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EVALUATION OF ANTIBODY RESPONSE AND ANTIGENIC CHARACTERIZATION OF TOXOPLASMA GONDII IN EXPERIMENTALLY INFECTED PRIMATES

(AVALIAÇÃO DA RESPOSTA DE ANTICORPOS E CARACTERIZAÇÃO ANTIGÊNICA DO TOXOPLASMA GONDII EM PRIMATAS EXPERIMENTALMENTE INFECTADOS)

(EVALUACIÓN DE LA RESPUESTA DE ANTICUERPOS Y CARACTERIZACIÓN ANTIGÉNICA DEL Toxoplasma gondii EN PRIMATES INFECTADOS EXPERIMENTALMENTE)

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SUMMARY

This study investigated the presence of *Toxoplasma gondii* antibodies in experimentally infected New World primates, by means of an enzyme linked immunosorbent assay (ELISA) for IgG and IgM, and the pattern of polypeptide recognition using Western-blotting. Seven days after infection all primates yielded positive results in both ELISA-IgG and ELISA-IgM. Western blotting documented four major polypeptides with apparent molecular weight of 160, 120, 80 and 35-30 kDa. The 35-30 kDa polypeptide was identified by antibodies from all infected primates and should be further evaluated for use in specific diagnosis of *T. gondii* infection.

KEY-WORDS: *Toxoplasma gondii*. ELISA-test. Western-blotting. Antigenic characterization. Primates.

RESUMO

O presente estudo investigou a presença de anticorpos para *Toxoplasma gondii* em primatas do Novo Mundo experimentalmente infectados, por meio de um ensaio imunoenzimático (ELISA) para as classes de imunoglobulina IgG e IgM, e o padrão de reconhecimento de polipeptídeos no soro desses mesmos animais utilizando a técnica de "Western-blotting. Todos os primatas mostraram-se positivos aos sete dias de infecção tanto no ELISA-IgG como no ELISA-IgM. O "Western blotting" revelou quatro principais polipeptídeos com pesos moleculares aparentes de 160, 120, 80 e 35-30 kDa. O polipeptídeo de 35-30 kDa foi identificado pelos anticorpos de todos os primatas infectados e deve ser melhor investigado para uso no diagnóstico específico de infecções por *T. gondii*.

PALAVRAS-CHAVE: *Toxoplasma gondii*. ELISA-teste. "Western-blotting". Caracterização antigênica. Primatas.

RESUMEN

En el presente estudio se investigó la presencia de anticuerpos para *Toxoplasma gondii* en primates del Nuevo Mundo infectados experimentalmente. Fue utilizado un ensayo inmunoenzimático (ELISA) para las clases de inmunoglobulina IgG e IgM, y para el patrón de reconocimiento de polipéptidos en el suero de estos mismos animales se usó la técnica de Western blot. Todos los primates fueron positivos a los siete días de la infección, tanto en el ELISA-IgG

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como en el ELISA-IgM. El Western blot reveló cuatro polipéptidos principales con pesos moleculares aparentes de 160, 120, 80 y 35-30 kDa. El polipéptido de 35-30 kDa fue identificado por los anticuerpos de todos los primates infectados y debe ser investigado mejor para su uso en el diagnóstico específico de infecciones por *T. gondii*.

PALABRAS-CLAVE: *Toxoplasma gondii*. ELISA-test. Western blot. Caracterización antigénica. Primates.

INTRODUCTION

Toxoplasma gondii is an Apicomplexa protozoan parasite that infects man and other warm-blooded animals (DUBEY, 1993). It is an obligate intracellular parasite, with an enteroepithelial cycle in the definitive host (Felids), and an extraintestinal cycle in intermediate hosts. It may be transmitted by the consumption of undercooked infected meat, by the oral fecal route, or congenitally (DUBEY et al., 1998).

Toxoplasmosis is one of the diseases of medical and veterinary interest that can affect the neotropical primates in their most various environment. There are many reports of toxoplasmosis in New World primates (HESSLER et al., 1971, ANDERSON e McCLURE, 1982, BORST e VAN KNAPEN, 1984, CUNNINGHAM et al., 1992, DIETZ et al., 1997, PERTZ et al., 1997, JUAN-SALLÉS et al., 1998, BOUER et al., 1999, EPIPHANIO et al., 2000, BOUER, 2001, EPIPHANIO et al., 2001, EPIPHANIO et al., 2003) wich are much more susceptible than the Old World primates (DUBEY e BEATIIE, 1988, ANDERSON e McCLURE, 1993), and for unknown reasons rarely survive the disease (DUBEY, 1986).

Few serologic surveys were performed to detect antibodies anti-*T. gondii* in primates naturally infected (McCONNELL et al., 1973, FERRARONI e MARZOCHI, 1980, CUNNINGHAM et al., 1992, BOUER, 2001) and also there are few studies with neotropical primates experimentally infected and in none of them the ELISA test was used (BOUER, 2001).

Few studies on antigenic characterization of *T. gondii* have been undertaken (TOMAVO et al., 1993, LAPPIN et al., 1994, SUAREZ-ARANDA et al., 2000) and litlle is known so far regarding the polypeptides recognition patterns by antibodies from neotropical primates.

The objective of the present study was to investigate the presence of *T. gondii* antibodies in primates experimentally infected and to evaluate the patterns of polypeptide recognition during the acute and chronic stage of the infection.

MATERIALS AND METHODS

Antigen preparation

Antigens were prepared from a *T. gondii* strain (RH strain) that was maintained through successive passages in Swiss webster mice. Soluble antigen used in Indirect ELISA and Western-blotting were prepared according to Domingues et al. (1998). The protein concentration of the soluble antigen was determined by the method of Hartree (1972).

Animals

Four adult monkeys (*Cebus apella*), clinically healthy and serologically negative for *T. gondii* and *Neospora caninum* were experimentally infected with a tachyzoite suspension (10^6/ml) of RH strain, by intraperitoneal route. Clinical examination and full blood count were performed during all the experimental infection. During a hundred and twenty days (120), after infection, the blood was collected in jugular or femoral vein, in the following days; 7^{th}, 15^{th}, 33^{th}, 45^{th}, 60^{th}, 90^{th} and 120^{th}. Negative control sera were obtained from the control animals (n=4). Two of these animals (animal 3 and 4) were reinoculated with the same strain and maintained for more 45 days, and the blood was collected with 150 and 165 days of infection.

Indirect ELISA

The ELISA was performed as methodology described by Bouer et al. (2001). Briefly, ELISA plates (Nunclon Surface, Nunc^{Ó} for IgG and Immulon 2, Dynex^{Ó} for IgM) were coated with 200 ml/well (IgG) and 100 ml/well (IgM) of antigen (concentration of 10 mg/ml and 5 mg/ml, respectively) diluted in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6, and incubated at 4°C for 18 hours in moist-chamber.

Between the various reaction phases the microplates were submitted to three washings of one minute each with 0.01 M PBS, pH 7.4, containing 0.05% Tween 20 (PBS-Tween). Sera were diluted at 1/100 in diluent buffer (PBS-Tween with 5% normal rabbit serum added). Anti-monkey IgG alkaline phosphathase conjugated (Sigma^{â}) and anti-monkey IgM labeled to peroxidase (KPL) were diluted at 1:10.000 and 1:1000 respectively. The substrates paranitrofenilphosphate (pNPP, Sigma^{â}) and peroxidase solution (H_2O_2) in ABTS System ("2,2 azino-di 3 ethyl-benzthiazoline sulfonate", KPL) were allowed to react for one hour (IgG) and 15 minutes (IgM), at room temperature. The reading was taken in an ELISA reader (Dynex^{â}) equipped with a 405 nm filter.

SDS-PAGE

T. gondii soluble proteins were separated by electrophoresis (Mini-Protean II, Bio-Rad) on a 7,5-17,5% gradiente polyacrylamide gel in the presence of 10% sodium dodecyl sulfate (LAEMMLI, 1970). Samples containing 10,0µg of soluble proteins were loaded into each well and a mixture of calibration proteins of molecular range between 250 kDa to 4 kDa (Invitrogen LC5725) was included into one well during each run. All gels were run at 200 volts for one hour and a half.

"Western-blotting"

Following SDS-PAGE, the polypeptides were electrophoretically transferred at 200V for 3 hours from the gel to a nitrocellulose membrane by the procedure described by Towbin et al. (1979). Membranes were blocked for 12 hours with 5% non-fat dry milk and 0,05% Tween-20 in Tris buffered saline (TBS: 20mM Tris, 500mM NaCl, ph 7,5).

For immuno-detection, strips of nitrocellulose containing individual runs were cut and incubated with test sera. Sera from experimental animals were tested along the acute and chronic phases of the infection (7 to 120 days). Sera obtained from the control animals (pool) served as the negative control of the reaction. Incubations were performed at room temperature. All sera were diluted 1:100 in blocking buffer (5% non-fat dry milk in TBS-Tween) and added to the membrane strips for 3 hours. Sera were removed and membranes were washed with blocking solution for 10 minutes, followed by two washes (5 minutes each) with TBS-Tween. The strips were then incubated for 90 minutes with appropriate dilution of alkaline phosphatase conjugated antibody against monkey IgG (Sigma) as already referred for serology. Conjugate was removed and three washes (5 minutes each) were performed using TBS-Tween. The polypeptide bands were visualized by the addition of the enzyme substrate 5 bromo-4-chloro-3 indoyl phosphate / nitroblue tetrazolium chloride (NBT-BCIP, SIGMA B-5655). Colorimetric reactions were stopped by washing the blots in distilled water. Relative mobility of immunorecognized polypeptides was estimated from a calibration curve based on mobility of proteins standards.

RESULTS

ELISA test

Antibodies of diagnostic value (OD ³ 0,212) were detected at day seven after infection (IgG) until the end of the experiment. All the animals showed similar humoral immune response, presenting increasing titers during all the infection, although there is a difference in intensity response among them. For ELISA IgM antibodies of diagnostic value (OD ³ 0,237) were also detected at day seven after infection and for three animals they were detected until the end of the infection; antibodies of one of them were only detected up to 15 days after infection (Table 1). The reactivity (positive or negative) was calculated as being 2 times and a half the average (x= 0,090) of the negative control sera (n=10).

Antigenic characterization of *T. gondii*

The electrophoresis on a 12% gradiente polyacrylamide gel characterized the polypeptides of *T.gondii* soluble antigens showing bands with molecular weights ranging from 220 kDa to 15 kDa.

IgG antibodies to *T. gondii* polypeptides were first detected, although very poorly, in serum of one of the primates experimentally infected at 15 days of infection. The molecular weight was approximately of 35 kDa. At 30 days of infection, it was possible to detect polypeptides of 30 kDa and 25 kDa. As the infection progresse increasing numbers of toxoplasma antigens were recognized with the staining of most individual bands becoming more evident. After 45 days of infection (chronic phase), the polypeptides of 160 kDa, 120 kDa and 100 kDa were identified and the bands with 35-30 kDa were more intense stained. The profile of polypeptides revealed in chronic phase (45-120 dias) was the same until the end of the experiment (Figure 1).

DISCUSSION

Our data show that ELISA for primates IgG specific antibodies against *T. gondii* is a useful test for the determination of the kinetic of humoral immune response of toxoplasmosis in primates, as also confirmed by Western blotting detection. ELISA is a sensitive serological test, able to detect low antibody titers either in recent infections (DUBEY *et al*., 1996), or in animals experimentally infected for long periods of time (DUBEY et al., 1997). Althoug few reports are available, ELISA is extremely efficient for the detection of IgG and IgM antibodies against *T gondii* in primates naturally and experimentally infected (BOUER, 2001).

All samples tested by ELISA recognized several *T. gondii* antigens by Western blotting, and the intensity of stained bands correlated well with IgG antibody titers. One of the infected primates (animal 3) showed a weak and undefined reaction in this test, probably due to low titers presented in ELISA, and the other three animals showed clearly positive bands coresponding to higher titers in ELISA.

In the present study, four major polypeptides with apparent molecular weights 160, 120, 80 and 35-30 kDa were recognized by antibodies of all experimental animals. Potasman et al. (1986) also recognized antigen of 35 kDa. Lappin et al. (1994) studying felines recognized antigens

Table 1 - Values of optic density (OD) obtained for 4 *Cebus apella* experimentally infected by *Toxoplasma gondii* through IgG and IgM ELISA in Jaboticabal, São Paulo, Brazil.

	Animal 1		Animal 2		Animal 3		Animal 4	
Period	**IgG**	**IgM**	**IgG**	**IgM**	**IgG**	**IgM**	**IgG**	**IgM**
0	0,155	0,163	0,143	0,199	0,148	0,149	0,143	0,135
7 dai	0,303	0,520	0,646	0,775	0,196	1,192	0,234	0,242
15 dai	0,307	0,461	0,746	0,725	0,232	0,818	0,438	0,690
30 dai	0,510	0,404	0,910	0,756	0,288	1,098	0,353	0,402
45 dai	0,610	0,599	1,080	0,492	0,377	0,614	0,498	0,246
60 dai	0,576	0,449	0,752	0,357	0,360	0,590	0,599	0,270
90 dai	0,760	0,454	1,012	0,352	0,392	0,618	0,506	0,199
120 dai	1,371	0,304	1,046	0,379	0,370	0,563	0,672	0,256
150 dai	___	___	___	___	1,026	0,905	1,463	0,253
165 dai	___	___	___	___	0,996	0,894	1,802	0,252

"0"= indicates sera taken before experimental inoculation
dai=days after infection
cut off IgG ≥ 0,212; IgM ≥ 0,237

during chronic infection of 65, 55, 51, 33, 31, 28, 26 and 19 kDa. Some of these are similar to the ones recognized in our study with apparent molecular weight of 53, 35, 30, 25 and 21 kDa. A group of high molecular weight (160, 145, 120 and 100 kDa) were recognized by most serum samples in the IgG Western blotting after 45 days of infection. The greater number of polypeptides detected in chronic phase of infection is probably due to the release of internal antigens after parasite destruction by variable surface glycoprotein (VSG) specific antibodies.

The consistent serorecognition of the polypeptides of 35-30 kDa and 160-120 kDa would indicate them as good candidates to be assessed for diagnosis purpose. However, further studies must evaluate additional parameters related to the proposed antigen before it may be considered a reliable tool in serodiagnosis of *T.gondii* infection.

Additional detailed studies using the Western blotting in characterization of different bands in primates may enhance the understanding of the host/parasite

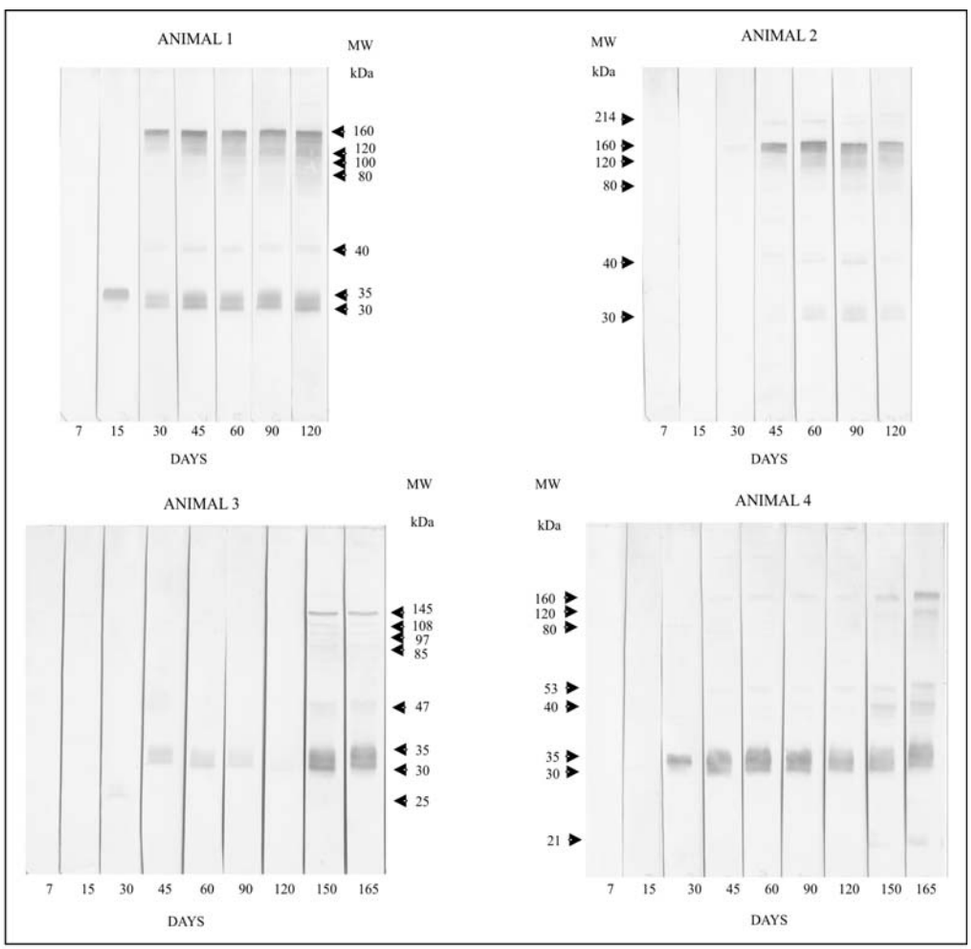

Figure 1 - Immunoreactivity of sera from *Cebus apella* (animais 1-4) experimentally infected by *Toxoplasma gondii*, obtained in different days along the infection period (7, 15, 30, 45, 60, 90, 120, 150 ,165).

relationship and give some insight into the pathogenicity and immunogenicity of this parasite. Specific parasite polypeptides identified could be further evaluated for use in the diagnosis of toxoplasmosis.

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