



Revista Brasileira de Parasitologia  
Veterinária

ISSN: 0103-846X

zacariascbpv@fcav.unesp.br

Colégio Brasileiro de Parasitologia  
Veterinária  
Brasil

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Revista Brasileira de Parasitologia Veterinária, vol. 25, núm. 1, enero-marzo, 2016, pp. 99  
-104

Colégio Brasileiro de Parasitologia Veterinária  
Jaboticabal, Brasil

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# Kinetics and avidity of anti-*Toxocara* antibodies (IgG) in rabbits experimentally infected with *Toxocara canis*

Cinética e avidéz de anticorpos (IgG) anti-*Toxocara* em coelhos experimentalmente infectados com *Toxocara canis*

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Received March 2, 2015

Accepted June 19, 2015

## Abstract

An evaluation was made of the kinetics and avidity of anti-*Toxocara* antibodies (IgG) in rabbits experimentally infected with embryonated *Toxocara canis* eggs. Seventeen four month old New Zealand White rabbits were distributed into two groups. In the experimental group, twelve rabbits were infected orally with 1,000 embryonated *T. canis* eggs. A second group (n = 5), uninfected, was used as a control. Serum samples were collected for analysis on days 7, 14, 21, 28 and 60 post-infection (DPI). An indirect ELISA test was performed to evaluate the reactivity index (RI) of IgG anti-*T. canis* antibodies and to calculate the avidity index (AI). The animals showed seroconversion from the 14<sup>th</sup> DPI, with high AI (over 50%) except for one animal, which presented an intermediate AI. At 60 DPI, all the animals were seropositive and maintained a high AI. The data indicated that specific IgG antibodies formed early (14 DPI) in rabbits infected with *T. canis*, with a high avidity index that persisted throughout the course of the infection.

**Keywords:** Toxocariasis, antibody avidity, immune response, ELISA.

## Resumo

O objetivo deste estudo foi o de avaliar a cinética e a avidéz de anticorpos anti-*Toxocara canis*, em coelhas infectadas experimentalmente com ovos embrionados de *Toxocara canis*. Foram utilizados 17 coelhos New Zealand de linhagem branca, com quatro meses de idade, distribuídos em dois grupos. No grupo experimental, doze coelhas foram infectadas, oralmente, com 1.000 ovos larvados de *T. canis*. Um segundo grupo (n=5), não infectado, foi utilizado como controle. Nos dias 7, 14, 21, 28 e 60 pós-infecção (DPI), foram coletadas amostras de soro para análise. O teste de ELISA indireto foi realizado para avaliar o índice de reatividade (IR) de anticorpos IgG anti-*T. canis* e para cálculo do índice de avidéz (IA). A soroconversão nos animais ocorreu a partir do 14<sup>o</sup> DPI, com verificação de alto IA (superior a 50%), com exceção de um animal, que apresentou médio IA. Aos 60 DPI, todos os animais foram soropositivos e mantiveram alto IA. Os dados mostram que em coelhos infectados por *T. canis*, anticorpos IgG específicos formam-se precocemente (14 DPI), apresentando alto índice de avidéz e que se mantém durante o curso da infecção.

**Palavras-chave:** Toxocaríase, avidéz de anticorpos, resposta imune, ELISA.

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## Introduction

Toxocariasis, although little recognized as a public health problem, is considered an important worldwide zoonosis (RUBINSKY-ELEFANT et al., 2010). In humans, the principal route of transmission is by ingestion of embryonated *Toxocara canis* or *T. cati* eggs, which are parasites of dogs and cats, respectively (DESPOMMIER, 2003).

Humans act as paratenic hosts of *Toxocara* spp. After hatching in the intestine, the larvae migrate through the organism and can cause hemorrhage, necrosis and eosinophilic inflammation, or be encapsulated in granulomas, either being destroyed or remaining viable for many years (MAGNAVAL et al., 2001).

Human toxocariasis is diagnosed based on clinical, epidemiological and laboratory data, primarily using the ELISA test, whose sensitivity ranges from 78.3% (GLICKMAN et al., 1978) to 100% (RUBINSKY-ELEFANT et al., 2006; ROLDÁN et al., 2006), and specificity from 86% (JACQUIER et al., 1991) to 97.4% (SAVIGNY et al., 1979), for the detection of IgG anti-*Toxocara* immunoglobulins. In clinical studies on humans, Hubner et al. (2001) and Dziemian et al. (2008) reported that, in chronic cases of the disease, there is high IgG avidity, while low avidity is noted in acute infections. However, the persistence of the antibodies over a long period, due to antigenic stimulation caused by the maintenance of the *Toxocara* spp. larvae in the tissues of paratenic hosts, can make it difficult to classify the infection as acute or chronic (FENOY et al., 1992; RUBINSKY-ELEFANT et al., 2006).

The literature on the avidity of anti-*Toxocara* antibodies in hosts is scanty and limited to mice (FENOY et al., 2008; SCHOENARDIE et al., 2014). Therefore, studying the avidity of anti-*Toxocara* spp. antibodies in other animal models may help shed light on the profile of the humoral immune response to toxocariasis.

This study involved an investigation into the kinetics and avidity of anti-*Toxocara canis* antibodies in experimentally infected rabbits.

## Materials and Methods

### Animals

Seventeen 4-month old New Zealand White rabbits from the UNOESTE vivarium were used. To confirm the negative diagnosis of enteroparasites, three fecal samples collected in three consecutive weeks prior to infection were analyzed using the Willis-Mollay and Hoffman techniques (HOFFMANN, 1987). The project was approved by the Animal Research Ethics Committee of the University of Western São Paulo – UNOESTE in Presidente Prudente, SP, Brazil (under Protocol 132/09).

### Experimental groups

The rabbits in this study were divided into two groups. The first group consisted of 12 females artificially infected with 1000 *T. canis* eggs, and the second comprised five uninfected females, which served as control.

Throughout the experimental period, the animals received commercial rabbit feed pellets and water *ad libitum* and were housed individually in suspended galvanized wire mesh cages in a biotery.

### Obtaining *Toxocara canis* eggs

*T. canis* eggs were obtained from adult females which were released from naturally infected puppies kept in the kennel at UNOESTE.

To obtain the eggs, the anterior third of the uteruses of the female nematodes were dissected (FAN et al., 2003) and stored for five weeks at 27 °C for embryonation. After this period, the material was washed three times in saline solution by centrifugation (922 x g) for three minutes. A total of 1000 embryonated eggs were counted in a Neubauer chamber, and diluted in 3.0 mL of phosphate buffered saline (PBS) solution for experimental infection.

### Infection of the animals

Prior to experimental infection, the animals were sedated by intraperitoneal administration of 30mg/kg of combined tiletamine/zolazepam (Zoletil 5%, Virbac), diluted in saline (KANASHIRO & CASSU, 2008).

The animals were infected using the procedure described by Pecinali et al. (2005), with minor modifications. The animals were orally inoculated with 3.0 mL of the solution containing embryonated eggs, using a gastric gavage. Another 3.0 mL of PBS was administered to the animals via the same route to ensure the passage of the eggs. The control group received only PBS, following the same procedures for the administration of the anesthetic and inoculum.

To obtain the serum, blood samples were collected through puncture of the central auricular artery at the following time points; 0 (pre-infection), 7, 14, 21, 28 and 60 days post-infection (DPI).

### Excretory-secretory *T. canis* antigen (TES) production

The *T. canis* excretory-secretory larval antigen (TES) for ELISA was prepared as described previously (SAVIGNY, 1975), with some modifications (RUBINSKY-ELEFANT et al., 2006). Briefly, *T. canis* eggs collected from the uterus of adult female worms were embryonated after incubation in 2% formalin for approximately 1 month at 28 °C. Following, the material was washed by centrifugation (3 minutes at 1,200 g) in 0.9% NaCl until the formalin solution was completely removed. Eggshells were broken by slow homogenisation in an Erlenmeyer flask, and L3 larvae were collected by using a Baermann apparatus in serum-free Eagle's medium. The supernatant of the larvae culture was weekly collected, and the protease inhibitor phenylmethylsulphonyl fluoride (PMSF, Sigma, 0.2 M, 5.0 µL/mL) added. The collected supernatant was concentrated in Amicon Ultrafiltration units (Millipore, Danvers, MA), dialysed against distilled water, centrifuged (15,000 g for 30 minutes at 4 °C), and filtered using 0.22 µm Millipore membranes. The Lowry method (LOWRY et al., 1951) was employed to determine the protein content.

### ELISA (enzyme-linked immunosorbent assay)

The ELISA test was performed to evaluate the production of anti-*T. canis* antibodies, using the protocol described by Savigny et al. (1979) with modifications (RUBINSKY-ELEFANT et al., 2006).

To perform the test, 96-well polystyrene microplates (Corning, Costar, New York, NY) were coated (1.9 µg/mL antigen/well) with excretion-secretion antigens produced by the *T. canis* (L<sub>3</sub>) larvae (TES) and tested for 2 hours at 37 °C, followed by 18 hours at 4 °C. The microplates were then washed three times for 5 minutes with phosphate buffered saline (PBS) 0.01M, pH 7.2, containing 0.05% Tween-20 (PBS-T). The microplates were blocked with 2.5% skimmed milk (Molico, Nestlé) in PBS-T (200 µL/well) for 1 hour at 37 °C and then washed three times with PBS-T.

The serum samples (100 µL/well) were diluted at 1:200 and incubated for 40 minutes at 37 °C, in duplicate. After three washing cycles, the plates were incubated with conjugated goat anti-rabbit IgG (100 µL/well; Sigma-Aldrich A0545, USA) at a dilution of 1:40,000 in PBS-T for 40 minutes at 37 °C. After another round of washing, the plates were incubated with chromogenic solution (100µL/well; OPD Fast-Sigma, St Louis, EUA), comprising ortho-phenylenediamine (0.4 mg/mL) and H<sub>2</sub>O<sub>2</sub>-urea (0.4 mg/mL) in 0.05M of phosphate-citrate buffer for 15 minutes at 37 °C. The reaction was stopped with H<sub>2</sub>SO<sub>4</sub> 2N (50 µL/well), and the optical densities (OD) were measured at 492 nm (Titertek Multiskan MCC/340, Lab-System, Finland).

The cut-off point was calculated from the mean optical densities of sera from 17 uninfected animals (5 animals in the control group and 12 pre-infection animals) plus two standard deviations. The antibody levels were expressed as reactivity indices (RIs), calculated as the ratio between the absorbance value of the optical density of each tested sample and the optical density at the cut-off point (0.292). The samples with RIs above 1 were considered positive.

### Avidity of antibodies (IgG)

The dissociative method was used to study avidity, with urea as the denaturing agent (HEDMAN et al., 1989). To evaluate the possibility of the denaturing agent acting on the absorbed antigen in the solid phase and interfering in the results, the sensitized and blocked ELISA plate was first treated for 5 minutes with 8M urea prior to performing the assay. To determine the avidity of antibodies, serum samples and conjugate dilutions were the same for ELISA assay, according to the previous description, except that sera were applied in duplicate to the microtiter wells. After incubation for 40 min at 37 °C, one well of the doublet was washed with PBS-T three times for 5 min; likewise, the other well was washed with 8M urea dissolved in PBS-T. The avidity index (AI) was calculated as the ratio between the OD sample treated with urea and the OD sample not treated with urea, multiplied by 100, according to the formula:

$$AI(\%) = (OD \text{ with urea} / OD \text{ without urea}) \times 100$$

Only the samples with RIs above 1 were considered for AI calculation. The avidity indices were established according to

Hedman & Rousseau (1989), where: AI lower than 30% indicates predominance of low-avidity antibodies; AI between 30 and 50% indicates intermediate-avidity antibodies; and AI above 50% indicates high-avidity antibodies.

### Statistical analysis

The mean values of the avidity and reactivity indices were calculated from the data obtained. The AI and RI of the sera of the females were compared using the paired t-test, while the correlation between the two indices was evaluated via a simple linear regression analysis. The adequacy of the model was tested using residual analysis and adjustment of statistics, with a coefficient of determination (R<sup>2</sup>). The significance level used in all the comparisons was 5%. All the analyses were performed with the aid of SPSS v. 13.0 software for Windows (FIELD, 2009).

## Results

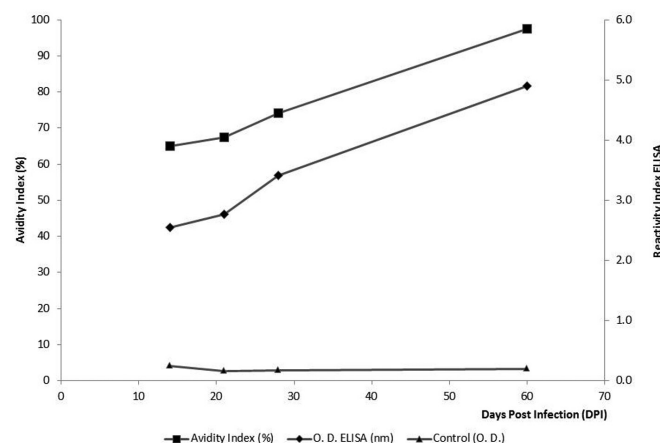
Seroconversion of the infected animals was observed on the 14<sup>th</sup> DPI in 41.7%, followed by a gradual increase in RI. Positive RI frequencies of 83.3%; 91.7% and 100% were observed on the 21<sup>st</sup>, 28<sup>th</sup> and 60<sup>th</sup> DPI, respectively.

Only one animal demonstrated seroconversion on the 60<sup>th</sup> DPI, with a slightly lower RI than that of the other animals (RI = 4.457).

The average RIs were 1.064, 2.264, 3.081 and 4.895 on the 14<sup>th</sup>, 21<sup>st</sup>, 28<sup>th</sup> and 60<sup>th</sup> DPI, respectively (Figure 1). The average RIs on the 28<sup>th</sup> and 60<sup>th</sup> DPI presented a statistically significant difference ( $p = 0.0019$ ).

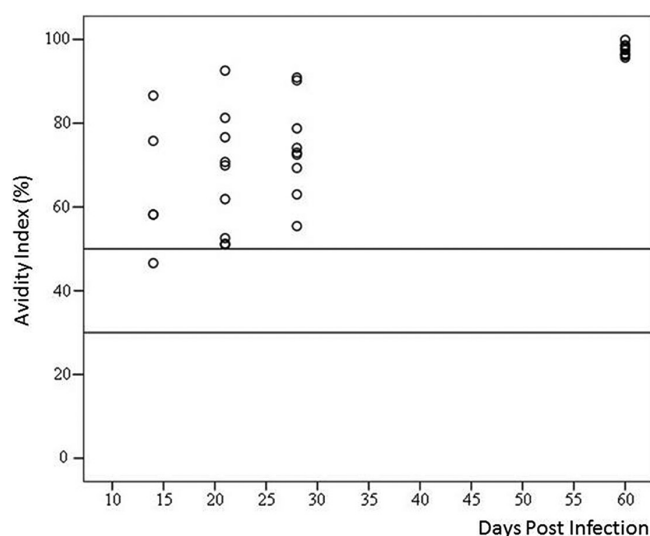
Figure 2 shows the classification of the Avidity Index (AI) of the animals. All the animals that seroconverted had high-avidity antibodies, except for one animal which presented intermediate-avidity antibodies (IA = 46.6%) on the 14<sup>th</sup> DPI.

The AI ranged from 46.6% to 86.6% (mean AI = 65.1%) at 14 DPI, 51.0 to 92.6% (mean AI = 67.5%) at 21 DPI, and



**Figure 1.** Avidity index (AI; %) and Reactivity index (RI) for antibodies (IgG) detected by enzyme linked immunosorbent assay (ELISA) in rabbits experimentally infected with *Toxocara canis* at different post-infection time points (DPI).





**Figure 2.** Avidity index (%) of IgG anti-*Toxocara canis* in experimentally infected rabbits, detected by enzyme linked immunosorbent assay (ELISA) at different post-infection time points (DPI). Classification of AI: less than 30: predominance of low avidity; higher than or equal to 30 and less than 50: predominance of intermediate avidity; equal to or higher than 50: predominance of high avidity.

**Table 1.** Correlation coefficient between reactivity and avidity indices for detection of IgG antibodies by enzyme linked immunosorbent assay (ELISA) in rabbits experimentally infected with *Toxocara canis* at different time points after infection.

Post-infection Time Points (days)	R	CI 95%	P
7	NC	-	-
14	NC	-	-
21	0.3567	-0.64-0.91	0.4876
28	0.4597	-0.56-0.93	0.3590
60	0.8765	0.36-0.98	0.0096

NC: Not calculated: Reactivity Index of IgG <1.0. R: Correlation coefficient. p: Level of significance. P values < 0.05 indicates statistical significance.

55.4% to 90.9% (mean AI = 74.1%) at 28 DPI. At 60 DPI, the AI ranged from 95.7% to 99.9% (mean AI = 97.4%).

The correlation between the RI and AI was evaluated 21, 28 and 60 DPI, since the RIs on the 7<sup>th</sup> and 14<sup>th</sup> DPI were inconsistent for calculation. A statistically significant correlation (R = 0.8765; p = 0.0096) was observed at 60 DPI (Table 1).

## Discussion

The novelty of this study was to evaluate the kinetics and avidity of anti-*Toxocara* antibodies (IgG) in rabbits experimentally infected with a single dose of embryonated *T. canis* eggs.

Seroconversion was observed from the 14<sup>th</sup> DPI, followed by a gradual increase in positive animals over the infection time. Similarly, Morales et al. (2002) verified the same response pattern using Western blot for detecting anti-*Toxocara* antibodies in rabbits

(the same strain and age). Variations in seroconversion time have been observed in paratenic hosts experimentally infected with *Toxocara* spp. In pigs, anti-*Toxocara* antibodies were observed at 7 (SOMMERFELT et al., 2001) and 14 DPI (TAIRA et al., 2003) and in sheep at 21 DPI (SANTARÉM et al., 2011). This phenomenon has also been observed in murines, which are considered the experimental model for human toxocariasis. In mice, seroconversion was observed at 7 (OLLERO et al., 2008), 14 (KOLBEKOVÁ et al., 2011) and 23 DPI (LESCANO et al., 2012).

There are a number of factors that may determine the pattern of immune response and pathogenesis in paratenic hosts, including individual characteristics (CUELLAR et al., 2001), the infective dose and number of reinfections (KAYES et al., 1985; OLLERO et al., 2008). Our findings reinforce the statement that individuality may explain a difference in responsiveness to antigens by each individual animal, since some of the rabbits (41.7%) seroconverted in the early days of infection (14 DPI), followed by observation of seroconversion at different time points, including one registered at 60 DPI.

Experimental studies have shown the influence of infective dose on the immune response to toxocariasis. In pigs experimentally infected with doses of 1000 or 2000 eggs, significant differences were observed in titer values related to dose, with higher titers for the group dosed with 2000 eggs during the period between 2 and 7 weeks post-infection (SOMMERFELT et al., 2001). Havasiová-Reiterová et al. (1995) evaluated the effects of 5 to 2,500 infective *Toxocara canis* eggs, observing a faster production of antibodies in mice infected with a higher dose of eggs. In our study, the rabbits were infected with a single dose of 1000 eggs, and interestingly, the observed response pattern of the animals was very similar to that described by Morales et al. (2002), using an infective dose of 5000 eggs.

Early antibody response in experimentally infected paratenic hosts may be detected by ELISA (SOMMERFELT et al., 2001), however the detection of specific IgG antibodies, does not allow differentiation between acute and chronic phases of toxocariasis (RUBINSKY-ELEFANT et al., 2010). The use of antibody avidity measurements has been widely employed to distinguish the acute and chronic phases of several parasitic diseases that affect humans, such as schistosomiasis (MOSTAFA et al., 2002), fascioliasis (ABOU-BASHA et al., 2000), neurocysticercosis (MANHANI et al., 2009), toxoplasmosis (CANDOLFI et al., 2007; GAY-ANDRIEU et al., 2009) and toxocariasis (HUBNER et al. 2001; DZIEMIAN et al., 2008).

Regarding human toxocariasis, the chronic phase is characterized by high IgG avidity, while low avidity is observed in acute infections (HUBNER et al., 2001; DZIEMIAN et al., 2008). Some authors, however, find it difficult to classify the infection as acute or chronic, due to the persistence of antibodies over a long period as a consequence of the antigenic stimulation caused by *Toxocara* spp. larvae (GLICKMAN et al., 1986; FENOY et al., 1992; RUBINSKY-ELEFANT et al., 2006). Therefore, experimental studies, particularly in murines, have been carried out to evaluate whether antibody avidity is a useful model for identification of acute and chronic toxocariasis.

In mice infected with a single dose or with multiple infections of *T. canis* eggs, a high AI (about 50%) was observed 40 to 60 DPI

(FENOY et al., 2008). In BALB/c mice infected with a single dose of 1000 eggs, a percentage drop (low avidity: 7.25 to 27.5%) was verified at 15 DPI, an intermediate avidity for IgG up to 45 DPI (27.5 to 31.4%), and IgG AI between 31.4 and 58% at 60 DPI (SCHOENARDIE et al., 2014). In our study, however, high avidity IgG was detected early after infection (mean AI = 65.1%) and gradually increased for at least 2 months (mean AI = 97.4%), resulting in a strong association between the Avidity and Reactivity Indices at 60 DPI.

The present study has some limitations. Several experimental studies have evaluated the subclasses of immunoglobulins involved in toxocariasis (KAYES et al., 1985; OLLERO et al., 2008). We used the indirect ELISA based on the detection of total IgG anti-*Toxocara*, which is currently the standard serological method for the diagnosis of human toxocariasis (RUBINSKY-ELEFANT et al., 2010; MACPHERSON, 2013). In our study, we evaluated a small sample size to minimize the number of evaluated animals. In terms of challenge, the infection of the rabbits was limited to a single dose of 1000 eggs, which has been used in other studies with murine models (XI & JIN, 1998; CHO et al., 2007; SCHOENARDIE et al., 2014). Thus, future studies are needed, designed to evaluate different subclasses of immunoglobulins and IgG avidity in rabbits subjected to different doses of infection in a long term study, and a larger sample size, to precisely explain the wide individual variations in immune response and to demonstrate the possibility of using rabbits as an animal model for toxocariasis.

Given these considerations and limitations our data suggest that in rabbits infected with *T. canis*, specific IgG antibodies formed early after infection and a high avidity index was maintained at relatively high levels.

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