Revista da Sociedade Brasileira de Medicina Tropical 41(2):142-147, mar-abr, 2008

Avidity of IgG antibodies against excreted/secreted antigens of *Toxoplasma gondii*: immunological marker for acute recent toxoplasmosis

Avidez de anticorpos IgG anti-antígeno de secreção e excreção de *Toxoplasma gondii*: marcador imunológico de toxoplasmose aguda recente

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ABSTRACT

Detection of anti-toxoplasma IgM antibodies has frequently been used as a serological marker for diagnosing recently acquired toxoplasmosis. However, the persistence of these antibodies in some patients has complicated the interpretation of serological results when toxoplasmosis is suspected. The purpose of the present study was to evaluate the avidity of IgG antibodies against excreted/secreted antigens of *Toxoplasma gondii* by means of immunoblot, to establish a profile for acute recent infection in a single serum sample and confirm the presence of residual IgM antibodies obtained in automated assays. When we evaluated the avidity of IgG antibodies against excreted/secreted antigens of *Toxoplasma gondii* by means of immunoblot, we observed phase-specific reactivity, i.e. cases of acute recent toxoplasmosis presented low avidity and cases of non-acute recent toxoplasmosis presented high avidity towards the 30kDa protein fraction, which probably corresponds to the SAG-1 surface antigen. Our results suggest that the avidity of IgG antibodies against excreted/secreted antigens of *Toxoplasma gondii* is an important immunological marker for distinguishing between recent infections and for determining the presence of residual IgM antibodies obtained from automated assays.

Key-words: Toxoplasmosis. Serology. Avidity. Excreted/secreted antigens.

RESUMO

A detecção de anticorpos IgM antitoxoplasma tem sido freqüentemente utilizada como marcador sorológico para o diagnóstico de toxoplasmose de aquisição recente. Entretanto, a persistência destes anticorpos em alguns pacientes tem complicado a interpretação dos resultados sorológicos quando a toxoplasmose é suspeitada. A proposta deste trabalho foi avaliar a avidez de anticorpos IgG contra antígenos de secreção e excreção de *Toxoplasma gondii* por immunoblot, para estabelecer um perfil de infecção recente aguda em uma única amostra de soro e confirmar a presença de anticorpos IgM residuais obtidos nos testes automatizados. Quando a avidez de anticorpos IgG contra antígenos de secreção e excreção *Toxoplasma gondii*, por immunoblot, foi avaliada, observou-se reatividade estágio específica, ou seja, casos de toxoplasmose aguda recente apresentaram baixa avidez e os casos de infecção recente não aguda apresentaram alta avidez para a fração protéica de 30kDa, que corresponde provavelmente ao antígeno de superfície- SAG-1. Nossos resultados sugerem que a avidez dos anticorpos IgG contra antígenos de secreção e excreção *Toxoplasma gondii* é um importante marcador imunológico para distinguir doença recente de infecção e determinar a presença de anticorpos IgM residuais detectados nos testes automatizados.

Palavras-chaves: Toxoplasmose. Sorologia. Avidez. Antígenos de secreção-excreção.

Toxoplasmosis is caused by an intracellular parasite, *Toxoplasma gondii*, which presents global distribution and varying epidemiological characteristics among different populations. The course of infection by *Toxoplasma gondii* is generally benign, since the vast majority of infected patients remain asymptomatic or only present mild symptoms. However, the infection can cause significant morbidity and mortality in developing fetuses and in immunocompromised individuals[4].

Toxoplasmosis is generally diagnosed by demonstrating specific antibodies to *Toxoplasma* antigens in serum samples from infected patients. Serological diagnoses of acute toxoplasmosis have traditionally been made by detecting IgM antibodies or

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Recebido para publicação em: 04/06/2007
Aceito em: 11/03/2008

by demonstrating significant increases in specific IgG antibody levels, or both. However, because of the prevalence of high IgG *Toxoplasma* antibody titers in normal subjects and IgM antibodies detected several months or even years after acute infection in some individuals, interpretation of serological tests may be troublesome when acute toxoplasmosis is suspected[13]. Since IgG avidity evaluations for *Toxoplasma* infection serodiagnosis were introduced, this method has proved to be a highly useful procedure, especially in combination with conventional serological assays[19,20]. However, low-avidity results do not rule out the possibility of recently acquired infection, because low avidity may persist for as long as one year following the acute infection and low or borderline avidity can occur in significant numbers of patients without specific IgM antibodies[2]. Several reports have emphasized the value of detecting specific antibodies against excreted/secreted antigens (ESAs) of *Toxoplasma gondii* for diagnosing acute toxoplasmosis[5,9,12,20]. *Toxoplasma gondii* ESAs constitute 90% of the circulating antigens in infected humans and thus are one of the first targets of the host immune response[13,19]. In our laboratory, we have recently developed a technique to obtain ESAs by incubating tachyzoites in human fibroblast cultures for a short period of time. In the present study, we evaluated IgG avidity against *Toxoplasma gondii* ESAs by means of immunoblot, in order to establish a profile for acute recent infection in single serum samples and to determine the presence of residual IgM antibodies detected in automated assays.

MATERIAL AND METHODS

Serum samples. One hundred and eighty serum samples were obtained from individuals during routine screening for toxoplasmosis and were analyzed using commercially available kits (VIDAS and AxSYM) to detect IgM and IgG antibodies against *Toxoplasma gondii* and specific IgG antibody avidity. These human serum samples were characterized using the classical serological methods of indirect immunofluorescence (IIF) and indirect hemagglutination (IHA) and by means of an automated system (VIDAS) to define study groups as follows: group I: 36 serum samples from patients suspected of recently acquired (acute) Toxoplasma *gondii* infection; group II: 61 serum samples from patients suspected of recently acquired but non-acute *Toxoplasma gondii* infection; group III: 39 serum samples from patients with chronic toxoplasmosis; and group IV: 44 serum samples with negative serology, which were taken to be healthy controls. In addition, group V consisting of 20 patients characterized serologically and clinically with rubella, mononucleosis and cytomegalovirus was included in this study in order to test the specificity of the method. Details of the specific serological tests for characterizing study groups I, II, III, IV and V are described in Table 1. The institution's ethics committee approved the project.

Serological tests. The serum samples had been routinely tested using two automated assays: AxSYM IgG and IgM (Abbott Laboratories, USA) and VIDAS Toxo IgG, IgM and IgG avidity (BioMérieux, Brazil); and using two non-automated methods: indirect immunofluorescence (IIF) (BioMérieux, Brazil) and indirect hemagglutination (IHA) (BioMérieux, Brazil). The avidity of *Toxoplasma gondii*-specific IgG antibodies was determined as previously described by Hedman et al[9]. All commercially available reagents were used in accordance with the manufacturer's instructions.

Table 1 - Details of the specific serological tests for characterizing study groups I, II, III, IV and V.

Group	Number	Description
Group I (acute)	36	Serum samples from these patients exhibited specific IgM antibodies (titer ≥64), IgG antibodies (titer ≥256) using IIF and IHA, and low avidity of *T. gondii*-specific IgG. Serum samples were positive for *T. gondii*-specific IgG and IgM using VIDAS. Three serum samples exhibited an "acute" serological profile without clinical manifestation.
Group II (recent non-acute)	61	Samples had specific IgM antibodies (titer≥64), associated with positive results for IgG (titer of ≥64) using IIF and IHA, and high avidity of *T. gondii*-specific IgG. Serum samples were positive for *T. gondii*-specific IgG and IgM using VIDAS.
Group III (chronic)	39	Serum samples exhibited specific IgG antibodies (titer ≥64), negative results for *T. gondii*-specific IgM-(titer <16) using IIF and high avidity of *T. gondii* specific IgG. Serum samples were positive for IgG and negative for IgM *T. gondii*-specific using VIDAS.
Group IV (healthy controls)	44	All serum samples in this group were negative for Toxoplasma-specific IgM and IgG using IIF and IHA.
Group V (other diseases)	20	Seven serum samples from individuals infected with Epstein-Barr, six serum samples from patients infected with rubella virus and seven serum samples from individuals infected with cytomegalovirus were tested.

IIF: indirect immunofluorescence assay, IHA: indirect hemagglutination.

Excreted/secreted antigen. RH-strain tachyzoites of *Toxoplasma gondii* were obtained from the peritoneal cavities of Balb/c mice that had been infected two days earlier, and were inoculated into human fibroblast cell cultures, for 3h at 37°C. The culture supernatants were centrifuged at 1,000g for 10 minutes and were filtered through membranes of pore size 0.22μm (Millipore Corporation, Bedford, MA, USA). The ESAs were stored at -70°C with 100U/ml aprotinin (Sigma Chemical Co, St. Louis, MO, USA). To determine the mean number of intracellular parasites in host cells after 3h at 37°C, fibroblast cell cultures were trypsinated and counted in a Neubauer chamber.

Kinetics of ESA production. After washing three times with PBS at pH 7.2, the human fibroblast cultures were inoculated with 1 x 10^8 tachyzoites and incubated at 37°C. Five-milliliter aliquots of culture were taken at various times from one to 24 hours post-infection. Supernatants obtained at various times from the cultures were evaluated regarding the numbers and viability of tachyzoites by means of trypan blue and the protein concentrations were determined using the Bio-RAD DC Protein Assay, France.

Immunoblotting of IgG antibodies against ESA antigens. The ESAs were subjected to electrophoresis on polyacrylamide gels using a discontinuous sodium dodecyl sulfate (SDS) buffer system as described by Laemmli[15], with 18% running gel and 3% stacking gel. The running buffer contained 25mM TRIS,

192mM glycine, and 0.1% SDS at pH 8.3. Gels were run at 5-6 V/cm until the bromophenol blue marker reached the bottom. Molecular weight markers (Full Range Rainbow™, Amersham Life Sciences, England) were used to plot the calibration curve. Proteins separated by gel electrophoresis were further transferred to a 0.45μm (pore size) nitrocellulose membrane overnight, using a semidry transfer system. After transfer, nitrocellulose strips were blocked with 5% skimmed milk (Molico, Nestlé) in PBS plus 0.05% T20 for 2h at room temperature, in order to saturate protein-binding sites. Strips were further incubated for 18h at 4°C with serum samples, diluted 1:25 in 1% skimmed milk with PBS T20. After incubation, the strips were washed with PBS plus 0.05% Tween 20 three times for 5 min and incubated for 2h at room temperature with a 1:500 dilution of conjugate anti-human IgG-peroxidase (Sigma Chemical Co, St Louis, MO, USA) in PBS T20. After six washings with PBS plus 0.05% Tween 20, the strips were treated with 3mg of 4-chloronaphthol (Sigma Chemical Co, St Louis, MO, USA) in 10ml of methanol and 4μl of hydrogen peroxide.

IgG-avidity of ESA antigen by means of immunoblotting. Strips were incubated with serum samples, in duplicate, diluted 1:25 into 1% skimmed milk in PBS plus 0.05% Tween 20 overnight at 4°C. One strip of the duplicate was washed with PBS plus 0.05% T20 three times for 5 min. The second nitrocellulose strip was washed with 8M urea solutions in PBS, three times for 5 min. Both strips were incubated with conjugate anti-human IgG-peroxidase (Sigma Chemical Co, St Louis, MO, USA) diluted at 1:500 in PBS plus 0.05% Tween 20 for 2h at room temperature. After a further washing step, repeated six times for 5 min in PBS, the strips were treated with 3mg of 4-chloronaphthol (Sigma Chemical Co, St Louis, MO, USA) in 10ml of methanol and 4μl of hydrogen peroxide.

Avidity of *Toxoplasma gondii*-specific IgG by means of ELISA. The avidity of *Toxoplasma gondii* IgG antibodies was determined as described by Hedman[9], with some modifications. Microtiter plates were previously coated with 10^5 tachyzoites/well obtained from peritoneal exudates of Balb/c mice that had been infected two days earlier with the RH strain of *Toxoplasma gondii*. Serum samples were then added in duplicate, diluted 1:16 in PBS on separate plates. After incubation for 45 min at 37°C, the plates were subjected to differential washing methods as follows: one plate was washed with 8M urea solution in PBS for 5 min, while the other plate was washed only with PBS T20 for 5 min. In addition, both plates were washed twice with PBS T 20 for 5 min. Conjugate anti-human IgG-peroxidase (Sigma Chemical Co, St Louis, MO, USA) diluted at 1:3,000 in PBS T20 and incubated for 45 min at room temperature was added. After three washes in PBS, the reaction was developed using a substrate solution consisting of orthophenylenediamine at 0.5mg/ml in 0.1M citrate-phosphate buffer (pH 5.0) and 0.012% H_2O_2. After incubation for 15 min at room temperature, the reaction was stopped with 2N H_2SO_4. The absorbance was measured at 492nm by using a plate reader system (Titertek Multiskan Plus, Geneva, Switzerland). The avidity index was calculated as the ratio between the absorbance obtained for the plate washed with PBS plus urea and the absorbance for the plate washed with PBS without urea and was expressed as a percentage.

RESULTS

Non-automated serological assays. Out of the 200 human serum samples, 133 samples were positive for IgG-IIF and IgG-IHA, 97 samples were positive for IgM-IIF and IgM-IHA, both with antibody titers ≥16. All other serum samples from patients with heterologous infections (rubella, cytomegalovirus and mononucleosis) had negative specific IgG and IgM antibodies.

Automated serological assays and IgG avidity by means of ELISA. Out of the 200 samples, 136 were positive for IgG-VIDAS and 144 samples were positive for IgG-AxSYM. Ninety-seven serum samples were positive for IgM-VIDAS and 104 serum samples were positive for IgM-AxSYM. All other serum samples from patients with heterologous infections had negative specific IgG and IgM antibodies. For IgG avidity from VIDAS, out of 136 human serum samples, 91 had presented high-avidity IgG, nine had borderline-avidity IgG and 36 had low-avidity IgG. Among the IgG avidity results from ELISA, out of 136 human serum samples, 90 samples had high-avidity IgG, eight had borderline-avidity IgG and 38 had low-avidity IgG.

Kinetics of ESA production. During the first 12 hours of infection, the number of extracellular tachyzoites declined, and no host cells could be detected. During the next 12 hours, there was an increase in parasite numbers, and dead host cells were observed. The tachyzoites forms remained intact before and after inoculation, as observed by phase contrast microscopy. The ESA concentration in supernatants harvest from cultures at each study time was estimated and detected as early as one hour after infection. Levels of ESA only rose significantly in cultures after three hours, reaching a maximum at the end of the study period.

Immunoblotting of IgG antibodies against ESA antigens. Out of the 200 serum samples, 136 were reactive and revealed the same reactivity against fractions of different molecular weights. The eight discordant serum samples with regard to specific IgG antibodies did not occur between VIDAS and AxSYM, and the serum samples from patients with heterologous infection did not present any reactivity to protein fractions of *Toxoplasma gondii* ESA.

Avidity of IgG antibodies against ESAs by means of immunoblotting. Out of 136 human serum samples, 103 had high-avidity IgG and 33 samples had low avidity IgG against the 30kDa fraction protein. Serum samples from acute recently infected patients had low avidity and the samples from non-acute and chronic patients had high avidity against the 30kDa fraction protein.

DISCUSSION

Serological evaluation of the time when *Toxoplasma* infection was acquired is of fundamental importance for pregnant women, since infection during pregnancy requires intervention and treatment. During this phase, both clinical manifestations and congenital toxoplasmosis may occur[3]. Several methods have been used to measure specific IgM antibodies as markers for toxoplasmosis infection[4]. However, in clinical practice, it is not unusual to observe persistent IgM antibodies for prolonged periods after the acute phase of infection[1 4 6 20 22].

Table 2 shows that, in our study, residual IgM antibodies were detected by VIDAS and AxSYM, and this was confirmed by the negative results found for IIF, IHA, high avidity tests (VIDAS) and clinical follow-up information (data not shown). IgM-VIDAS assays showed better performance (residual IgM n=64) than seen with AxSYM (residual IgM n=71). VIDAS showed high specificity and positive predictive values with regard to detecting specific IgM antibodies[11]. IgG avidity determination is an important serological marker that can be used to distinguish between acute and non-acute disease in single serum samples, through confirming the presence or absence of residual IgM antibodies[4 10 13 17].

Table 2 - Performance of automated assays for detecting residual IgM antibodies in the samples studied.

	IgM antibodies		
Automated assay	positive (nº)	true positive (nº)	residual (nº)
VIDAS	97	33	64
AXSYM	104	33	71

True positive: Serum sample exhibited specific IgM antibodies (titer ≥64) using IIF and IHA, low avidity of specific IgG antibodies and clinical compatibility with *acute* recent toxoplasmosis.

However, recent studies on the kinetics of IgG avidity in pregnant women have observed high avidity in pregnant women who had been infected for at least three to five months and low avidity one year after the acute phase[18]. In addition, significant numbers of patients present borderline avidity results[22].

Based on clinical information (data not shown) and conventional serological assays, we analyzed ELISA avidity and VIDAS avidity to determine residual IgM antibodies. For this, 71 serum samples were used to determine residual IgM and were subjected to avidity assays. The VIDAS and ELISA avidity assays showed similar performance in the panel of 71 residual IgM samples: respectively 60/71 and 59/71 samples presenting with high avidity. Borderline avidity results were observed in nine samples with VIDAS and in eight samples with ELISA (Table 3).

Problems in measuring specific IgG antibodies have only rarely been observed in laboratory practice[7]. In our study too, we only observed false positive results for specific IgG antibodies in eight out of 200 samples analyzed, as confirmed by negative results found with IgG-IIF and IgG-IHA. In this panel of eight false positive IgG samples, 3/8 (37.5%) yielded positive results with IgG-AxSYM and 5/8 (62.5%) were borderline IgG according to both automated assays (VIDAS and AxSYM) (Table 4). False positive results in relation to specific IgG antibodies may lead to wrong interpretation and diagnosis of congenital toxoplasmosis[6]. Development of different serological techniques has increased the difficulty in defining diagnoses, even in the presence of several markers in single serum samples, due to persistency in distinguishing between acute recent phases and non-acute recent phases.

In the *Toxoplasma* serology laboratory of the Palo Alto Medical Foundation, which is a diagnostic reference center, positive IgM results in pregnant women are confirmed by combining measurements of IgG, IgA, IgE antibodies and IgG avidity in serum samples obtained at various times during pregnancy. In public clinics and some laboratories in Brazil, residual IgM antibodies are determined in single serum samples in combination with specific IgG antibodies and rarely with IgG avidity, due to the cost of equipment and reagents for performing this additional test.

Table 3 - Comparison of residual IgM antibodies obtained from automated assays and IgG avidity tests for 71 serum samples.

	IgM residual serum samples					
IgG avidity result	VIDAS avidity		ELISA avidity		ESA avidity	
	nº	%	nº	%	nº	%
Low	2	2.8	4	5.6	0	
Borderline/indeterminate	9	12.7	8	11.3	0	
High	60	84.5	59	83.1	71	100.0

Table 4 - Discordant results obtained using IIF, IHA, VIDAS and AXSYM for measuring* Toxoplasma*-specific IgG antibodies.

	Toxoplasma gondii- specific IgG antibodies				ESA-specific IgG antibodies
Sample	IIF	IHA	VIDAS	AXSYM	Immunoblot
	Titer/cutoff	Titer/cutoff	UI/cutoff	UI/cutoff	
1	<1:16 (<1:16)	<1:16 (<1:16)	< 4.0 (<4.0)	20(<3.0)	NR
2	<1:16 (<1:16)	<1:16 (<1:16)	< 4.0 (<4.0)	20(<3.0)	NR
3	<1:16 (<1:16)	<1:16 (<1:16)	< 4.0 (<4.0)	27(<3.0)	NR
4	<1:16 (<1:16)	<1:16 (<1:16)	6.0 (<4.0)	10(<3.0)	NR
5	<1:16 (<1:16)	<1:16 (<1:16)	7.0 (<4.0)	3(<3.0)	NR
6	<1:16 (<1:16)	<1:16 (<1:16)	4.0 (<4.0)	3.5(<3.0)	NR
7	<1:16 (<1:16)	<1:16 (<1:16)	4.0 (<4.0)	4.0(<3.0)	NR
8	<1:16 (<1:16)	<1:16 (<1:16)	4.0 (<4.0)	3.0(<3.0)	NR

NR = non-reactive, UI = international units, IIF= indirect immunofluorescence assay, IHA=indirect hemagglutination, ESA= excreted/secreted antigen.

In this respect, the present study focused on the potential for diagnoses in single serum samples by using the avidity of IgG antibodies against tachyzoite ESAs. These constitute most of the circulating antigens in acutely infected humans, and thus are one of the first targets of the immune response[5 12]. Based on different studies, we have recently developed a technique for obtaining ESAs by incubating tachyzoites in human fibroblast cell cultures for a short period of time[2 14]. At the outset of our studies on *Toxoplasma gondii* ESA, our concern was to rule out the possibility that the antigens could have been obtained from lysis of parasites during the procedure to produce the antigen. The results relating to ESA kinetics have indicated that the antigen obtained has a secretory or excretory origin. The kinetics of secretion-excretion show that ESAs can be detected within the first hour and reach their maximum concentrations within three hours of inoculation. This was similar to what had previously been observed by Hughes[13] when they detected ESAs in serum during the initial period of infection.

Protein fractions, including P30, were recognized by IgG antibodies with the same reactivity patterns in patients with acute and non-acute disease, in agreement with the findings of Acebes[1] and Meek[18] (Figure 1). However, the samples that presented false positive results in relation to specific IgG antibodies in automated assays, and also the healthy individuals and individuals with other diseases, did not present reactivity of IgG antibodies against protein

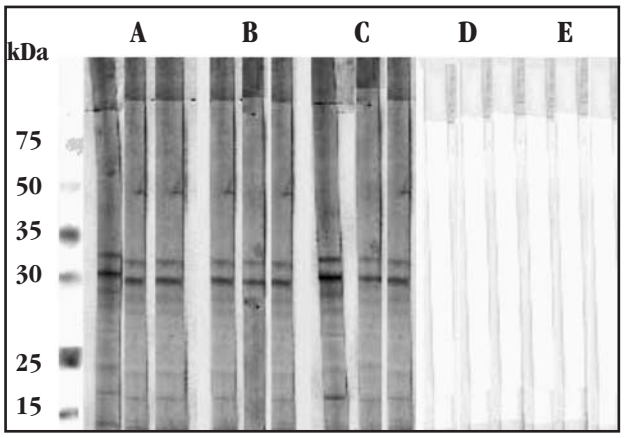

Figure 1 - Antigenic profile of excreted/secreted antigens of* Toxoplasma gondii *recognized by specific IgG antibodies in serum samples from acute recent cases (A), non-acute recent case (B), cases of chronic infection (group C), healthy individuals (group D) and cases of other diseases (group E), as determined by immunoblot.

fractions of *Toxoplasma gondii* ESA, as seen using immunoblot (Figure 1). These results differed from those of Hafid[8] and Meek[18], which were based on ESA obtained from African green monkey kidney cell cultures and from infected mice.

The avidity performance of IgG against the protein fraction of ESA-P30 in the panel of 33 acute disease samples and 61 non-acute samples was reliable: 33/33 present low avidity and 61/61 present with high avidity, respectively. Three out of 36 serum samples with serological characteristics of acute diseases but without compatible clinical features present high avidity in assays of IgG avidity against ESA by means of immunoblot (Figure 1). In addition, no indeterminate results for assays of IgG avidity against ESA by means of immunoblot were observed in the present study (Figure 2).

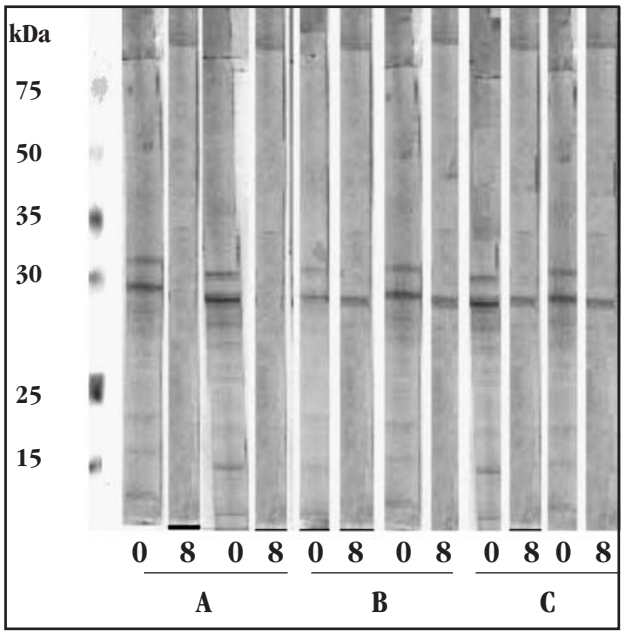

Figure 2 - Antigenic profile of excreted/secreted antigens of* Toxoplasma gondii *recognized by specific IgG antibodies in serum samples from acute recent cases (A), non-acute recent cases (B) and chronic infection cases (group C), as determined by immunoblot without or with 8M urea treatment.

0 = without urea; 8 = 8M urea.

Our data do not agree with those in the study by Marcolino[15], which could not distinguish between patients with acute and non-acute disease through analyzing IgG avidity against P30 obtained from the peritoneum of infected mice. Most commercial kits and in-house methods use tachyzoites grown in mouse or other cell cultures and this factor is probably associated with the intrinsic proprieties of the technique used. This would explain the better performance of assays of IgG avidity against ESA protein fractions obtained from human fibroblast culture in distinguishing between acute and non-acute disease. Our date suggest that IgG avidity against P30 (probably SAG-1) can be considered to be an immunological marker for distinguishing acute disease from non-acute disease, thereby confirming the presence of residual IgM antibodies obtained for automated assays in single serum samples.

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