

IDENTIFICATION OF *Toxocara canis* ANTIGENS BY WESTERN BLOT IN EXPERIMENTALLY INFECTED RABBITS

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SUMMARY

Toxocariasis is a frequent helminthiasis that can cause visceral and ocular damage in humans specially in children. The identification of specific antigens of *Toxocara canis* is important in order to develop better diagnostic techniques. Ten rabbits were infected orally with a dose of 5000 *Toxocara canis* embryonated eggs. Rabbits were bled periodically and an ELISA assay was performed to determine levels of specific *Toxocara* IgG antibodies. ELISA detected antibodies at day 15 after infection. Western blot (WB) assay was performed using excretory/secretory antigens (E/S) of *T. canis* second stage larvae. Different antigen concentrations were evaluated: 150, 200, 250 and 300 µg/mL. The concentration of 250 µg/mL was retained for analysis. Rabbit sera were diluted 1:100. Secondary antibody was used at a dilution of 1:1000. Results of WB indicated that in the first month after infection specific antibodies against the 200 KDa, 116 KDa, 92 KDa and 35 KDa antigens were detected; antibodies against the 92 KDa, 80 KDa, 66 KDa, 45 KDa, 31 KDa and 28 KDa antigens appeared later. All positive sera in the ELISA test were also positive in WB. Two antigen bands, 92 KDa and 35 KDa, were identified since the beginning and throughout the course of infection. These antigens merit further evaluation as candidates for use in diagnosis.

KEY WORDS: *Toxocara canis*; Immunoblot; ELISA; Antibodies; Diagnosis.

INTRODUCTION

Toxocariasis is one of the parasitic diseases which constitute a public health problem in Colombia¹. Its causal agent, the nematode helminth *Toxocara canis*, is an ascaridae frequently present in dogs²⁰. Toxocariasis is a disease whose clinical manifestations are unspecific, characterized by larval migration to inner organs of humans and some animals¹⁰. In the absence of parasitological evidence of infection, immunological methods are required for its diagnosis¹⁰.

Currently, an ELISA technique is being used to detect IgG antibodies against the parasite^{9,22}; this test uses an excretory / secretory antigen (E/S) and provides a high sensitivity. The parasite proteins of this antigen which elicit an immune response can be identified by immunological techniques. Therefore, this paper includes the detection of anti-*Toxocara* IgG antibodies in rabbit sera by an ELISA test and the characterization and identification of the antigenic components of *T. canis* present in the excretory / secretory antigen using the Western Blot technique.

MATERIALS AND METHODS

Experimental infection in rabbits: Twenty-seven puppies aged from one to three months were killed with a 5 ml lethal intracardiac injection

of a mixture of magnesium and potassium sulfate solutions. An incision on the abdominal wall was performed on every puppy, and the small intestine, where the adult parasites were located, was removed. The recovered worms were classified and the gravid uteri were extracted from the females. This material was digested for 30 minutes in acid-pepsin solution (pepsin 1%, HCl 0.1%), washed and incubated in a 1% formalin solution at 28 °C. After 28 days, the embryonated eggs were hatched and one part of them was used for antigen preparation and the other for production of antibodies in rabbits.

Ten white New Zeland male rabbits of two months of age, were inoculated orally with 5000 infective eggs of *T. canis*. The infected rabbits, and three additional control rabbits, were bled periodically, every month, during seven months.

Preparation of the excretory/secretory larval antigen (E/S): *Toxocara canis* larvae were cultured *in vitro* according to the method described by DE SAVIGNY⁶. The culture medium used was Dulbecco's Modified Eagle's Medium (DEM) SIGMA^R supplemented with antibiotics (penicillin and streptomycin) and sodium bicarbonate, pH 7.2. The concentration of larvae, determined following BADLEY *et al.* method³, was of 10⁴/ 10 ml of medium. The supernatant was divided into aliquots and stored at -20 °C. One of these antigen lots was dialyzed and the

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remaining were processed without dialysis.

IgG antibody detection in rabbits by ELISA: The ELISA was performed following the guidelines by VOLLER *et al.*²², and DE SAVIGNY *et al.*⁸. Antibodies against *Toxocara canis* antigens were determined in all the blood samples obtained periodically from the rabbits. Microplates of polystyrene (Immunolon I^R) were covered with 100 µl of a 1.0 µg of protein / ml E/S antigen solution in carbonate – bicarbonate buffer, pH 9.6. The plates were incubated for three hours at room temperature to allow for antigen adsorption. 100 µl of diluted sera 1:800 in phosphate buffered saline (PBS) plus 0.5% Tween 20 solution and three wells were used per sample. Sera were incubated during 2 hours at room temperature. An alkaline phosphatase-anti-rabbit IgG conjugate was then added in a 1:1000 dilution and allowed to incubate overnight (18 hours) at 4 °C. The reaction was revealed by adding 100 µl of substrate, p-nitrophenyl phosphate in diethanolamine buffer (1 mg/ml), and stopped with 25 µl of 3N NaOH. The reading was performed at 405 nm and the cut-off value was established at 0.300.

Western blot: Electrophoresis in polyacrylamide gels and immunoblot were performed following the method by MAGNAVAL *et al.*¹³. Polyacrylamide gels were prepared in a concentration of 10%, with 2% sodium persulfate and Temed. For the estimation of molecular weights, a broad range molecular weight ladder standard (Bio-Rad[®]) was used which included the following proteins: myosin, 200 KDa; β-galactosidase, 116 KDa; phosphorilase B, 97 KDa; bovine serum-albumin, 66 KDa; ovum-albumin, 45KDa; carbonic anhydrous, 31KDa; soy trypsin inhibitor, 21 KDa; lysozyme 14 KDa and bovine pancreatic trypsin inhibitor 6.5 KDa. The antigen solution was mixed with Laemmli buffer 2X (Tris 0.125M, SDS 5.1%, glycerol 20%, EDTA 0.01 M, β-mercaptoetanol 10%, bromophenol blue 0.04%) in a 1:1 V/V proportion and the mixture was allowed to reduce for 5 minutes. The running conditions were: 75 volts (V) for 15 minutes followed by 125 V during 80 minutes; in running buffer (Tris 0.025 M, Glycin 0.192M, methanol 2%). Finally, the nitrocellulose membranes were stained with red Ponceau and washed with distilled water until the excess colouring was completely withdrawn.

The nitrocellulose membrane was blocked at 4 °C overnight with a blocking solution (Tris 0.020 M pH 7.4, NaCl 0.500 M, Tween 20 0.1% and skimmed milk 5%) to avoid nonspecific binding. Serum samples were diluted 1:400 in blocking solution, incubated with the nitrocellulose strips during 1 h at room temperature and washed with PBS/Tween 20 0.1%. The alkaline phosphatase-antirabbit IgG conjugate, diluted 1:10,000 in blocking solution, was added and allowed to incubate at room temperature for 1 h. After five washes, the NBT/BCIP substrate was added (Nitro Blue Tetrazolium 0.3 mg / ml, Bromine – 4 Chlorine – 3 Indol Phosphate 0.15 mg/ml) in alkaline phosphatase (Tris 0.050 M, MgCl₂ 0.004 M, pH 9.8) activator solution; the reaction was then stopped by washing with distilled water.

RESULTS

Detection of anti - IgG antibodies in rabbit sera by ELISA: Each of the 15 serum samples obtained from bleeding periodically each rabbit during a six month period was processed for IgG antibodies by the ELISA test. Different positive and negative controls were used, thus verifying the reproducibility of the technique.

Antibody titers were first detected on day 15 after inoculation of the infective eggs. The cut-off point was 0.30; the mean of the titers of the control rabbits was 0.039 with a range between 0 and 0.154. The infected rabbits presented high titers, between 0.344 and 1.889. During the course of the infection, the positive titers increased rapidly between day 21 and day 58, remaining high and constant, with slight variations from then on. When averaging the obtained absorbances for each bleeding, from both the infected and control rabbits, the behavior of the humoral immune response to *T. canis* infection can be seen: at the beginning, titers tend to increase rapidly until day 60 and then they stabilize (Fig. 1).

Identification of specific *T. canis* proteins by Western blot: The immunoblot to detect anti-IgG antibodies was done for all the serum samples collected from rabbits during the six-month observation period. Thus, it was possible to assess the behavior of the immune response against the antigenic determinants of *T. canis* in every rabbit.

The antigenic determinants which react with antibodies vary during the course of infection: at the beginning four proteins of molecular weights 200 KDa, 116 KDa, 92 KDa and 35 KDa reacted strongly; at around day 30 seven characteristic bands of molecular weights 92 KDa, 80 KDa, 66 KDa, 45 KDa, 35 KDa, 31 KDa and 28 KDa are recognized; two bands that remain constant during all the course of infection were identified, the 92 KDa and 35 KDa proteins. This pattern remained constant for all the infected rabbits. On the other hand, sera from control rabbits (negative sera) did not present any reaction (Fig. 2).

DISCUSSION

Components of E/S *T. canis* antigen are identified by Western blot when they are confronted with sera of previously infected rabbits. A characteristic pattern of seven bands is observed, which can be grouped into one low molecular weight fraction (28-45 KDa) and one high molecular weight fraction (66-200 KDa). However, except for two proteins (the 92 and 35 KDa proteins), not all of these antigens are recognized by sera during the whole course of the infection.

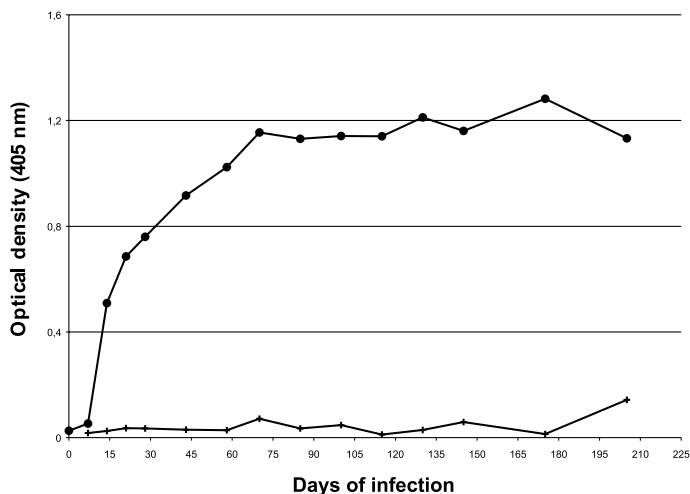


Fig. 1 - Course of the humoral immune response of rabbits against experimental *Toxocara canis* infection during a six month period. Average of optical densities: Infected rabbits (●); and non infected rabbits (+).

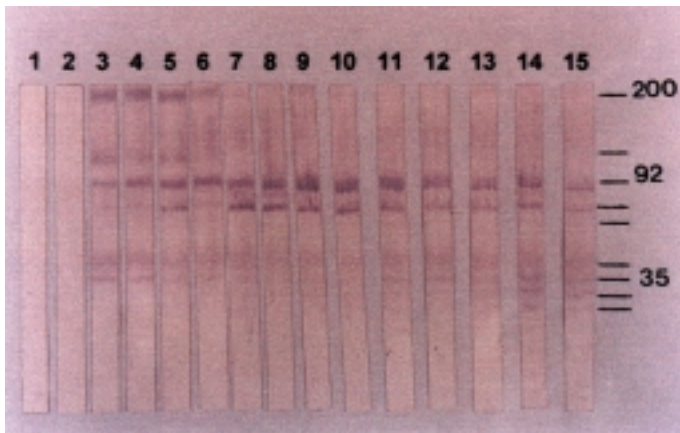


Fig. 2 - Antigenic components detected by antibodies of *Toxocara canis* of an experimentally infected rabbit throughout a 7 month follow-up. Lane 1: Day 0 (before experimental infection); Post-infection: Lane 2: day 7; Lane 3: day 14; Lane 4: day 21; Lane 5: day 28; Lane 6: day 43; Lane 7: day 58; Lane 8: day 70; Lane 9: day 85; Lane 10: day 100; Lane 11: day 115; Lane 12: day 130; Lane 13: day 145; Lane 14: day 175; Lane 15: day 205. The molecular weights of each band were estimated; two of them, the 92 KDa and 35 KDa bands, remained constant throughout the course of the infection.

AKAO *et al.*² identified eight bands with sera of infected rabbits, while SPEISER & GOTTSTEIN²¹ found 10 different antigenic components, common to two antigen preparations, and also noted cross-reaction with filariae. BADLEY *et al.*³ detected 15 bands using sera obtained from infected rabbits and monkeys and from patients with visceral larva migrans (VLM) and ocular larva migrans (OLM). In a radio-iodine ES-Ag analysis MAIZELS *et al.*^{14,15}, found five principal components, 32, 55, 70, 120 and 400 KDa and established that the 400 KDa band corresponds to a polysaccharide fraction related to blood group antigens.

MAGNAVAL *et al.*¹³, classified into two groups the seven antigenic components detected, the high molecular fraction (132, 147 and 200 KDa) and the low molecular fraction (24, 28, 30 and 35 KDa); they suggest that the high molecular weight fractions can account for cross-reactivity while the low molecular weight fractions seem to be more specific of the genus *Toxocara*.

In our study, a correlation between the band pattern and the stage of infection can be observed: as infection progresses, the antigen-antibody reaction becomes visible on immunoblot. On the other hand, the concordance between the results in the ELISA test and the Western blot must be highlighted: positive or negative sera on the ELISA test yielded the same results in the Western blot. Similarly, MAGNAVAL *et al.*¹³ (1991) in the study about application of the Western blot for diagnosis of toxocaríasis, demonstrated a correlation between these two immunological methods.

JACOB¹¹ observed four bands with molecular weights between 29 and 210 KDa and noted that the 66 KDa band was also identified in some patients whose sera were used as negative controls. NUNES *et al.*¹⁷, observed numerous bands in patients with signs of VLM, amongst which five principal components can be distinguished: (above 205 KDa, around 205 KDa, 116 – 97 KDa, 55 – 50 KDa and 35 – 29 KDa), and

agree in that the low molecular weight fractions do not present any cross reactivity with other ascarid antigens.

During the antigen preparation process no protease inhibitors were used; however, the antigen obtained was of high quality and remained stable for a long period. ROBERTSON *et al.*¹⁹ in a study of proteolytic enzymes secreted by *in vitro* cultured *T. canis* larvae, state that some nematode species, including *Toxocara*, have protease activity in their excretion/secretion products, which under natural conditions are probably involved in the invasion and migration into their vertebrate hosts. Additionally, these *in vitro* secreted proteases are capable of degrading antigenic components causing loss of its reacting capacity in immunoassays. The optimal quality of the antigen, observed so far in this study, can probably be explained by the optimum conditions in the process of obtaining and storing; however, the appearance of protease activity in any moment cannot be excluded.

Amongst the new perspectives for the immunodiagnosis of toxocaríasis, the development of new methodologies which make this diagnosis easier and more practical can be foreseen. YAMASAKI *et al.*²³, proposes the development of a highly specific recombinant *T. canis* antigen which yields greater specificity as compared to the conventional E/S antigen.

RESUMO

Identificação de antígenos de *Toxocara canis* por Western blot em coelhos experimentalmente infectados

O *Toxocara canis* é responsável por uma helmintíase freqüente, causando danos viscerais e oculares no ser humano, especialmente em crianças. A identificação de antígenos específicos de *Toxocara canis* é importante para se obter melhores técnicas de diagnóstico. Neste trabalho, dez coelhos foram infectados, por via oral, com um inóculo de 5000 ovos embrionados de *T. canis*. Periodicamente, foi coletado sangue dos coelhos para determinação dos níveis de anticorpos IgG, pela técnica ELISA, que revelou anticorpos no 15º dia após a infecção.

A técnica de Western blot foi realizada utilizando os antígenos excretores-secretores (E/S) com larvas de *T. canis* de segundo estágio. Foram avaliadas diferentes concentrações de antígeno: 150, 200, 250 e 300 µg/mL.

No final, a concentração ótima de antígenos para análise foi 250 µg/mL. Cada soro foi diluído 1:100 e o anti IgG de coelho, ligado a fosfatase alcalina, foi utilizado a 1:10000. Os resultados do Western blot indicaram que no primeiro mês após a infecção apareceram anticorpos específicos contra os determinantes antigênicos 200 KDa, 116 KDa, 92 KDa e 35 KDa; os anticorpos contra 92 KDa, 80 KDa, 66 KDa, 45 KDa, 35 KDa e 28 KDa apareceram mais tarde. Todos os soros positivos pela técnica ELISA, também foram positivos pela de Western blot. Duas bandas estiveram presentes desde o começo da infecção. Estes antígenos justificam uma futura avaliação para uso no diagnóstico da Toxocaríase.

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