Humoral immune response of horses experimentally infected with *Trypanosoma evansi*[1]

Resposta imune humoral de eqüinos infectados experimentalmente com *Trypanosoma evansi*

Luiz Carlos Marques[2]*; Rosângela Zacarias Machado[2]; Antônio Carlos Alessi[2]; Lúcia Padilha Cury Thomaz de Aquino[3]

Abstract: Six adult horses were experimentally infected with *Trypanosoma evansi* (10^6 parasites). Three other adult horses served as negative control. Serum samples of the experimentally infected horses with *T. evansi* and non-infected controls horses were obtained before inoculation, and daily thereafter until 14 days post infection (DPI). After that time the serum samples were obtained weekly. Sera of the infected and non-infected control horses was tested by indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against *T. evansi.* Both ELISA and IFAT detected trypanosomal antibodies shortly after infection and showed progressive increases in antibodies levels during early stages of infection. The responses started on the eighth and eleventh DPI. Maximum IFAT and ELISA values were reached after four weeks of infection and were maintained at this level until the end of the period of study.
Key words: *Trypanosoma evansi,* trypanosomiasis, horses, immune response.

Resumo: Seis eqüinos foram inoculados com 10^6 tripomastigota sangüícolas de *Trypanosoma evansi.* Três outros animais foram mantidos como testemunhas. Amostras de soro sangüíneo foram obtidas de todos os animais, antes da inoculação, e diariamente até o 14º dia pós inoculação (DPI); após este período uma vez por semana. Pesquisa de anticorpos anti-*T. evansi*, foram realizadas através da reação de imunofluorescência indireta (RIFI) e do ensaio de imunoabsorção enzimática (ELISA). A resposta imune humoral, detectada através da RIFI e do ELISA, iniciou-se, em média, a partir do oitavo DPI, alcançando títulos máximos após quatro semanas de evolução, e os titulos de anticorpos anti-*T. evansi* mantiveram-se elevadas até o término das observações.
Palavras-chave: *Trypanosoma evansi,* tripanossomiase, eqüinos, resposta imune.

Introduction

Trypanosoma evansi causes a disease called, in South America, "mal de caderas" in horses. Traditionally, the diagnosis is usually carried out by direct examination of blood or buffy-coats and/or inoculation of blood into rodents such as rats or mice. During the acute stages of disease caused by *T. evansi* in livestock, large number of trypanosomes may appear in the peripheral bloodstream and diagnosis can be based on the detection of trypanosomes in wet blood smears. Later, in the chronic stage of the disease, the number of trypanosomes in the peripheral circulation may be low and difficult to be detected microscopically (RAE; LUCKINS, 1984).

Direct parasitological methods used in the diagnosis of trypanosomosis are effective only when large number of parasite are present in peripheral blood (LOSOS, 1980; MONZON *et al.*, 1983; MONZON *et al.*, 1984). Comparing six parasitological methods for *T. evansi* diagnosis in horses, Monzon *et al.* (1990) obtained sensibility of 88.2% by mouse inoculation, 71.1% by microhematocrit centrifugation technique, 63.3% by buffy coat method, 53.8% by wet blood film, 46.1% by Strout concentration method and 45.6% by Giemsa-stained smears. Even though, inoculation of suspect blood in laboratory animals proved to be one of the most sensitive parasitological methods, the reproducibility of the disease depends on the number of the inoculated trypanosomes and also on the virulence of the strain; detection of the parasite sometimes requires strict control of the parasitemia, detectable in variable times during the infection (MONZON; MANCEBO, 1986).

Considering this, a special attention has been given to the development of immunological diagnostic techniques based on antigen-antibody reaction. Indirect fluorescent antibody test (IFAT) has been widely employed in the diagnosis of man and animal trypanosomiasis. Monzon (1987) showed that the sensitivity of IFAT for detection of *T. evansi* infection in horses was 95%. Using the enzyme-linked immunosobent assay (ELISA) for detection of anti-*T. evansi* antibodies Luckins *et al.* (1979) detected Trypanosome antibodies in 95% of infected animals.

[1] Supported by FAPESP
[2] Professor da Faculdade de Ciências Agrárias e Veterinárias, UNESP/Jaboticabal. Via de Acesso Prof. Paulo Donato Castellane s/n. 144884-900 Jaboticabal, SP.
[3] Doutoranda do curso de pós-graduação em Medicina Veterinária (Patologia Animal), UNESP/Jaboticabal.
* Autor para correspondência.

The immune system plays a crucial role in the outcome of any parasite challenge (UCHE *et al*., 1993). Studies on the antibody response in horses infected with *T. evansi* are limited, what justifies greater efforts in the search and improvement of diagnostic methods for this trypanosomiasis that constitutes an important problem in horse-raising system. Effective diagnosis may provide valuable information, which is likely to be applicable in work with livestock production.

Material and Methods

Trypanosoma evansi strain

The strain used in this experiment was isolated from a naturally infected dog by Moreira and Machado (1985). The parasites have been collected in Alsever' solution (glucose 20.5 g, sodium citrate 8.0 g, sodium chloride 4.2 g, in 1,000 ml qs distilled water) and after addition of 10% dimethyl sulfoxide (DMSO), maintained at -196°C in liquid nitrogen. *T. evansi* strain was inoculated in an eight month-old mongrel dog in order to replicate trypanosomes. Blood from this dog with high parasitemia was used to inoculate horses and also for antigen preparation.

Experimental groups

Nine cross-bred adult clinically healthy horses were used. Three of them were kept as non-infected controls and each one of the other six were experimentally infected with approximate 10^6 trypomastigotes of *T. evansi* by intravenous route. Serum samples were obtained before inoculation, and daily thereafter until 14 days post infection (DPI). After that time the serum samples were obtained weekly until 11th week.

Indirect fluorescent antibody test (IFAT)

Trypomastigotes were isolated from infected blood according to technique described by Lanham and Godfrey (1970). Trypanosomes separated from the blood were placed in delimited areas previously prepared in microscope slides. Slides were dried at room temperature, properly wrapped and maintained in an air-tight recipient at -70°C. In the moment of use, slides were thawed at room temperature. Test sera were successively diluted (from 1:50 to 1:25,600) in 0.9% saline solution and distributed in the delimited areas containing the antigenic substrate, including also positive and negative control sera. Slides were incubated at 37°C in humid chamber for 45 minutes and then washed three times (five minutes each) in phosphate buffer saline (PBS) pH 7.2. After drying, the delimited areas were coated with goat anti-horse IgG fluorescein conjugate (KPL, 022106) diluted 1:40 in PBS containing 1% Evan's blue. After a new incubation in humid chamber at 37°C for 45 minutes, the slides were washed as mentioned above, dried, mounted with 9:1 glycerol/ 0.5M sodium bicarbonate (pH 9.5) and examined under an ultraviolet light microscope.

Enzyme-linked immunosorbent assay (ELISA)

Trypomastigotes were subjected to ultrasonication at maximum amplitude on MSE 100W for 20 seconds with 20 second intervals for 1 min. Resulting suspension was centrifuged at 10,000 g for 60 minutes and the supernatant was frozen at -70°C in 200ml aliquots until use. The protein content of the soluble antigen was determined as described by Hartree (1972).

ELISA plates were coated with 100ml of the antigen containing 10 μg/ml protein in carbonate-bicarbonate buffer pH 9.6. After overnight incubation at 4°C, the plates were washed three times in buffer (phosphate-buffered saline, pH 7.2 and 0,05% Tween 20). Amounts of 100 μl of test and control sera at 1:200 dilution in PBS-Tween containing 5% normal rabbit sera were added to the wells and tested in duplicate and the plates were incubated in humid chamber at 37°C for 90 minutes. Washing procedure was repeated as mentioned before, and 100ml of rabbit anti-horse IgG conjugated to alkaline phosphatase (Sigma, A-6063) at 1:30,000 dilution were added to each well. Incubation and washing were repeated as above and 5mg/ml of enzyme substrate p-nitrophenyl phosphate (Sigma, N-9389) was added. The plates were incubated at room temperature, in the dark, for 30 minutes and the reaction was stopped by the addition of 3M sodium hydroxide and the optical density was read photometrically (MRX Microplate Reader - Dynex Technologies) at 450 hm. Controls for each plate consisted of wells reacting with substrate only. The mean optical density for negative control sera was 0.171; values equal or above twice and half of the mean optical density of negative control sera were regarded as positive. The optimum dilution of serum and conjugate to give the largest difference between negative and positive was calculated considering a ratio of 2.5 as the cut-off point (0.427).

Results and Discussion

IFAT and ELISA results are shown in Figure 1. Anti - *T. evansi* antibodies (titers ≥ 1:50) was first detected from eighth to eleventh DPI in infected horses by IFAT. Antibody levels rose significantly after four weeks of infection and remained at high levels until the end of the study. Maximum antibody titers observed were 1:12,800. Antibodies of diagnostic value were not detected in control horses.

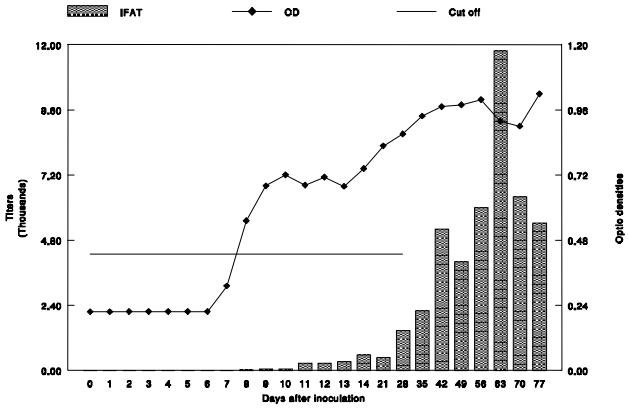

Figure 1 – Humoral IgG immune response obtained in serum samples of horses experimentally infected with *Trypanosoma evansi.* IFAT antibodies titers and ELISA optic densities.

Similarly to the result obtained by IFAT, *T. evansi* antibodies were demonstrated by ELISA in the serum of infected animals by the eighth day and maximal mean optical density was reached at the fourth week of infection.

There are few reports concerning to humoral immune response in *T. evansi* experimentally infected horses; however the result from the present investigation are similar to those reported by Monzon (1987) who detected antibodies in horses experimentally infected with *T. evansi* seven DPI by IFAT, followed by increasing antibodies levels and maximum values (1:2,048) at 50 DPI. In rabbits infected with *T. evansi* IgG antibodies were first detected in the serum seven DPI (UCHE *et al*, 1993). Results from the present study differ, however, from other reports using both IFAT and ELISA in sera of rabbits (LUCKINS *et al*., 1978), camels (LUCKINS *et al.,* 1979) and dogs (AQUINO *et al.,* 1999) in which antibodies were revealed within 10 to 19 DPI.

The serological tests employed in infected horses sera showed to be suitable for identifying *T. evansi* infection, since they detected 100% of positive sera. These results are similar to those reported by Aquino *et al*. (1999) in dogs experimentally infected with *T. evansi*. Nevertheless, a total of 61 parasite-positive camels, antibody-detection enzyme-linked immunosorbent assay (Ab-ELISA) detected 95% (OLAHO-MUKANI *et al*., 1993). Reyna-Bello *et al*., (1998) standardized the ELISA for detection of anti-*T. evansi* antibodies in naturally and experimentally infected horses. The results obtained were compared to those from an IFAT, with a relative sensibility of 98.3%, a specificity of 95.12% and a predictive value of 96.83% were determined.

Little is known about the mechanisms by which trypanosomes may survive in an immune organism; the protective role of antibodies is very discussed, and there are evidences that they participate on the limitation of the infection (BRENER; ANDRADE, 1979). The immunity conferred in chronic stages of the infection is not absolute and the evolution course of the disease leads to progressive emaciation and death, except in rare cases of spontaneous recovery (MARQUES, 1996).

The ELISA and IFAT detected anti-*T. evansi* antibodies in horses shortly after infection and showed progressive increases in antibodies levels during early stages of infection.

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