999.

YAMAMOTO, Y. I.; SHIMIZU, S. H.; CAMARGO, M. E. A novel IgM-indirect hemagglutination test for the serodiagnosis of acute toxoplasmosis. **Journal of Clinical Laboratory Analyses**, New York, v. 5, p.127-132, 1991.

ZARNKE, R. L.; DUBEY, J. P.; KWOK, O. C.; VER HOEF, J. M. Serologic survey for *Toxoplasma gondii* in lynx from interior Alaska. **Journal of Wildlife Diseases**, Ames Ia, v. 36, n. 2, p. 219-224, 2000.