

4.2 Estudo II

Heterologous antibodies to evaluate the kinetics of the humoral immune response in dogs experimentally infected with *Toxoplasma gondii* RH strain

Enquanto anticorpos anti-*T. gondii* são freqüentemente encontrados em cães, a doença clínica é menos comum e geralmente vista em animais jovens ou associada com infecções concorrentes e condições de imunossupressão (RHYAN; DUBEY, 1992; DUBEY, 1987). O diagnóstico clínico é difícil porque os sinais clínicos são variados e não-específicos, presentes em muitas outras doenças infecto-contagiosas, incluindo a infecção pelo parasito estreitamente relacionado *N. caninum*. O diagnóstico laboratorial torna-se mais dificultado porque os testes sorológicos disponíveis geralmente não distinguem entre infecção ativa e crônica, pois a presença de anticorpos detectados por estes testes indica somente exposição ao parasito. Para definir infecção ativa com *T. gondii* por testes sorológicos é necessário a demonstração de altos e crescentes títulos de anticorpos IgG específicos em amostras de soros pareadas (intervalos de 2 a 4 semanas) (DUBEY, 1987). Em cães, pouco é conhecido sobre a produção de anticorpos na fase aguda da infecção por *T. gondii*, e portanto, um teste que possa detectar anticorpos IgM específicos em uma única amostra de soro seria de grande valia para diferenciar entre infecção ativa e latente na toxoplasmose canina, como é amplamente utilizado para determinar infecções agudas em humanos (CAMARGO et al., 1978).

Os testes sorológicos convencionais utilizados para detecção de anticorpos IgM em soros de pacientes com toxoplasmose aguda apresentam várias limitações. IFAT e ELISA para detecção de anticorpos IgM específicos geralmente requerem processamento adicional das amostras de soro para bloquear fator reumatóide (IgM anti-IgG) que produz reações falso-positivas ou remover anticorpos IgG competitivos para produzir resultados mais sensíveis e confiáveis (CAMARGO et al., 1983). IHAT utilizando 2-ME para o tratamento das amostras de soro tem evidenciado a presença de anticorpos IgM específicos quando uma significativa queda no título de anticorpos é vista nas amostras tratadas com 2-ME em relação às amostras não-tratadas (CAMARGO et al., 1989). Entretanto, este procedimento apresenta limitações para a detecção de baixos níveis de anticorpos IgM. Por outro lado, ELISA de captura IgM é baseado em uma separação imunológica de anticorpos IgM da amostra de soro-teste antes da reação com o antígeno, eliminando assim anticorpos IgG competitivos e minimizando reações não-específicas devido a fatores reumatóides em soros humanos (MINEO et al., 1986). Além disso, a baixa sensibilidade observada em outras técnicas ELISA-IgM convencionais, devido

à competição entre diferentes classes de anticorpos para determinantes antigênicos, pode ser revertida com ELISA de captura IgM.

Anticorpos anti-IgM humana reagem cruzadamente com IgM canina. Esta reatividade cruzada foi demonstrada anteriormente por imunoeletroforese em “foguete” (HAU et al., 1990) e por um ELISA de captura IgM para a detecção de anticorpos IgM específicos ao vírus da cinomose em soros de cães (BLIXENKRONE-MÖLLER et al., 1991).

No estudo II, nós desenvolvemos um ELISA de captura IgM (McELISA) usando anticorpos heterólogos (anti-IgM humana) ou homólogos (anti-IgM canina) como anticorpos de captura para determinar a resposta de IgM anti-*T. gondii* em cães experimentalmente infectados com a cepa RH do parasito. Além disso, a cinética da resposta imune humoral foi avaliada por outros três testes sorológicos (IHAT, IFAT-IgG e ELISA-IgG) e a detecção do parasito em tecidos dos cães inoculados foi investigada por bioensaio em camundongos e análises imunohistoquímicas.

Todos os quatro cães inoculados mostraram soroconversão como determinado pelos vários testes sorológicos, mas somente um animal exibiu sinais clínicos graves da infecção, como febre, anorexia, diarreia, dispnéia e morreu após 55 dias da inoculação. Os outros animais apresentaram sintomas moderados e caracterizados por febre e ligeira inapetência após 7 a 14 dias, e foram acompanhados até 62 dias pós-inoculação.

Resultados de sensibilidade e especificidade mostraram que McELISA foi capaz de detectar anticorpos IgM anti-*T. gondii* em todos soros de cães com infecção ativa (grupo I) e não mostrou qualquer reatividade nos grupos com infecção crônica (grupo II) e negativos (grupo III) a *T. gondii* ou com soros IgG positivos a *N. caninum* (grupo IV) (Fig. 1). A reatividade cruzada entre anticorpos IgM humana e canina foi demonstrada ser funcional para o diagnóstico de infecção ativa por *T. gondii* como evidenciado pela alta e significativa correlação positiva ($r = 0,9689$; $p < 0,0001$) entre estes anticorpos heterólogos (Fig. 2).

A cinética de anticorpos IgM foi obtida por McELISA usando anti-IgM humana ou anti-IgM canina (Fig. 3) e mostrou uma detecção precoce a partir de 7 dias, alcançando títulos máximos entre 10 e 20 dias e decrescendo a partir de 27 dias pós-inoculação (p.i.). No final do experimento (55 a 62 dias p.i.), dois animais ainda apresentavam títulos detectáveis de IgM por McELISA. A cinética da resposta de IgM obtida por IHAT (Fig. 4) mostrou uma detecção entre 7 e 10 dias com títulos detectáveis de IgM até 34 a 41 dias p.i.. Assim, anticorpos IgM foram detectados por McELISA mais precocemente e por um maior período que o IHAT com 2-ME, embora mostrando um perfil transitório de anticorpos (IgM de curta duração). Ao contrário, anticorpos IgG avaliados por IFAT e ELISA foram detectados precocemente (7 dias

p.i.), alcançando os mais altos títulos entre 20 e 40 dias p.i. e por todo o período de observação (IgG de longa duração) (Fig. 5 A,B). Achados clínicos e resposta de anticorpos IgG semelhantes foram encontrados por Domingues e colaboradores (1998) em infecção experimental de um cão com a cepa N de *T. gondii*. Por outro lado, nossos resultados mostraram uma resposta imune mais precoce (7 a 10 dias p.i.) e duradoura (> 60 dias p.i.) comparado aos resultados obtidos em cães experimentalmente inoculados com cistos teciduais de uma cepa avirulenta (LINDSAY et al., 1996).

Parasitos foram isolados por bioensaio em camundongos de todos os tecidos do cão com doença clínica fatal (Tabela 1). Análise imunohistoquímica revelou a presença de taquizoítas livres e parasitos dentro de vacúolos parasitóforos no citoplasma de células do baço, coração e pulmão apenas do cão que apresentou toxoplasmose fatal (Fig. 6).

Os resultados deste estudo demonstraram que a infecção aguda por *T. gondii* em cães pode ser caracterizada por um perfil transitório de IgM que pode constituir em importante marcador da infecção ativa. Além disso, McELISA mostrou ser um potencial método para o diagnóstico da toxoplasmose aguda em cães, particularmente pelo uso de anticorpos heterólogos, como anti-IgM humana, que são reagentes comercialmente disponíveis com alto grau de purificação e qualidade.



Heterologous antibodies to evaluate the kinetics of the humoral immune response in dogs experimentally infected with *Toxoplasma gondii* RH strain

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Abstract

An IgM capture ELISA using heterologous antibodies was developed to evaluate the kinetics of the humoral immune response in dogs experimentally infected with *Toxoplasma gondii* RH strain. Detection of parasite in tissues from inoculated dogs was evaluated by mouse bioassay and immunohistochemical techniques. Serum samples were obtained at regular intervals up to 62 days post-inoculation (p.i.), when the animals were necropsied and their tissues examined. Antibody levels were measured by IgM capture ELISA (McELISA), indirect hemagglutination (IHA), indirect fluorescent antibody test (IgG-IFAT) and indirect immunoenzymatic assay (IgG-ELISA). All dogs seroconverted but only one exhibited severe clinical signs of infection. IgM antibodies were detected by McELISA from the seventh day on, with decreasing IgM levels around the 27th day. Similar results were obtained from IHA, although McELISA showed earlier and longer detection of IgM antibodies. IgG antibodies were detected from the seventh day on, and throughout the period of observation. Immunohistochemical findings and mouse bioassay revealed the presence of free tachyzoites in tissues of the clinically affected dog only. These results suggest that *T. gondii* acute infection in dogs shows a remarkably transient IgM synthesis, and this feature may constitute an

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important marker of active infection. Furthermore, McELISA was shown to be a potential tool to diagnose canine toxoplasmosis.

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1. Introduction

Toxoplasmosis is one of the most common zoonoses, caused by the apicomplexan protozoan *Toxoplasma gondii*, which affects a wide range of hosts, including humans and domestic animals. Dogs are highly predisposed to *T. gondii* infection due to their dietary habits, which facilitate ingestion of cyst-contaminated tissues, and to the close contact with soil containing sporulated oocysts (Germano et al., 1985). Several epidemiological surveys have demonstrated that *T. gondii* infection in dogs is distributed worldwide with seropositivity rates ranging from 19.6 to 91.0% (Germano et al., 1985; Svoboda, 1987; Bjorkman et al., 1994). However, clinical illness is rare, it is found in fetuses affected by congenital transmission, young dogs with immature immune systems, or associated with concomitant infections, such as canine distemper virus (CDV) (Fontaine et al., 1986; Rhyan and Dubey, 1992). Clinical signs of toxoplasmosis in dogs are variable and nonspecific, usually characterized by respiratory, digestive, ocular, neurological and muscular disturbances (Ahmed et al., 1983).

Serology is an alternative method to the direct demonstration of *T. gondii* (e.g. by mouse inoculation, and immunohistochemical analyses), which are time- and animal-consuming procedures (Dubey et al., 1995b). Serological diagnosis of *T. gondii* infection in dogs has been evaluated by various tests, such as indirect hemagglutination (IHA), indirect fluorescent antibody test (IFAT), and enzyme-linked immunosorbent assay (ELISA) (Uggla et al., 1990; Guimarães et al., 1992; Rajamanickam et al., 1995), which are based on the detection of the IgG isotype. Thus, the presence of antibodies detected by these tests indicates only previous contact with the parasite. To define active infection with *T. gondii* by serological assays, it is necessary to demonstrate high and increasing titers of specific antibodies in paired serum samples taken 2–4 weeks apart (Dubey, 1987). On the other hand, the demonstration of specific IgM antibodies is widely used to determine acute infections in humans and cats (Camargo et al., 1978; Dubey et al., 1995a). Indeed, the IgM measurement in a single serum sample for diagnosis of recent infection might have potential to elucidate the different phases of canine toxoplasmosis.

We have developed an IgM capture ELISA (McELISA) using anti-human IgM (heterologous) or anti-dog IgM (homologous) as capture antibodies to measure IgM antibody response to *T. gondii*. Experimental infection in dogs with *T. gondii* RH strain was performed in order to evaluate the kinetics of the humoral immune response by four serological tests (McELISA, IHA, IgG-IFAT and IgG-ELISA). In parallel, the detection of *T. gondii* in tissues from inoculated dogs was investigated by mouse bioassay and immunohistochemical techniques.

2. Materials and methods

2.1. Parasite and soluble antigen

T. gondii RH strain tachyzoites were grown intraperitoneally in Swiss mice for 48–72 h as previously described (Mineo et al., 1980). Mice were killed by cervical dislocation according to the 2000 Report of the AVMA Panel on Euthanasia and parasites were obtained from mouse peritoneal exudates, washed three-times by centrifugation at $1000 \times g$ for 10 min at 4°C with 0.01 M phosphate buffered saline (PBS) pH 7.2 and the suspension adjusted to 1×10^4 tachyzoites/ml to use as inoculum for the dogs.

T. gondii excreted–secreted antigen was obtained as previously described (Decoster et al., 1988) with modifications. Briefly, the parasite suspension was diluted in Hanks' saline (1×10^8 tachyzoites/ml), incubated for 45 min at 37°C , and centrifuged for 10 min at $10,000 g$ at 4°C . The supernatant was collected, filtered through a $0.45 \mu\text{m}$ membrane and used as *T. gondii* excreted–secreted antigen in ELISA, after its protein concentration was determined (Lowry et al., 1951). Antigen aliquots were stored at -70°C .

2.2. Animals and serum samples

Four dogs (mixed gender, breeds and ages) serologically nonreactive to *T. gondii* by IHA and IgG-IFAT were inoculated intraperitoneally with 10^4 RH strain tachyzoites. Animals were individually housed in cages and given food and water ad libitum, with daily observation for clinical signs or mortality up to 62 days post-inoculation (p.i.). These dogs had shown low or undetectable levels of specific IgM antibodies in our preliminary studies. The surviving dogs were sedated and then killed by an intravenous injection of euthanasia solution (Thiopental[®], Cristália—Produtos Químicos Farmacêuticos Ltda, Itapira, SP, Brazil) according to the 2000 Report of the AVMA Panel on Euthanasia. As soon as the animals died or were killed, they were necropsied and organs, such as spleen, lung, brain, heart, kidney, skeletal muscle, eye and tongue were collected for mouse bioassay and immunohistochemical analysis. Serum samples were obtained prior to and after the inoculation at about 3-day intervals during the first three weeks and then weekly until 62 days after infection to determine the kinetics of IgG and IgM antibody response.

To evaluate the sensitivity and specificity of McELISA, four groups were analyzed: (I) 35 serum samples collected from the dogs between 7 and 55 days after experimental inoculation with *T. gondii*, (II) 35 dog sera with IgG antibodies to *T. gondii* in both IFAT and ELISA, (III) 35 dog sera nonreactive to *T. gondii* in both IgG-IFAT and IgG-ELISA, and (IV) 35 dog sera with high IgG-ELISA titers to *N. caninum*. Sera from groups II–IV were obtained from dogs admitted at the Veterinary Hospital, Federal University of Uberlândia (UFU), Brazil, and from dogs captured by the Zoonosis Control Center of Uberlândia (CCZU). Serum samples were stored in aliquots at -20°C .

2.2.1. Reference sera

Two positive-IgM control sera were obtained from a dog, which was bled 10 and 17 days after infection with *T. gondii*, and previously determined by a conventional serological

test (IHA with 2-mercaptoethanol (2-ME); HAP Toxoplasmoses, Salck Ind. Com. Prod. Biológicos, Brazil). Another positive-IgM reference serum was obtained from a human with acute toxoplasmosis (provided by Fleury Laboratories, São Paulo, Brazil), as previously determined by IHA, IgM-IFAT and IgM-ELISA. Positive-IgG serum samples from three chronically infected dogs and three chronically infected humans, previously determined by IHA, IgG-IFAT and IgG-ELISA also were used as controls. Negative controls were obtained from a panel of sera from three healthy dogs and three healthy humans with consistently negative serological results by IHA, IgG-IFAT, and IgG-ELISA. Furthermore, the four pre-inoculation serum samples were included among the negative control sera.

All experiments were performed according to the Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation.

2.3. Serological assays

2.3.1. McELISA

Optimal conditions for IgM capture ELISA (McELISA) were established in preliminary experiments through block titration of the reagents (capture antibodies, sera, antigen and conjugate). Microtiter plates (Immulon 2, Dynex Technologies, USA) were coated with capture antibodies at 5 µg/ml in 0.06 M carbonate buffer, pH 9.6, overnight at 4 °C. Two different preparations were tested: goat anti-dog IgM (The Binding Site Limited, Birmingham, UK) or sheep anti-human IgM, µ-chain specific (Kirkegaard & Perry Laboratories, USA). Subsequent steps were carried out using PBS plus 0.05% Tween 20 (PBS-T) containing 5% nonfat milk as diluent buffer, and washing with PBS-T was done between the steps of the reaction. Plates were incubated with sera diluted 1:16 for 2 h at 37 °C. Subsequently, *T. gondii* excreted–secreted antigen (5 µg of protein per well), prepared as described by Decoster et al. (1988), was added and incubated for 2 h at 37 °C. The bound antigen was detected with a peroxidase-rabbit F(ab')₂ anti-*T. gondii* (Wilson and Nakane, 1978) diluted 1:50 and incubated for 1 h at 37 °C. The assay was developed by adding the enzyme substrate (0.1 M citric acid, 0.2 M sodium phosphate, 0.01 M 2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), 0.03% H₂O₂) and the optical density (OD) was read at 405 nm (Titertek Multiskan, Flow Laboratories, USA). Controls on each plate included serum control (PBS-T substituting sera), antigen control (PBS-T substituting antigen), and reference sera. The cut-off for a positive test was determined as the mean OD for the negative control plus five standard deviations. Antibody titers were arbitrarily expressed as ELISA Indexes (EI), according to Turunen et al. (1983) with some modifications, as follows: $EI(\%) = (OD_{\text{sample}} / OD_{\text{cut-off}}) \times 100$, where values of $EI \geq 100$ were considered as cut-off titers.

2.3.2. IHA

An IHA was performed using a commercial kit (HAP Toxoplasmoses, Salck Ind. Com. Prod. Biológicos, Brazil), according to the manufacturer instructions. Sera were diluted two-fold from 1:32 to 1:2048 and all positive samples (titer $\geq 1:64$) were retested after treatment with 2-ME to identify IgM antibodies (Camargo et al., 1978). A two titers or higher decrease in antibody levels was indicative of specific IgM antibodies.

2.3.3. *IgG-IFAT*

IFAT to detect canine IgG antibodies to *T. gondii* was similar to that used for diagnosis of human infections (Camargo, 1964). Slides with formalized tachyzoites attached were incubated with dog sera diluted two-fold from 1:16 to 1:8192, and then with fluorescein isothiocyanate-labeled rabbit anti-dog IgG (provided by Zoonosis Control Center, Brazil) in parallel with positive and negative control sera. A bright, uniform and entire peripheral fluorescence of the organisms is required for an IFAT positive result.

2.3.4. *IgG-ELISA*

An ELISA was carried out to detect canine IgG anti-*T. gondii* as described by Mineo et al. (1980), with modifications. Briefly, microtiter plates (Corning Laboratories, USA) coated with *T. gondii* soluble antigen (10 µg/ml) were incubated with dog sera diluted 1:64 in PBS-T plus 1% bovine serum albumin (PBS-T-BSA) for 1 h at 37 °C. After repeated washing, peroxidase-protein A (Sigma, USA) diluted 1:25,000 in PBS-T-BSA was added and incubated for 1 h at 37 °C. Enzyme substrate, reading of OD values and antibody titer calculations were performed as described for McELISA.

2.4. *Detection of T. gondii*

2.4.1. *Mouse bioassay*

Tissue samples were collected, homogenized and inoculated orally and/or intraperitoneally (100–200 mg/ml in a final volume of 0.5 ml) into five mice for each tissue as previously described (Jacobs et al., 1960). Smears of peritoneal exudates from mice that died were examined in light microscopy for the presence of free tachyzoites. Surviving mice were bled 15 days after infection and sera examined for seroconversion by IgG-ELISA as above described, using peroxidase-labeled anti-mouse IgG (Sigma, USA).

2.4.2. *Immunohistochemical assay*

Tissues collected were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of 3 µm were processed on glass slides, deparaffinized and hydrated by conventional techniques. Next, they were incubated with 5% acetic acid for blocking endogenous alkaline phosphatase and then with 2.5% goat normal serum to block nonspecific binding sites. Subsequently, sections were incubated with primary antibody (rabbit IgG anti-*T. gondii*) and then with secondary antibody (biotinylated goat anti-rabbit IgG). The reaction was amplified by a streptavidin-biotinylated alkaline phosphatase complex (Dako A/S, Denmark), revealed by Fast Red/Naphthol (Sigma, USA) and counterstained by Meyer's hematoxylin.

2.5. *Statistical analysis*

Statistical analysis consisted of determinations of geometric means (GMs) with 95% confidence intervals (CIs) and the differences between the means were analyzed by the unpaired Student's *t*-test. The correlation between anti-human IgM and anti-dog IgM used

as capture antibodies in McELISA was determined by Pearson's correlation test. Values of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Clinical signs

All inoculated dogs showed a slight increase in body temperature on days 7–14 p.i. Only dog 1 presented severe clinical signs, such as anorexia, diarrhoea, and respiratory distress and died on day 55. The other animals presented moderate symptoms and were followed up to 62 days p.i.

3.2. Sensitivity and specificity of McELISA

Sensitivity and specificity of McELISA were evaluated by using the four serum groups (Fig. 1). All 35 dog sera from group I (acute infection) had EI > 100 (GM: 246.9, 95% CI: 233.8–300.5). In contrast, all 35 sera from chronic infection (group II) and 35 nonreactive samples (group III) showed EI < 100 (GM: 75.6, 95% CI: 74.0–77.7 and GM: 68.0, 95% CI: 66.2–70.3, respectively). On the other hand, from 35 sera (group IV) with high IgG titers to *N. caninum* (GM: 83.2, 95% CI: 79.9–88.3), 33 (94.3%) showed EI < 100 , and only two (5.7%) samples had EI = 100. IgM titers measured in group I were significantly higher than those obtained in the other groups ($P < 0.05$). In addition, a significant positive correlation ($r = 0.97$; $P < 0.0001$) was found between anti-human IgM and anti-dog IgM used as capture antibodies in McELISA (Fig. 2).

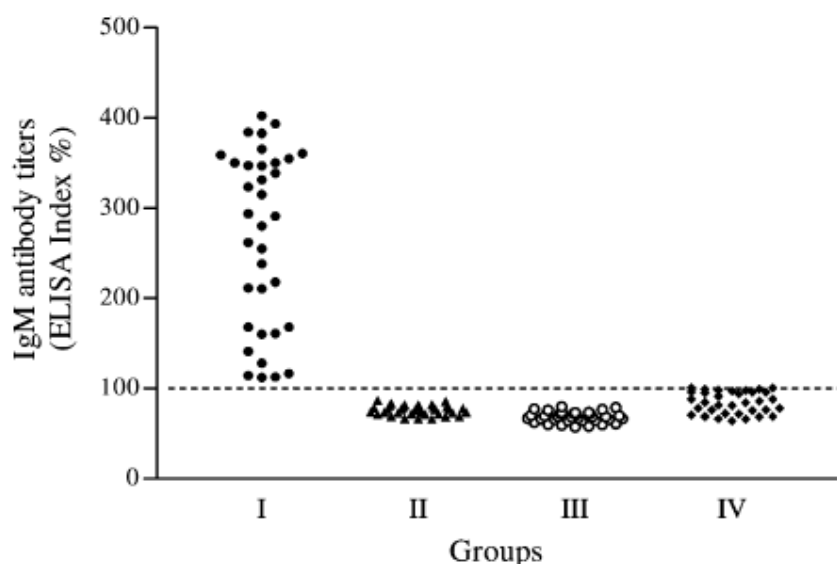


Fig. 1. IgM antibody titers to *T. gondii* measured by McELISA (expressed as ELISA Index, %) in four groups: (I) 35 sera from dogs with acute infection, (II) 35 sera from dogs with chronic infection, (III) 35 sera from dogs nonreactive to *T. gondii*, and (IV) 35 dog sera with high IgG-ELISA titers to *N. caninum*. The dashed line indicates the cut-off value for the reaction.

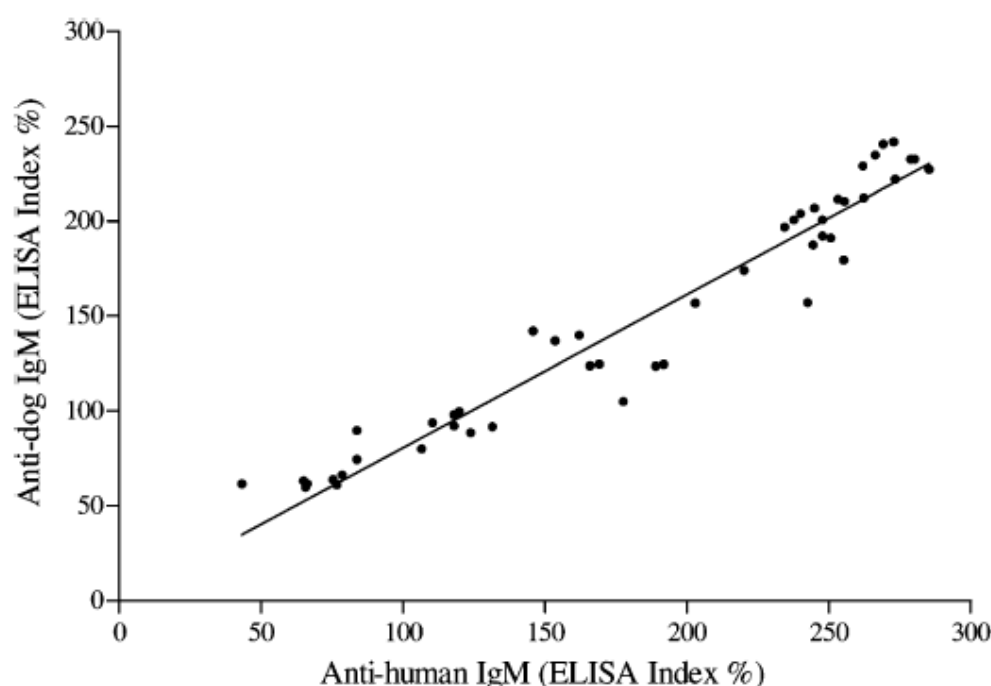


Fig. 2. Correlation between anti-human IgM and anti-dog IgM used as capture antibodies in McELISA in sera from dogs experimentally infected with *T. gondii* (Pearson's correlation coefficient: $r = 0.9689$; $P < 0.0001$).

3.3. IgM antibody synthesis by McELISA

The kinetics of IgM antibody synthesis from dogs experimentally inoculated with *T. gondii* was obtained by McELISA using anti-human IgM or anti-dog IgM as capture antibodies (Fig. 3). *T. gondii*-specific IgM antibodies were detected in all dogs from the seventh day on, reaching maximum titers mostly between 10 and 20 days p.i. Dogs 2 and 3 showed an accentuated drop in IgM titers around the 27th day, with no significant IgM level on the 48th day. Dog 4 exhibited a slow decrease on the IgM levels with low titers at the end of the experimental period. Similarly, dog 1 had a less abrupt decrease in IgM titers, although he had detectable levels when he died on the 55th day after infection.

3.4. Humoral immune response by IHA

The kinetics of the humoral immune response obtained by IHA without 2-ME treatment is demonstrated in Fig. 4. *T. gondii* infection was detected in all experimentally infected dogs as determined by their seroconversion. *T. gondii*-specific antibody was detected on day 7 (dogs 3 and 4) and day 10 (dogs 1 and 2). The antibody titers showed a significant drop around the 40th day after infection in the majority of the dogs. When analyzing the antibody titers in the 2-ME treated sera, a significant decrease on the antibody levels was observed up to 34 days p.i. in all dogs, indicating the presence of high levels of IgM antibodies. Detectable IgM levels still remained until the 41st day (dog 1) and up to the 62nd day (dog 4).

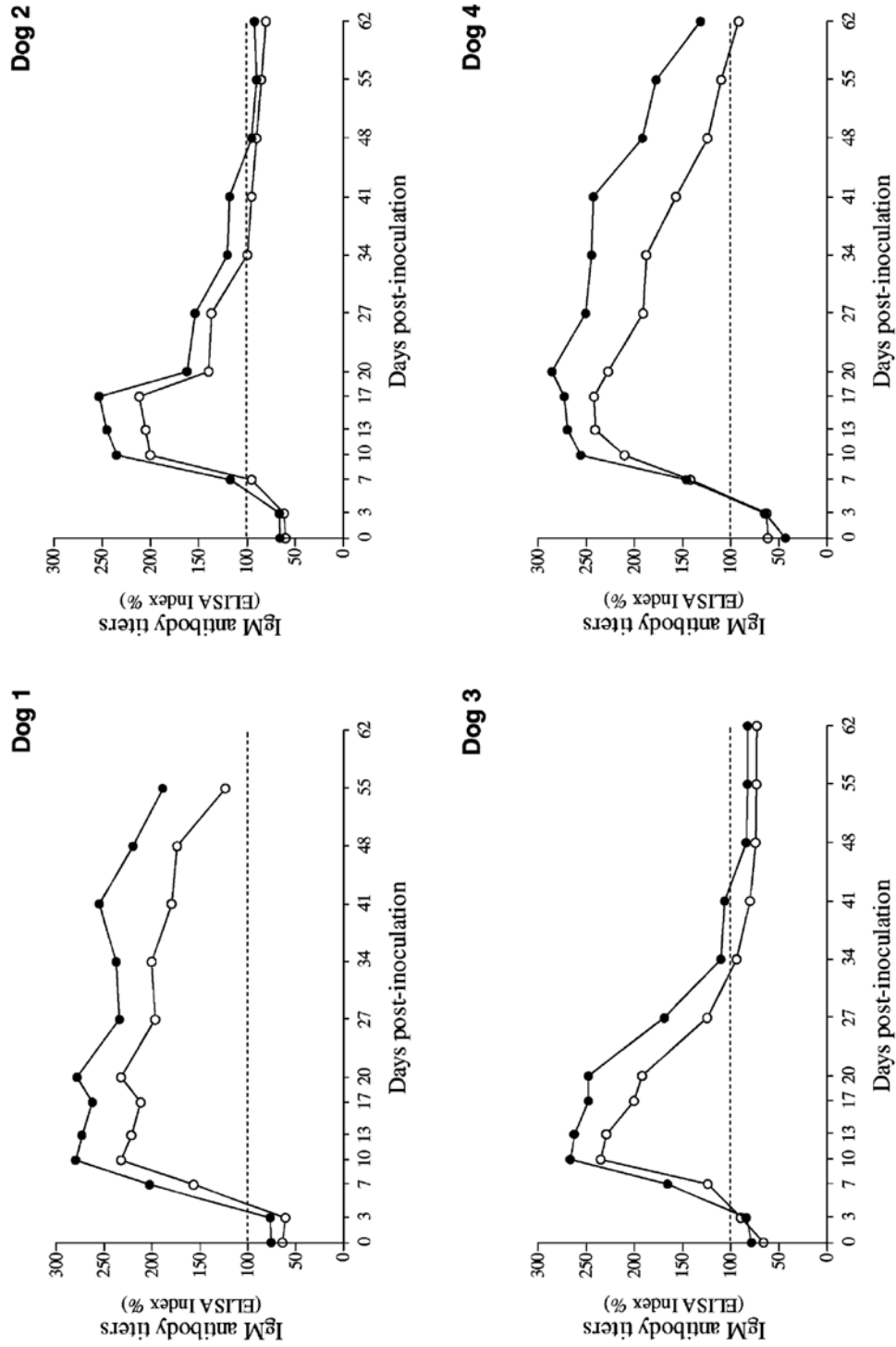


Fig. 3. Kinetics of IgM antibody synthesis obtained by McELISA in sera from dogs experimentally infected with *T. gondii*, using anti-human IgM (●) or anti-dog IgM (○) as capture antibodies. The dashed lines indicate the cut-off value for the reaction (EI $\geq$ 100).

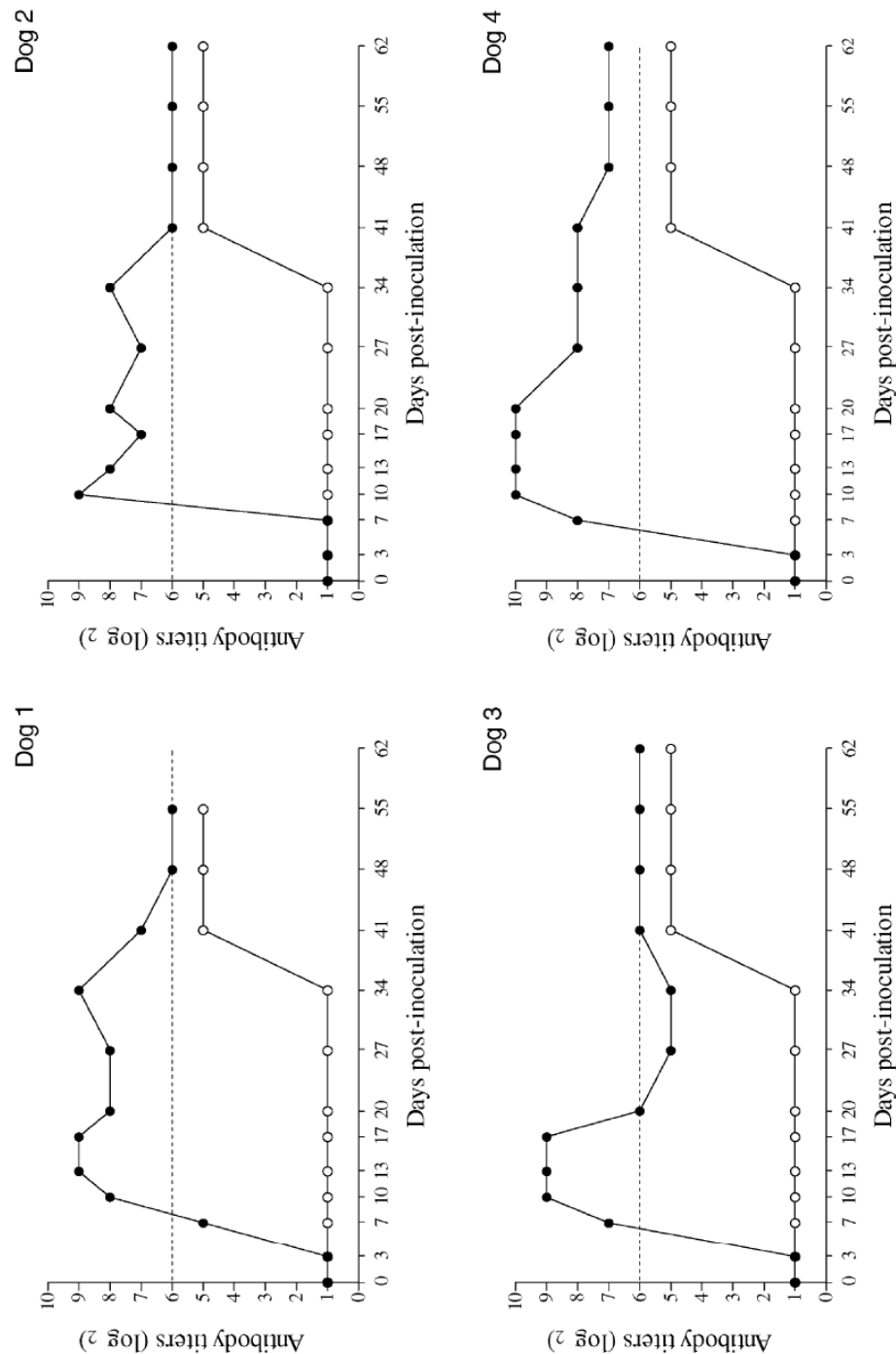


Fig. 4. Kinetics of the humoral immune response obtained by IHA without 2-ME (●) and with 2-ME (○) in sera from dogs experimentally infected with *T. gondii*. The dashed lines indicate the cut-off values for the reaction (1:64).

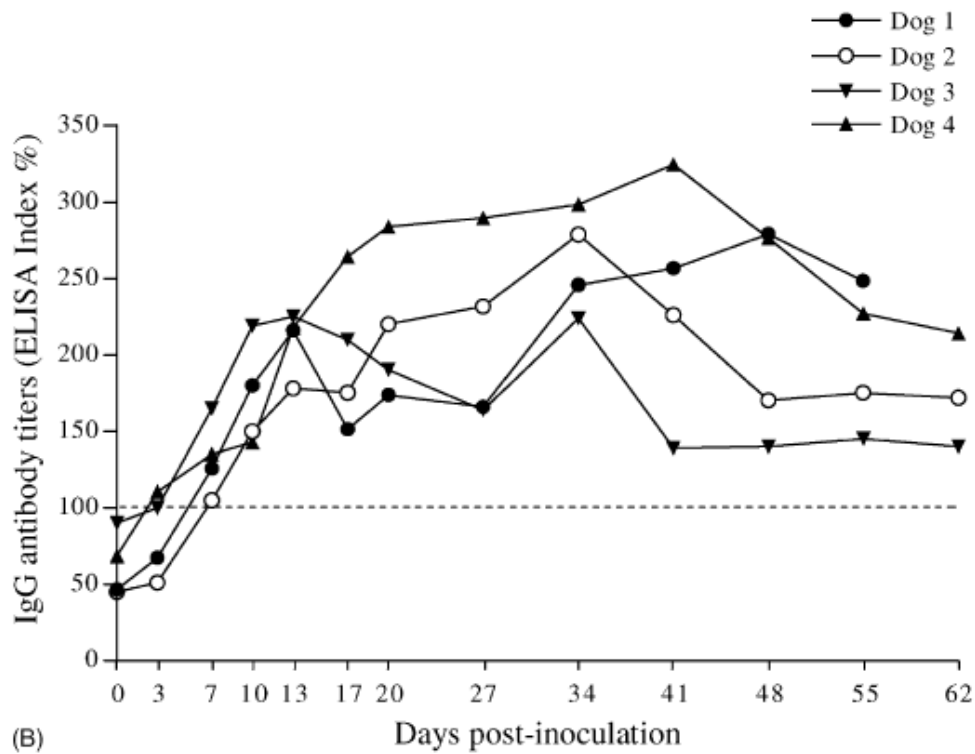
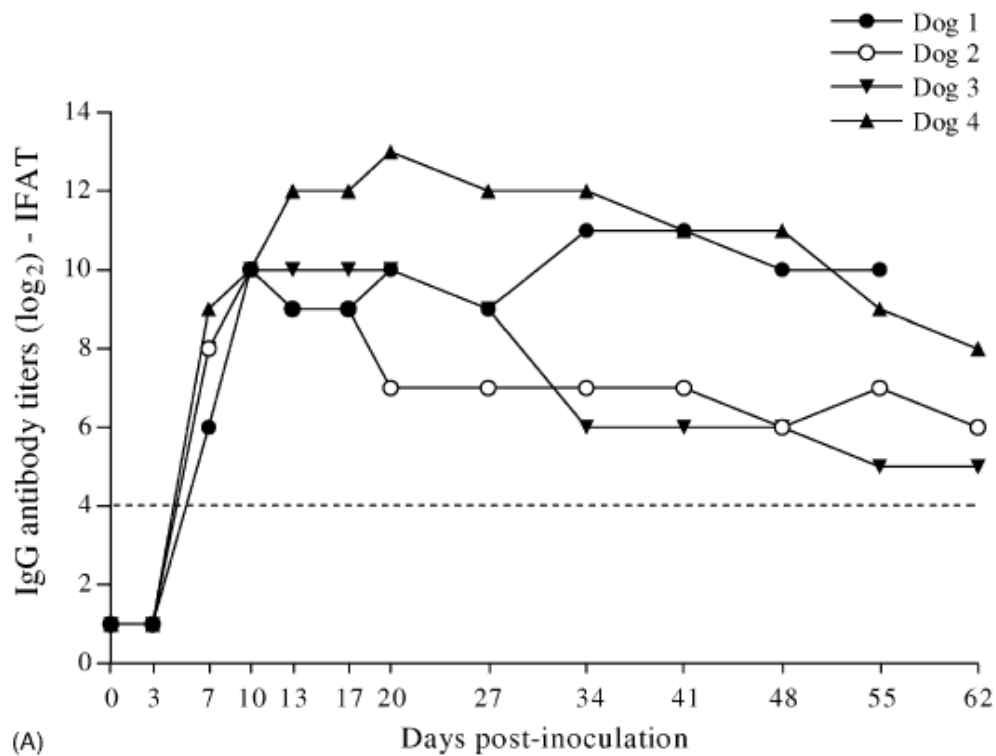


Fig. 5. Kinetics of the humoral immune response obtained by (A) IgG-IFAT and (B) IgG-ELISA in sera from dogs experimentally infected with *T. gondii*. The dashed lines indicate the cut-off values for IgG-IFAT (1:16) and IgG-ELISA (EI $\geq$ 100).

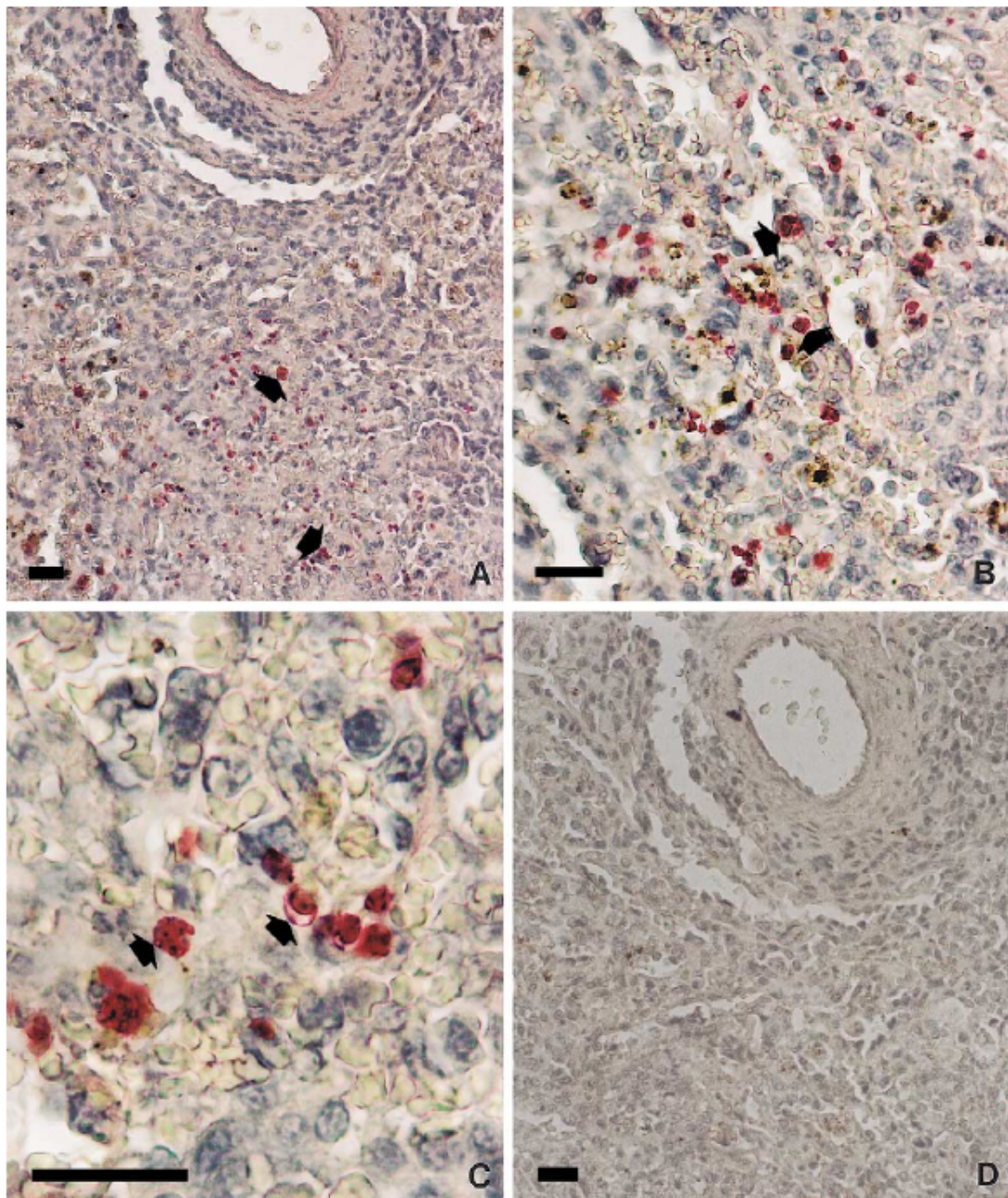


Fig. 6. Photomicrographs from immunohistochemical assay in the spleen of dog 1 experimentally infected with *T. gondii*. (A–C) Spleen cells show free tachyzoites and parasitophorous vacuoles in the cytoplasm containing parasites strongly stained (arrows). (D) Negative control in the absence of primary antibody. Preparations were stained by fast red/naphthol and counterstained by Meyer's hematoxylin. Bar scale = 100 μ m.

3.5. IgG antibody synthesis by IFAT and ELISA

The profile of IgG antibody synthesis was evaluated by IgG-IFAT and IgG-ELISA (Fig. 5). All dogs had IgG seroconversion by IFAT starting on the seventh day after infection, achieving the highest level around the 20th–40th days, with antibody titers ranging from 1:64 to

Table 1
Detection of *T. gondii* in tissues from infected dogs by mouse bioassay

Dog number	Free tachyzoites in mouse peritoneal exsudate smears (tissues from infected dogs*)							
	Brain	Heart	Spleen	Muscle	Lung	Kidney	Eye	Tongue
1	+	+	+	+	+	+	+	+
2	–	–	ND	–	–	–	–	–
3	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–

(+) Presence; (–) absence; ND: not done.

* Tissue samples from experimentally infected dogs with *T. gondii* were inoculated orally and/or intraperitoneally into five mice for each tissue.

1:8192 (Fig. 5A). IgG-ELISA showed similar kinetics with seroconversion at seventh day after infection (Fig. 5B). In both assays, all dogs exhibited detectable IgG levels throughout the experiment, although dogs 2 and 3 had lower IgG titers.

3.6. *T. gondii* detection by mouse bioassay and immunohistochemistry

Immunohistochemical assay revealed the presence of free tachyzoites and parasites inside parasitophorous vacuoles in the cytoplasm of cells from spleen, heart and lung in dog 1 only (Fig. 6). In addition, *T. gondii* was also isolated from the brain, heart, spleen, skeletal muscle, eye, kidney, lung, and tongue after bioassay in mice (Table 1). *T. gondii* was found neither by immunohistochemical nor mouse bioassay in the remaining dogs, and all surviving mice were considered seronegative after being tested by IgG-ELISA.

4. Discussion

High rates of infection with *T. gondii* are observed in dogs, however clinical toxoplasmosis is rare. It is usually seen in young animals, often associated with concurrent infections such as canine distemper (Fontaine et al., 1986; Rhyen and Dubey, 1992). Diagnosis of canine clinical toxoplasmosis is difficult because the available serological tests do not distinguish between chronic and active infection, and clinical signs are vague and resemble the newly recognized *N. caninum* infection (Mineo et al., 2001). In dogs, little is known about the antibody production in the acute phase of *T. gondii* infection, and a test that could detect IgM antibody is necessary to differentiate active from latent infection.

Conventional serological tests still present drawbacks in assessing IgM antibodies in sera from patients with acute toxoplasmosis. IgM-IFAT and IgM-ELISA require additional serum processing to block rheumatoid factor or remove IgG antibodies in order to produce more sensitive and reliable results (Camargo et al., 1983). A remarkable difference in the antibody profile between 2-ME treated and untreated serum samples indicates the presence of high levels of IgM antibodies. Nevertheless, the serum treatment with 2-ME used in the conventional IHA test fails to detect low levels of IgM antibodies (Camargo et al., 1989). On the other hand, IgM capture ELISA is based on an immunological separation of IgM from the test sample before the reaction with antigen, eliminating competitive IgG antibodies

and minimizing nonspecific reactions due to rheumatoid factors in human sera (Duermeier et al., 1979; Mineo et al., 1986). In contrast to the other conventional IgM-ELISA techniques, the decrease of sensitivity due to competition between different classes of antibodies for antigenic determinants may be frequently minimized when using capture ELISA.

In our investigation, specific IgM antibody kinetics was followed in *T. gondii*-infected dogs. In all animals, McELISA detected IgM isotype early on and for a longer period than IHA with 2-ME, emphasizing the potential role of IgM capture ELISA in diagnosing active canine toxoplasmosis. In addition, sensitivity and specificity results showed that McELISA was able to detect specific IgM antibodies in all sera from the dogs with *T. gondii* active infection, with no reactivity in both negative and positive IgG serum groups. Due to the unavailability of *N. caninum*-positive IgM sera, we have tested group IV sera with high IgG-ELISA titers to this parasite. Our results showed that although GM values with 95% CI were higher in this group than groups II and III, only two samples had EI values on the borderline.

Anti-human IgM antibodies cross-react with canine IgM. This cross-reactivity has been demonstrated by rocketimmunoelectrophoresis (Hau et al., 1990), and by a capture ELISA for the detection of CDV specific IgM antibodies, which were also detected in dog and mink sera (Blixenkrone-Møller et al., 1991). In the present study, the cross-reactivity between canine and human IgM antibodies was also demonstrated to be functional to diagnose *T. gondii* active infection as shown by high and significant positive correlation between these heterologous antibodies.

As previously shown by Jacobs et al. (1955) and Sharma et al. (1973), we observed that all dogs inoculated with the highly virulent RH strain of *T. gondii* seroconverted as determined by various serological assays. Although all animals exhibited moderate symptoms, parasites were found by immunohistochemistry and mouse bioassay only in dog 1. In contrast to an investigation in dogs experimentally inoculated with tissue cysts of an avirulent strain (Lindsay et al., 1996), our results indicated an early immune response since specific antibodies were observed by IHA and McELISA as early as 7–10 days and remained at detectable levels up to around 40 days p.i.

In contrast to the IgM profile, IgG antibody titers were detected throughout the experiment. Recently, we considered titers of 1:16 and 1:64 to be positive in IFAT and ELISA, respectively, due to the optimization of such cut-off titers based on the reactivity to SAG-1 antigen of *T. gondii* (Silva et al., 2002). Accordingly, the IgG response measured by IFAT and ELISA demonstrated that the antibody peak occurred between 20th and 40th days after infection, overlapping with the IgM peak. These data agree with a previous investigation, describing that the highest antibody titers were found about four weeks post-inoculation with *T. gondii* (Jacobs et al., 1955).

Additionally, in our investigation *T. gondii*-specific IgM and IgG antibodies were more precociously detected in experimentally infected dogs comparing to previous studies using experimentally infected cats (Dubey et al., 1995a), despite that both investigations have shown short-lived IgM and long-term IgG antibody detection.

Altogether, the results showed herein demonstrate that acute *T. gondii* infection in dogs can be characterized by a remarkably transient IgM isotype synthesis, and this feature may constitute an important marker of active infection. In addition, McELISA was shown to be a potential tool for the diagnosis of acute toxoplasmosis in dogs, particularly by using

heterologous antibodies, such as anti-human IgM, which are available as highly purified reagents.

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