

## Analysis of *Toxoplasma gondii* antigens with sera from toxoplasmosis patients

Análise de antígenos de *Toxoplasma gondii* em soros de pacientes com toxoplasmose

Maria de la Luz Galván-Ramírez, Cecilia Guillén-Vargas,  
Rafael Saavedra-Durán and Alfonso Islas-Rodríguez

**Abstract** Some proteins of the *Toxoplasma gondii* are recognized by IgG, IgM and IgA antibodies in patients with acute and chronic toxoplasmosis, depending on the strain and stage of the *Toxoplasma*. Sixty-nine sera from immunocompetent individuals were studied through the Western-Blot Test: 20 has an acute infection, 29 has a chronic toxoplasmosis infection and 20 were healthy (seronegatives). The protein analysis revealed by IgG and IgM antibodies were performed through the Immunoplot method in order to know their recognition frequency (f) and be valued as infection markers. In the acute phase, the IgM antibodies showed a recognition frequency (f = 0.60) for the 60kDa protein, and in the chronic phase the IgG antibodies showed a recognition frequency (f = 0.68) for the 12kDa protein. Seronegatives revealed no type of band. The protein of 12kDa can be a diagnostic marker of the chronic phase while protein 60kDa of the acute phase of toxoplasmosis.

**Key-words:** Toxoplasmosis. Acute. Chronic. Antigen. *Toxoplasma gondii*.

**Resumo** Demonstrou-se que algumas proteínas do *Toxoplasma gondii* são reconhecidas pelos anticorpos IgG, IgM e IgA em pacientes com toxoplasmose aguda e crônica. A cepa e o estágio do protozoário interferem na diferença. Sessenta e nove soros de indivíduos imunocompetentes foram submetidos à pesquisa de anticorpos pela técnica de Western-Blot, sendo 20 dos soros provenientes de pacientes com infecção aguda e 29 de infecção crônica de toxoplasmose e 20 sem doença (soronegativos). A análise das proteínas reveladas pelos anticorpos IgG e IgM foi feita pelo método de Immunoplot com a finalidade de se conhecer a frequência de reconhecimento (f), e serem valorizados como marcadores de infecção. Na fase aguda os anticorpos IgM apresentaram uma frequência de reconhecimento (f = 0.60) para a proteína de 60kDa. Na fase crônica, os anticorpos IgG apresentaram (f = 0.68) para a proteína de 12kDa. Soronegativos não revelaram nenhuma informação. A proteína de 12kDa pode ser um marcador diagnóstico da fase crônica, e a proteína de 60kDa pode ser um marcador diagnóstico da fase aguda da toxoplasmose.

**Palavras-chaves:** Toxoplasmose. Aguda. Crônica. Antígeno. *Toxoplasma gondii*.

*Toxoplasma gondii* is an obligate intracellular protozoan that causes chronic and acute forms of toxoplasmosis. Epidemiological studies indicates that 20% to 90% of adult population worldwide have been in contact with the parasite. The congenital infection is the most severe form of the disease, causing irreversible damage to the nervous system of the fetus, or abortion in pregnant women<sup>8 16</sup>.

There are several proteic antigens obtain from *Toxoplasma gondii* that are recognized by antibodies from patients with some of the forms of toxoplasmosis<sup>1 2 6 13 14 15 18 22 24</sup>, but none of the antigens, have been clearly identified which have the characteristics of a marker for acute or chronic toxoplasmosis.

Recently, several methods have been developed in order to detect seropositive subjects, not only with epidemiologic, but with diagnostic and prognostic purposes. Western Blot technique have been used in order to discriminate subjects with the chronic infection from subjects with the acute form of the disease<sup>4 5 7 9 13 20</sup> but without conclusive results<sup>5 13</sup>.

Here we report, using the tachyzoite stage antigen extract from *Toxoplasma gondii* in polyacrilamide gel electrophoresis (SDS-PAGE) and Western Blot technique, the presence of antigenic markers for acute and chronic forms of toxoplasmosis, using the frequency analysis described by Larralde<sup>10</sup>.

## MATERIAL AND METHODS

*Subjects (Sera).* We study forty-nine female patients, between 18-42 years old, from the Hospital of Obstetrics and Gynecology of the Mexican Social Security Institute at Guadalajara; Jalisco, Mexico. Twenty of them, were adult patients with acute toxoplasmosis and twenty-nine were adult patients with chronic toxoplasmosis. 20 normal subjects were used as the control group. The three groups: chronic, acute and subjects normal were accurately classified by criteria universally accepted<sup>8</sup>; such as the presence of lymphadenopathy for acute toxoplasmosis patients, or retinochoroiditis for chronic patients. Also we consider the presence of IgG antibodies for chronic patients and high titer of IgM antibodies for acute patients, by indirect immunofluorescence technic. All sera were divided in three parts and frozen at -20°C until used.

*Preparation of Toxoplasma antigen.* The strain of *Toxoplasma gondii* used in this study was the

"RH" (Sabin, 1941); maintained by serial passage every 2 days in the peritoneal cavity of Balb/c mice (donated in the Institute of Biomedical Research of the Autonomous University of México, A.P. 70228, 04510 México, D.F. by Doctor Saavedra). Tachyzoites were obtained from the peritoneal cavity of the mice and placed in phosphate-buffer saline, pH 7.2 (PBS), containing heparin 0.1%, finally the pellet was washed in PBS twice.

Contaminated mouse cells were removed by differential centrifugation at 300g followed by 3 washes with 0.15M NaCl solution. The pellet was suspended in 1ml of PBS, and stored in 100µl aliquots at 70°C<sup>17</sup>. Protein concentration was determined following Lowry's method<sup>12</sup>.

*SDS-PAGE and Western-Blot.* The antigen obtained (1mg protein/ml) was mixed in a 1:2 dilution with the Laemmli sample buffer (0.02M Tris-HCL pH 6.8, 20% glycerol, 2% 2-mercaptoethanol and 0.05% bromophenol-blue), and boiled at 100° for 5 minutes. The antigen was separated on a vertical slab gel with 15% acrylamide by electrophoresis technic, at a constant current of 25mA, in buffer which contained 5ml 30/.88% acrylamide/bisacrylamide, 1.8ml of 2M 8.8 Tris-base, 200µl 10% sodium dodecilsulfate, 3.1ml distilled water, 500µl of 10% ammonia persulfate, 5µl of TEMED.

Markers of molecular weight (Mw), were run in the same gel with bovine serum albumin (Mw = 69kDa), glyceraldehyde 3-phosphate dehydrogenase (Mw = 36kDa), carbonic anhydrase (Mw = 30kDa), trypsinogen inhibitor (Mw = 21.5kDa) and lactalbumin (Mw = 14kDa); Sigma Chemical House.

The proteins already separated, were transferred on to a nitrocellulose paper (Transbolt Transfer Medium, Bio-Rad, Richmond CA) using 25mM Tris-HCL buffer pH 8.2 containing 192mM glycine and 20%(v/v) methanol in an electrophoretic transfer chamber (Bio-Rad) overnight at 0.25mAmps<sup>21</sup>. The nitrocellulose strips were soaked for 30min in 0.01M phosphate-buffer saline; containing 0.1%(v/v) Tween-20 (PBST), washed and then exposed for 90min, to 1:50 diluted human sera in PBST containing 5% non-fat dried milk.

After washing two times in PBST, the nitrocellulose strips were incubated for 90min with peroxidase-conjugated rabbit anti-human IgG or IgM (DAKO, Copenhagen), diluted 1:1000 in the same buffer. After washing, in the nitrocellulose strips, were developed the bands

color, with 3,3 diaminobenzidin tetrahydrochloride (DAB, DAKO, Copenhagen), DAB 2.5mg made in 10ml PBST plus CaCl 1% with 0.03% (v/v) H<sub>2</sub>O<sub>2</sub>. All incubations were carried out at room temperature and the reaction was stopped with H<sub>2</sub>O<sup>19</sup>.

*Immunoplot.* A graphic method to measure from 0 to 1, the frequency of recognition (f) of each band from an individual antigen recognized by specific antibodies in sera of sick and normal subjects through Western Blot thecnic, identifying with these diagnostic markers of chronic and acute forms. Where (f)= The number of times that a band were recognized was divided with the total number of samples. The proteins candidates for diagnostic markers were considered, when their recognition frequency were > than 0.5.

The bands recognized in sera from control subjects, were made into graphics according with their frequency (f) on their ordinates (Y), and the bands recognized in sick subjects in the abscissa (X).

Interpretation of the graphic were necessary to rely on the regression line or the quadrants

lines with the frequency > 0.5. The proteins situated near the regression line or in quadrants II and III were considered immunodominant and immunogenic, but they were not useful as diagnostic markers, on the contrary, proteins situated near the X and Y axes, in quadrants I and IV are candidates for diagnostic markers<sup>11</sup>.

## RESULTS

Sera from patients with chronic and acute form of toxoplasmosis were selected by their clinical characteristics plus the presence of specific antibodies against *Toxoplasma gondii*. This selection help us to investigate the presence of an specific protein in order to differentiate each form of the disease as well as to be used as a diagnostic marker.

*Western Blots.* Illustrative Western Blots of the *Toxoplasma gondii* antigens reacting with sera from patients with acute or chronic toxoplasmosis as well as with sera from normal subjects are shown in Figure 1. Twenty sera from acute toxoplasmosis patients recognized 8 bands of molecular weights ranging from 19

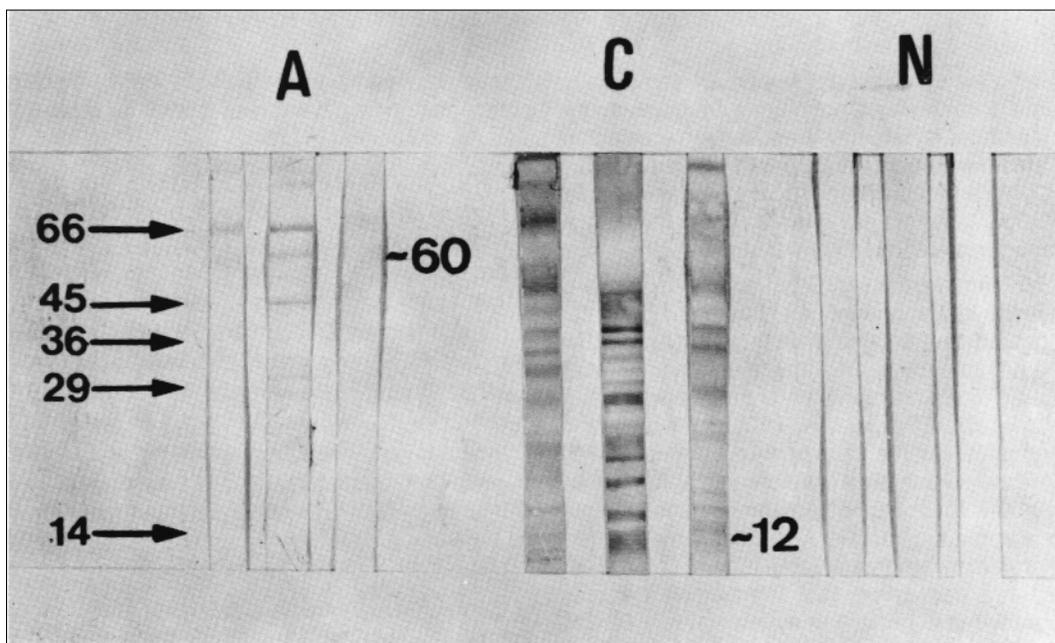


Figure 1 - Western Blot of sera from patients with acute (A) and chronic (C) toxoplasmosis, and normal subjects (N) reacting with *Toxoplasma gondii*.

to 90kDa. Twenty-nine sera, from chronic toxoplasmosis subjects, recognized 20 bands

from 12 to 90kDa and finally, sera from normal subjects did not recognize any bands (Table 1).

Table 1 - Molecular weights and frequency of recognition (f) of *Toxoplasma gondii* antigens by Western Blot toxoplasmosis acute and chronic.

Proteins kDa	Chronic n. bands	n = 29 (f)	Acute n bands	n = 20 (f)
12	20	0.68		
14	11	0.37		
19	7	0.24	4	0.2
20	5	0.17		
24	12	0.41		
27	14	0.48		
29	7	0.24		
30	11	0.37		
34	10	0.34		
35			1	0.05
36	14	0.48		
38	10	0.34		
45	14	0.48		
46	4	0.13		
48	8	0.27		
50	13	0.44	2	0.1
60			12	0.60
66	16	0.55	10	0.5
68			8	0.40
70	13	0.44	3	0.15
75	4	0.13		
80	13	0.44		
90	7	0.24	4	0.2

*Immunoblots.* The immunoblots of frequencies showed that sera containing IgM from acute toxoplasmosis patients frequently recognized two proteins: one of approximately 60kDa (f = 0.60) and the other of approximately 66kDa (f = 0.50). Normal sera did not recognize any of the antigenic bands (Figure 2A).

The immunoblots of frequencies from sera containing IgG of chronic toxoplasmosis patients, recognized one protein of approximately 12kDa (f = 0.68) and other of approximately 66 kDa (f = 0.55) and normal sera did not recognized any protein, Figure 2B. In Figure 2C, the immunoblot results shows the frequencies of data obtained with sera from chronic against sera from acute toxoplasmosis. Sera containing IgG from chronic individuals recognized more often one protein of approximately 12kDa (f = 0.68); while sera containing IgM from acute patients recognized one protein of approximately 60kDa (f = 0.60); other bands were recognized by antibodies presents in both chronic and acute patients but could not be selected as a diagnostic

markers since they did not have significant recognition and were less than 0.5 (Table 1).

## DISCUSSION

It is difficult to observe through the Western-Blot method, bands recognized by antibodies present in the sera from patients with chronic and acute toxoplasmosis. Using the immunoblot, described by Larralde in 1989<sup>11</sup>, makes it possible to order the results of the electroimmunotransference in such a way that the complex mix of antigens in general and those of *Toxoplasma gondii* in particular, can be classified quantitatively and specifically. Thus, this method constitutes a potential tool for bands discrimination, that possess diagnostic and prognostic information.

According with this analysis, three proteins of approximately 12, 60 and 66kDa were often recognized by the antibodies present in the sera from patients with different states of toxoplasmosis.

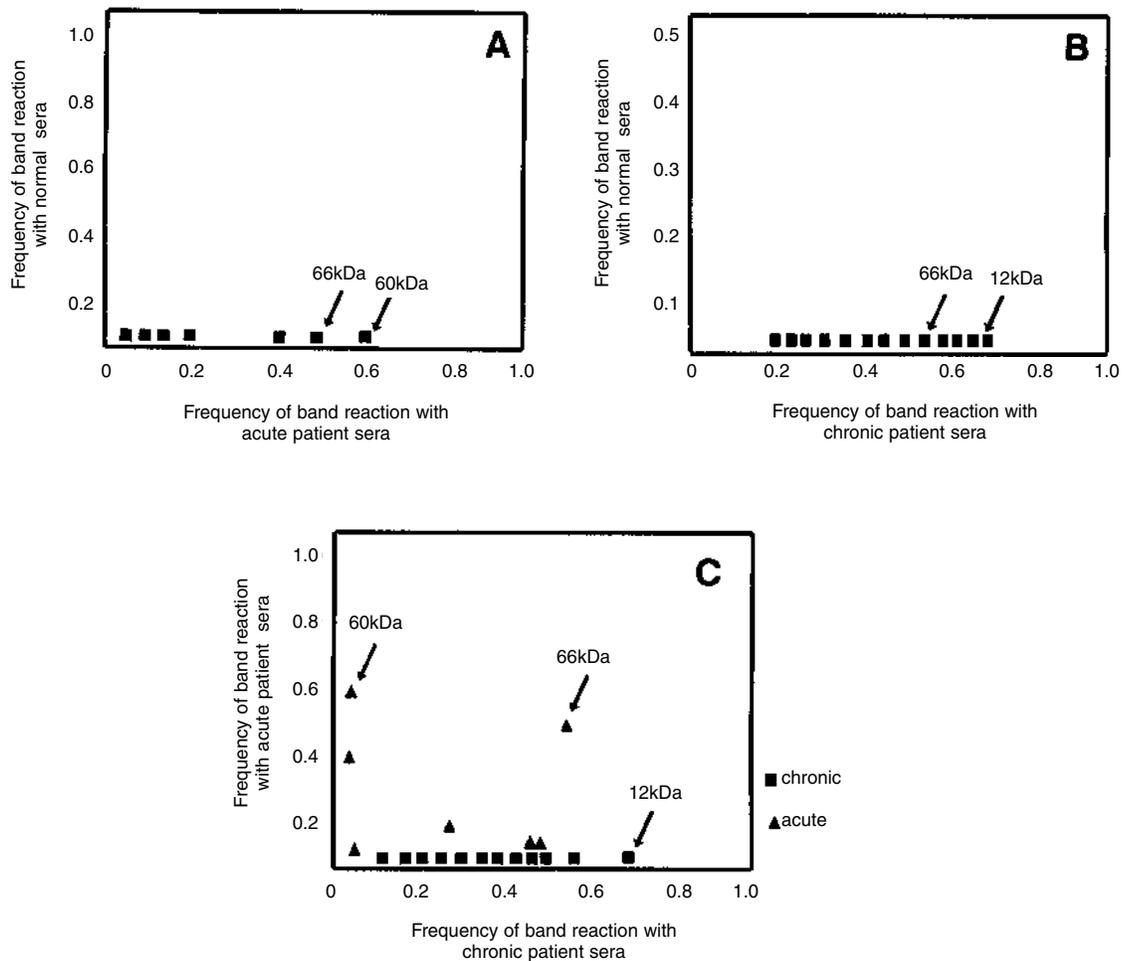


Figure 2 - Immunoplots of (A) acute *Toxoplasmosis* patients versus normal individuals; (B) chronic versus normal, and (C) acute versus chronic. In (A) it can be observed the approximately 60kDa and 66kDa frequently recognized antigens as markers of acute *Toxoplasmosis*. In (B) it can be observed the approximately 12kDa and 66kDa frequently recognized antigens as markers of the chronic form of the disease. In (C) it can be observed the above mentioned specific markers for acute and chronic forms and 66kDa protein.

Due to the highest frequency recognition of 60kDa protein by IgM antibodies in acute patients, we consider this protein a good marker for this form of infection. Different authors had reported proteins with similar molecular weight recognized by IgM antibodies in patients with the acute form<sup>22 23</sup>.

In chronic infections the protein of 12kDa was recognized by the IgG antibodies with a frequency greater than 0.5. Since this protein was not recognized neither by normal or acute patients, we consider this as a marker protein when Immunoplot method is used.

The presence of the 66kDa protein in chronic as well as in the acute patients point to this protein as a marker for every persons that have been in contact with *Toxoplasma gondii*; for the same reason it can not be used as a marker in order to differentiated between the two forms of disease<sup>2 7</sup>.

It's important to mention that with the immunoplot analysis, the proteins recognized with frequencies lower than 0.5 it can't be consider as a diagnostic markers for toxoplasmosis because of their low specificity.

Although there are other quantitative diagnostic methods for primary toxoplasmosis based in IgG antibodies avidity<sup>3</sup>; the serologic diagnosis of acute toxoplasmosis can be fortified by the production of monoclonal antibodies, highly specific, against the 60kDa marker protein and employing simpler methods and less expensive as the ELISA.

Further research is needed to confirm if the proteins of 12 and 60kDa can be considered as diagnostic markers for the acute and chronic stages of Toxoplasmosis.

#### ACKNOWLEDGEMENTS

The authors would like to acknowledge MD Patricia Paredes Casillas for her technical assistance in the preparation of this paper.

#### REFERENCES

1. Boulanger F, Bunhomme A, Lebonvallet S, Bummet N, Pluot M. Quantitative immunolocalization of our immunodominant antigens of *Toxoplasma gondii*. *Biology Cellular* 73:897, 1991.
2. Decoster A, Darcy F, Capron A. Recognition of *Toxoplasma gondii* excreted and secreted antigens by human sera from acquired and congenital toxoplasmosis: identification of markers of acute and chronic infection. *Clinical Experimental Immunology* 73:376-382, 1988.
3. Camargo EM, Da Silva SM, Lesser PG, Granato CH. Avidéz de anticorpos IgG específicos como marcadores de infecção primária recente pelo *Toxoplasma gondii*. *Revista do Instituto de Medicina Tropical de São Paulo* 33: 213-218, 1991.
4. Handman E, Goding JW, Remington JS. Detection and characterization of membrane antigens of *Toxoplasma gondii*. *Journal of Immunology* 124:778-783, 1980.
5. Hassl A, Auer H, Dermetin K, Piucher O, Aspoch H. Experimental studies on circulating antigen of *Toxoplasma gondii* in intermediate host: criteria for detection and structural properties. *Zentralblatt für Bakteriologie, Mikrobiologie Und Hygiene. Series A, Medical Microbiology, Infection Diseases, Virology Parasitology* 263:625-634, 1987.
6. Huskinson J, Thulliez P, Remington JS. Toxoplasma antigens recognized by human immunoglobulin A antibodies. *Journal Clinical Microbiology* 28:2632-2636, 1990.
7. Kasper L, Ware PL. Recognition and Characterization of Stage-specific Oocyst/Sporozoite Antigens of *Toxoplasma* antigens by Human antiserum. *Journal Clinical Investigation* 75:1570-1577, 1980.
8. Kociecka W, Mrozewicz B, Simon E, Pakula M. Clinical criteria and evaluation of the pathology and progress of lympho-nodular toxoplasmosis. *Wiad-Parazytol* 36:99-119, 1990.
9. Koch G, Wohlfahrth A, Muller WA. Detection of antibodies against *Toxoplasma gondii* in human sera in immunoblot procedures. *Z-Gesamte-Hygiene* 36:555-558, 1990.
10. Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685, 1970.
11. Larralde C, Montoya RM, Sciutto E, Diaz ML, Govezensky T, Coltorti E. Deciphering Western Blots of Tapeworm Antigens (*Taenia Solium*, *Echinococcus Granulosus* and *Taenia Crassiceps*) reacting with sera from Neurocysticercosis and Hydatid Disease patients. *American Journal of Tropical Medicine and Hygiene* 40:282-290, 1989.
12. Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the Folin Phenol reagent. *Journal Biology Chemistry* 193:265, 1951.
13. Moir IL, Davison M, D.O. Ho-Yen. Comparison of IgG antibody profiles by immunoblotting in patients with acute and previous *Toxoplasma gondii* infection. *Journal Clinical Pathology* 44:569-572, 1991.
14. Partanen P, Turunen JH, Turunen J, Paasivuo R, Forsblom E, Suni J, Leniniikki PO. Identification of antigens components of *Toxoplasma gondii* by immunoblotting technique. *Federation of European Biochemical Societies* 158:252-254, 1983.
15. Partanen P, Turunen HJ, Paasivuo RTA, Leniniikki PO. Immunoblot Analysis of *Toxoplasma gondii* antigens by immunoglobulin G, M and A antibodies at different stages infections. *Journal Clinical Microbiology* 20:133-135, 1984.
16. Remington JS, Desmots G. Toxoplasmosis. In: Remington JS, Klein JO (eds) *Infectious diseases of the infant*, Saunders, Philadelphia p.243-249, 1980.
17. Saavedra R, Demeuter F, Decourt JL, Herion P. Human T-cell clone identifies a potentially protective 54kDa protein antigen of *Toxoplasma gondii* cloned and expressed in *Escherichia coli*. *Journal Immunology* 147: 1975-1982, 1991.
18. Sharma SD, Mullenax J, Araujo FG, Erilich HA, Remington JS. Western blot Analysis of the Antigens of *Toxoplasma gondii* recognized by human IgM and IgG antibodies. *Journal of Immunology* 2:977-983, 1983.
19. Scott DI. Immunoblotting and dot blotting. *Journal of Immunological Methods*. 119:153-187, 1989.
20. Suzuki Y, Thulliez F, Desmots G, Reminton JS. Antigen(s) responsible immunoglobulin G Responses

- specific for the acute stage of toxoplasmosis in humans. *Journal Clinical Microbiology* 28:1734-1738, 1990
21. Towbin H, Staehlin T, Gordon J. Electrophoretic transfer of proteins from polyacrilamide gel to nitrocellulose sheets: Procedure and some applications. *Biochemistry* 6:4350-4354, 1979.
  22. Verhofstede C, Van Gelder P, Rabaey M. Evaluation of immunoblotting for the detection of *Toxoplasma gondii* immunoglobulin M antibodies. *European Journal Clinical Microbiology Infection Diseases* 9:835-837, 1990.
  23. Woodinson G, Smith JE. Identification of the dominant cyst antigens of *Toxoplasma gondii*. *Parasitology* 100:389-392, 1990.
  24. Zotti C, Maiello A, Moiraghi RA, Sacro T, Parusso R, Ferrero L, Barale A. Reactivity of antigenic fractions obtained from *Toxoplasma gondii* evaluated with Western blotting. *Bulletin Delé Istituto Sieroterapico Milanese* 68:167-173, 1989.