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Determination of IgG avidity in BALB/c mice experimentally infected with *Toxocara canis*

Determinação da avidéz de IgG em camundongos BALB/c experimentalmente infectados com *Toxocara canis*

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Abstract

Toxocariasis is a zoonotic disease in that IgM titers can remain high for long periods making difficult to determine the stage of the disease. The aim of this study is to investigate the applicability of indirect ELISA, associated with urea, to discriminate between the acute and chronic toxocariasis. IgG avidity was evaluated in 25 BALB/c mice experimentally infected with 1000 *Toxocara canis* eggs. Blood samples were collected, and sera treated with 6 M urea and assayed by ELISA every two weeks. The percent IgG avidity was determined using the mean absorbance of sera treated with urea, divided by the mean absorbance of untreated sera. In the first 15 days post-inoculation, was observed a low percentage, between 7.25 and 27.5%, IgG avidity, characteristic of an acute infection. After 60 days of infection, all the mice showed between 31.4 and 58% IgG avidity, indicating a chronic infection.

Keywords: Visceral larva migrans, toxocariasis, acute and chronic infections, ELISA.

Resumo

A toxocaríase é uma zoonose na qual os títulos de IgM podem permanecer elevados por longos períodos, tornando difícil a determinação do estágio em que a doença se encontra. O objetivo deste estudo foi investigar a aplicabilidade de um teste indireto de ELISA, associado com ureia, para fazer a discriminação entre as fases aguda e crônica da toxocaríase. A avidéz de IgG foi avaliada em 25 camundongos BALB/c experimentalmente infectados com 1000 ovos embrionados de *Toxocara canis*. A cada duas semanas, amostras de sangue foram coletadas, o soro tratado com ureia 6M e realizado o ensaio pela técnica de ELISA. O percentual de avidéz de IgG foi determinado, usando-se a média das absorbâncias dos soros tratados com ureia dividida pela média das absorbâncias dos soros não tratados. Nos primeiros 15 dias pós-inoculação, foi observado um baixo percentual de avidéz de IgG, entre 7,25 e 27,5%, característico da fase aguda da infecção. Após 60 dias de infecção, todos apresentaram avidéz de IgG entre 31,4 e 58%, indicando a fase crônica da infecção.

Palavras-chave: Larva migrans visceral, toxocaríase, infecção aguda e crônica, ELISA.

Introduction

Toxocariasis is a zoonotic disease with worldwide distribution, caused by infection with larvae of *Toxocara canis* and *Toxocara cati*, whose definitive hosts are dogs and cats, respectively (DESPOMMIER, 2003). Children are more susceptible because of their habits of geophagy, onychophagy, poor hygienic conditions

and their larger risk of exposure to soil contaminated with parasite eggs (ALDERETE et al., 2003; SMITH et al., 2009). In tropical and developing countries, this parasite is considered a public health problem (HAYASHI et al., 2005). Clinical diagnosis is difficult due to the nonspecific signs and symptoms, making the use of immunological methods indispensable (FILLAUX; MAGNAVAL, 2013). Principally determinations of IgG, IgM and IgE are used, as hypergammaglobulinemia is an indicator of infection (DESPOMMIER, 2003). However, assessment of antibody levels alone does not determine the stage of the disease

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because, even though characteristic of acute infectious disease, IgM titers can remain high for long periods of time in *T. canis* infection (BOWMAN et al., 1987; HAVASIOVÁ-REITEROVÁ, 1995). Therefore, it is important to conduct studies to investigate IgG avidity when the serum is a reagent for both IgG and IgM (ALVARADO-ESQUIVEL et al., 2002).

Avidity is the term used to express the strength of the bond between the antibody and the multivalent antigen (HUBNER et al., 2001). The determination of IgG avidity is an important serologic marker because low and high avidity are found predominantly in early and late infection phases, respectively (FENOY et al., 2008). Studies using the ELISA immunoassay with urea, a denaturant agent that can dissociate the antigen-antibody interaction when the avidity is low (BERTOZZI et al., 1999), have been conducted to measure the IgG avidity percentage in human toxocariasis (HUBNER et al., 2001; ELEFANT et al., 2006). In experimental model, only two studies on avidity of specific IgG against TES were done (FENOY et al., 2008; KOLBEKOVÁ et al., 2011). The aim of this study is to investigate the applicability of indirect ELISA, associated with urea, to discriminate between the stages of toxocariasis infection in experimentally infected BALB/c mice.

Materials and methods

Parasites

Adult forms of *T. canis* were obtained by administration of pyrantel pamoate (15 mg/kg) to four- to eight-week-old dogs. Through hysterectomies performed on female worms, parasite eggs were obtained and incubated for 28 days in 2% formalin at 28 °C to allow embryonic development.

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

For production of TES, successive washings of the embryonated eggs, using centrifugation and phosphate-buffered saline (PBS) 0.15 M, pH 7.2, were performed. Subsequently, the eggs were treated with a solution of 5.6% sodium hypochlorite for 10 to 15 minutes. The eggs were washed again with PBS to remove the sodium hypochlorite and submitted, under aseptic conditions, to slow mechanical agitation in flasks with glass beads and RPMI 1640 medium at 37 °C (supplemented with HEPES 25 mM, Glucose 1%, penicillin 100 IU / ml, streptomycin 100 µg / ml, ofloxacin 0.4 µg / ml, and fungizone at 50 µg / ml). The larvae were then concentrated using the Baermann technique and cultured in RPMI 1640 medium at 37 °C and 5-6% CO₂ (DE SAVIGNY, 1975). The culture supernatant was harvested weekly, filtered through a 22-µm pore membrane (Millipore) (PAGE et al., 1991) and stored at -20 °C with protease inhibitors (PMSF - phenylmethylsulfonyl fluoride; 1 mM) (MAIZELS et al., 1991). The stored material was then mixed, concentrated by ultrafiltration (Sigma Stirred Cell - 10 kDa), dialyzed against ultrapure water (Milli-Q) at 4 °C for 24 hours, and then lyophilized. This procedure yielded TES, which was resuspended in ultrapure water and stored in aliquots at -70 °C. The TES antigen protein content was determined using a modified Bradford method (BCA kit, Pierce) (BRADFORD, 1976).

Mice

Twenty-five eight-week-old BALB/c mice were inoculated by a gastric tube with 1000 embryonated *T. canis* eggs. We evaluated the levels of anti-TES at different stages of infection. Blood samples were collected prior to inoculation (day zero) and at 15, 30, 45, 60, 75, 90, and 105 days post-inoculation. The study was approved by the Ethics in Research Committee at the Federal University of Pelotas. The experiments were carried out following the Federal Government Legislation on Animal Care.

ELISA test

Sera from mice on the different post-inoculation days were assayed (in duplicate) by ELISA, both with and without urea. Different concentrations of urea (4 M, 5 M and 6 M), plate wash times (one, two and three minutes) and numbers of washes (one, two and three times) were tested to standardize the technique. To sensitize the plate, we used TES antigen (1 µg/ml) in carbonate/bicarbonate buffer, pH 9.6 for three hours at 37 °C, followed by blocking for one hour with PBS 2% casein and adding the sera of the inoculated animals (1:50) in PBS-T for 45 minutes. Then, the plate was washed with PBS-T either containing urea or not containing urea, and the conjugate was added (anti-mouse IgG linked to peroxidase - 1:2000). The substrate orthophenylenediamine (OPD) was used at a concentration of 0.4 mg/ml in citrate-phosphate buffer (pH 4.0) with hydrogen peroxide 0.1%. Readings were performed on a spectrophotometer (450 nm). The ELISA avidity test was performed according to Fenoy et al. (2008), with modifications. The calculation of the IgG avidity was determined using the arithmetic mean absorbance obtained in sera treated with urea divided by the mean absorbance obtained from untreated sera and was expressed as a percentage (BERTOZZI et al., 1999). Statistical analysis was performed using Statistix 8. To compare the absorbance of sera (treated or untreated with urea) on different days after inoculation, we used variance analysis with repeated measurements and compared the averages using the "Least Significant Difference" (LSD).

Results and discussion

To assess the avidity of IgG against TES, the ELISA was first standardized with different concentrations of urea, obtaining the best result with a concentration of 6 M and a two-minute plate wash. IgG levels in the sera after treatment with urea were reduced significantly ($P < 0.01$) in all periods analyzed (Figure 1). When analyzing the percentages of IgG, in each of the twenty-five BALB/c mice, we observed a percentage drop (to low avidity) at 15 days post infection (7.25 to 27.5%), suggesting that acute infection had begun in this period. An intermediate avidity for IgG was observed until 45 days post-inoculation (between 27.5 and 31.4%), and at 60 days post-infection, all the animals presented an IgG avidity index of between 31.4 and 58% (Figure 2), which characterizes the chronic phase of toxocariasis (FENOY et al., 2008). The result of the present study are according to Fenoy et al. (2008) that found high-avidity of IgG antibodies (over 50%) 60 days post-infection

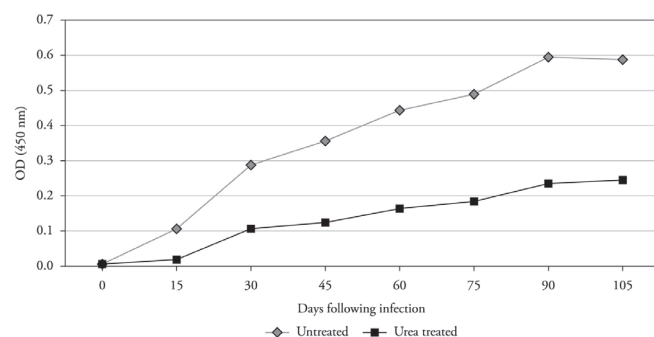


Figure 1. Mean absorbance of IgG in serum of mice experimentally infected with *T. canis*, untreated or treated with 6 M urea. Treated and untreated sera in the graph differ significantly ($P < 0.01$) for all post-inoculation days evaluated.

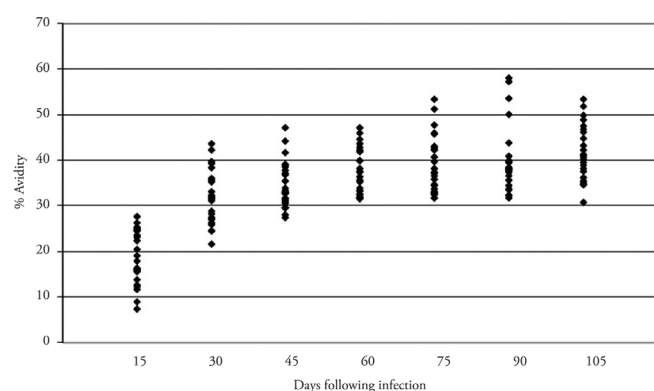


Figure 2. Percentage of avidity of anti-TES IgG using Indirect ELISA in 25 experimentally infected mice with 1000 eggs each of *T. canis* on different days post-inoculation.

of BALB/c with 1000 *T. canis* eggs. However, another study using BALB/c mice reported high-avidity of IgG antibodies at only 30 days post-infection of 1000 larvae (KOLBEKOVÁ et al., 2011).

Similar data were obtained from serological analysis of 1376 individuals in the Czech Republic, where an avidity index higher than 40% was presented, characterizing chronic infection (HUBNER et al., 2001). Although mice are the most widely used experimental models in the study of toxocariasis, they may exhibit some differences in the avidity of IgG antibodies for TES. This difference was observed in *Toxoplasma gondii* infection, in both humans and sheep, where chronic infection in humans was established when the percentage of IgG avidity was higher than 40%, which occurred after five months of infection (BERTOZZI et al., 1999). In sheep, the percentage of avidity was greater than 35%, which occurred after the fourth week of infection (SAGER et al., 2003).

The evaluation of IgG avidity has been widely used in the routine diagnosis of various infectious diseases, not only toxoplasmosis (LAPPALAINEN; HEDMAN, 2004; RAHBARI et al., 2012), but also rubella (REIS et al., 2004), varicella (ONO et al., 2004; KNEITZ et al., 2004) and cytomegalovirus (BONALUMI et al., 2011), been very useful for assessing seroconversion during gestation (BOBIC et al., 2009). The avidity of IgG antibody against TES

is potentially useful for immunodiagnostics of toxocariasis and should be included in the complementary diagnosis when IgM levels remain high throughout the infection. The present study demonstrated that the ELISA enzyme immunoassay with urea is an alternative for discriminating the stages of toxocariasis infections in BALB/c mice. The difficulty in diagnosing an active infection by *T. canis* is a serious problem, and many studies are still needed.

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