Analysis of IgG subclasses (IgG1 and IgG3) to recombinant SAG2A protein from Toxoplasma gondii in sequential serum samples from patients with toxoplasmosis

Silas S. Santana a, Deise A.O. Silva a, Letícia D. Vaz a, Carlos P. Pirovani b, Geisa B. Barros c, Elenice M. Lemos c, Reynaldo Dietze c, José R. Mineo a, Jair P. Cunha-Junior a,d

a Laboratory of Immunoparasitology, Institute of Biomedical Sciences, Federal University of Uberlândia, 38400-902 Uberlândia, MG, Brazil
b Laboratory of Proteomic, Center of Biotechnology and Genetics, State University of Santa Cruz, 45662-000 Ilhéus, BA, Brazil
c Infectious Disease Center, Federal University of Espirito Santo, 29040-091 Vitória, Espirito Santo, Brazil

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The kinetics of the humoral immune response was evaluated using the recombinant SAG2A protein comparatively to soluble Toxoplasma antigen (STAg) by ELISA in sequential serum samples of patients with toxoplasmosis up to 12 months of illness onset. The follow up of IgM and IgA levels to STAg showed a gradual decrease, with the majority of patients (88%) seropositive for IgM up to 12 months of infection, whereas IgA seropositivity was relatively low (78%) compared to IgM (100%) in the first 3 months of infection. The follow up of IgG and IgG1 antibodies showed a similar increasing profile for both SAG2A and STAg, with slightly higher seropositivity for STAg. The kinetics of IgG3 to STAg was similar to that of IgG1, contrasting with the kinetics of IgG3 to SAG2A that showed high levels up to 6 months of infection, with continuous decreasing over the time. Higher IgG3 seropositivity to SAG2A than STAg was also observed in the initial phases of infection. A higher IgG3/IgG1 ratio for SAG2A than STAg was detected in the first 3 months of infection, with decreasing profile over the time. The associations of IgG3/IgG1 ratio > 1.0 with positive IgM or IgA antibodies were predominantly found in the first 3 months of infection, whereas associations of IgG3/IgG1 ratio < 1.0 with positive IgM or negative IgA antibodies were mostly observed from 3 to 12 months of infection. In conclusion, our results demonstrate a differential kinetics of IgG3 antibodies to SAG2A and STAg in patients with toxoplasmosis up to 12 months of infection. Also, the IgG3/IgG1 ratio to SAG2A in association with classical serological markers of acute phase could be potential tools to distinguish early acute from convalescent phases of Toxoplasma gondii infection.

1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects a large number of hosts, including humans [1]. The acute phase of the infection is generally asymptomatic or with mild symptoms in immunocompetent hosts, and the parasite persists in the chronic phase [2,3]. Severe disease may occur in immunocompromised individuals and pregnant women that acquire the primary infection during gestation, where the passage of the parasite via placenta to the fetus may cause abortion, neonatal death or postnatal complications [4–7].

Toxoplasmosis is routinely diagnosed by the detection of specific antibodies to T. gondii. The detection of low titers of IgG antibodies with presence of IgM and IgA antibodies is suggestive of acute phase, while the absence of IgM and IgA antibodies and presence of high titers of IgG antibodies suggest chronic phase [8].

Unfortunately, the differentiation from acute and chronic phase is not easy to achieve. In some cases, the IgM antibody can persist more than one year after the initial infection, whereas the detection of IgA antibodies to T. gondii is not an accurate marker for diagnosis [9,10]. The detection of IgG antibodies indicates T. gondii infection, but it is difficult to estimate the time of infection. At present, the avidity of IgG antibodies represents a useful tool to differentiate phases of infection, although the detection of IgG avidity can be hampered, in some cases, since that the low-avidity IgG antibodies can be persistent for months, thus not estimating precisely the time of infection [11].

Sensitivity and specificity are essential parameters in diagnostic methods for toxoplasmosis to avoid positive- and negative-results that could difficult the diagnosis. In most cases, these parameters are dependent of the antigen utilized in each assay. In general, the serological kits utilize Toxoplasma lyssate antigens obtained from tachyzoites cultured in cell cultures or mouse peritoneal exudates, resulting in differences related to source of antigens causing considerable variation on the performance of diagnostic tests [12]. On the other hand, recombinant T. gondii
proteins have contributed for a reduction in the costs of production and purification of antigens for diagnostic tests, allowing the selection of specific antigens for each phase of infection. Thus, the assays based on recombinant antigens could be useful to establish the relative value of each isolated antigen in the determination of different phases of infection. This information is particularly important for the diagnosis of primary infection during pregnancy, mainly in cases of risk for transmission of the parasite to the fetus.

A number of studies have reported the potential use of various recombinant proteins in serological tests for the diagnosis of T. gondii infection, such as GRA1 (p24), GRA2 (p28), SAG1 (p30), SAG2 (p22), ROP1 (p66), ROP2 (p54) and MAG1 (p65, p68) [10,13–16]. Among them, the members of the SAG1 and SAG2 family are particularly attractive since the surface of T. gondii is mostly coated by these glycosylphosphatidylinositol-anchored antigens [10]. The protein SAG2A (22 kDa) is a surface antigen of T. gondii, expressed in tachyzoites of the parasite. It is known that SAG2A is important for the initial attachment and invasion of the parasite to cell hosts, as well as in the modulation of immune responses [17]. SAG2A is an immunodominant protein that elicits strong humoral response and our previous study showed that IgG and mostly IgG1 antibodies from patients with acute phase of toxoplasmosis reacted more strongly with SAG2A antigens than sera from patients with chronic phase, suggesting its potential use to characterize the acute phase of infection [15]. However, it has been little investigated the role of other IgG subclasses in the differential diagnosis of the phases of toxoplasmosis [6].

In the present study, we evaluated the kinetics of the humoral immune response to the recombinant SAG2A antigen in comparison with T. gondii soluble antigen (STAg) through the detection of specific IgG, IgG1 and IgG3 antibodies in serum samples of patients with toxoplasmosis in different time points of infection. Also, we verified the association of these serological markers based on SAG2A reactivity as potential tools to distinguish early acute from convalescent phase of toxoplasmosis.

2. Materials and methods

2.1. Patients and serum samples

This is a prospective longitudinal study that evaluated a total of 130 serum samples obtained from 19 patients with toxoplasmosis after different time points of illness onset. The patients enrolled in the study were attended at the Infectious Disease Center of the Federal University of Espirito Santo, Vitoria, ES, Brazil, after an initial screening by physicians of private clinics or public hospitals of the region that directed the patients that filled the following criteria of inclusion: male and female patients, age ranging from 7 to 80 years, presence of at least two from the clinical signals compatible with the acute phase of toxoplasmosis (weakness, fever, enlarged cervical lymph nodes, headache, arthralgia), and with serological evidence of infection characterized by the presence of IgM and/or IgG antibodies to T. gondii in conventional serological assays using a commercial kit (ELFA, VIDAS® Toxo IgM and IgG II, Biomérieux SA, Lyon, France). Exclusion criteria were pregnancy and/or human immunodeficiency virus (HIV)-positive patients. An average of seven blood samples were collected from each patient by venopuncture over the time for 12 months, and sera were stored at −20 °C in aliquots until serological assays. The study was approved by the Ethics Committee of the Institution and informed consent was obtained from all patients prior to study entry.

2.2. Production of soluble Toxoplasma antigen (STAg) and recombinant SAG2A antigen

Tachyzoites of the RH strain were maintained by serial passage in Swiss mice for 48–72 h [18]. The peritoneal exudates from infected mice were harvested and washed in phosphate-buffered saline (PBS, pH 7.2). Parasite suspensions were adjusted to 1 × 10⁸ tachyzoites/mL and submitted to freeze–thawing and sonication cycles in the presence of protease inhibitors [19]. The preparation was centrifuged at 10,000 × g for 15 min at 4 °C; the supernatants were collected, the protein content was determined by the method of Bradford [20] and aliquots were stored at −20 °C until used as T. gondii soluble antigen (STAg).

Recombinant SAG2A production was prepared as previously described [15]. Briefly, DNA from T. gondii RH strain tachyzoites were used as the template for amplification of the SAG2A gene by a single polymerase chain reaction (PCR) protocol, using the following primers containing Ndel and HindIII recognition sequences: 5’–CAAGTTGGCCTCATATTCACCCAG-3’ and 5’–ACTTTTGCAGAAGCTTCTCCGAAG-3’. The PCR product (607 bp) was digested and inserted in Ndel/HindIII sites of the pET28a vector. The resulting vector containing the sequence encoding SAG2A molecule in fusion with his-tag sequence (pET28a-SAG2A) was transformed in BL21 (DE) Escherichia coli. The transformed BL21 (DE) bacteria were grown in LB media supplemented with 50 μg/mL of kanamycin at 37 °C up to reach an optical density (OD) at 600 nm of 0.5. For SAG2A protein expression, the culture was induced with 0.5 mM isopropyl-β-d-thiogalactopyranoside (IPTG; Sigma Chemical Co., St Louis, USA), with slow shaking (90 rpm) at 20 °C for 16 h. Cells were harvested by centrifugation, the pellet was resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 200 mM sucrose, 0.2% Triton X-100 and 1 mM PMSF), and disrupted with freeze-and-thaw (six cycles) and sonication (ten 20-s cycles at 90% of power). Insoluble debris was removed by centrifugation at 10,000 × g for 20 min at 4 °C and the supernatant was loaded into a NTA-Ni column (Qiagen) previously equilibrated in 50 mM phosphate buffer, pH 8.0. The unbound proteins were removed by washing buffer (50 mM NaH₂PO₄ and 300 mM NaCl, pH 8.0). The SAG2A protein was eluted from the column with the same equilibrium buffer supplemented with 250 mM imidazole. SAG2A fractions were pooled and dialyzed against PBS, the protein concentration was determined by the method of Bradford [20] and aliquots were stored at −20 °C.

2.3. Capture ELISA for detection of IgM and IgA antibodies to T. gondii

Capture ELISA for IgM and IgA antibodies to T. gondii were performed in house as previously described [21]. Briefly, high-binding microtiter plates (Costar-Corning Incorporated, New York, USA) were coated overnight at 4 °C with goat anti-human IgM or anti-human IgA antibodies (10 μg/mL) (Sigma) diluted in 0.06 M carbonate buffer (pH 9.6). Plates were washed three times with PBS plus 0.05% Tween 20 (PBS-T) and blocked with PBS-T supplemented with 5% skim fat milk for 1 h at room temperature. Subsequently, wells were incubated with serum samples, in duplicate, diluted at 1:16 for 2 h at 37 °C and then with STAg (100 μg/mL) for 2 h at 37 °C. The bound antigen was detected with a peroxidase-labeled rabbit F(ab’)₂ anti-T. gondii conjugate (prepared as previously described [22]) diluted 1:50 and incubated for 1 h at 37 °C. The assay was developed by adding the enzyme substrate (0.03% H₂O₂ and 2,2’-azino-bis-3-ethylbenzothiazoline sulfinic acid [ABTS, Sigma] in 0.07 M citrate phosphate buffer, pH 4.2). The optical density (OD) was read at 405 nm in a plate reader (Titertek Multiskan Plus spectrophotometer, Flow Laboratories, McLean, USA). The cutoff of the
reaction was calculated as the mean OD of negative control sera plus 3 standard deviations. Antibody titers were expressed as ELISA index (EI), according to the following formula: EI = OD sample/OD cutoff, as described elsewhere [23]. Samples with EI values ≥ 1.2 were considered positive.

All serum samples were also analyzed for IgM anti- *T. gondii* by using a commercial kit (ELFA, VIDAS® Toxo IgM, Biomérieux SA) after different time points of illness onset, following the manufacturer’s instructions.

### 2.4. Indirect ELISAs for detection of IgG and subclasses (IgG1 and IgG3) to SAG2A and STAg

Indirect ELISAs for the detection of IgG antibodies and their subclasses (IgG1, IgG2, IgG3, and IgG4) to SAG2A and STAg were also performed in house. Briefly, high-binding microtiter plates (Costar-Corning Incorporated) were coated with 5 μg/mL of SAG2A or STAg diluted in 0.06 M carbonate buffer (pH 9.6) overnight at 4°C. Plates were washed with PBS-T and blocked with PBS-T supplemented with 5% skim fat milk for 1 h at room temperature. Subsequently, wells were incubated with serum samples, in duplicate, diluted at 1:64 for 2 h at 37°C. Plates were washed six times and incubated with peroxidase-labeled goat anti-human IgG (1:1000, Sigma) for 1 h at 37°C. Plates were washed again and the enzymatic activity was revealed by adding the enzyme substrate (0.03% H₂O₂ and 0.01 M ABTS). The OD values were determined at 405 nm. Results were expressed as ELISA index (EI) as described for capture ELISA for IgM and IgA antibodies.

All serum samples were also analyzed for IgG anti- *T. gondii* by using a commercial kit (ELFA, VIDAS® Toxo IgG II, Biomérieux SA) after different time points of illness onset, following the manufacturer’s instructions.

For the detection of IgG subclasses, microtiter plates were coated as described above, washed with PBS-T and blocked with PBS-T plus 1% bovine serum albumin (PBS-T–BSA) for 1 h at room temperature. Subsequently, wells were incubated with serum samples diluted at 1:64 in PBS-T–BSA for 2 h at 37°C. The wells were washed six times and incubated with the respective biotinylated secondary antibodies (Sigma) as follows: goat anti-human IgG1 (1:1000), anti-human IgG2 (1:1000), anti-human IgG3 (1:1000), or anti-human IgG4 (1:1000) for 1 h at 37°C. Plates were washed again and incubated with streptavidin–peroxidase (1:1000, Sigma) for 30 min at room temperature. Plates were washed and the reaction was revealed as described above.

### 2.5. Statistical analyses

Statistical analyses were performed using the GraphPad Prism v. 5.0 (GraphPad Software, San Diego, USA). Antibody levels were compared between the antigens in different time points using paired or unpaired Student’s t test, when appropriated. Association between IgG3/IgG1 ratio with IgA or IgM antibodies and positivity for both antigens were analyzed by the Fisher exact test or Chi-square test. The sensitivity and specificity of assay combinations were evaluated by serial tests using the multiple diagnostic tests from the Win Episcope 2.0 software (www.clive.ed.ac.uk). For this purpose, the sensitivity was defined as the percentage of positive results in samples from 0 to 3 months of infection (early acute phase) and the specificity as the percentage of negative results in samples from 3 to 12 months of infection (convalescent phase). The positive and negative predictive values were not possible to be calculated in the present study due to prevalence uncertainty as previously described [24]. P values <0.05 were considered statistically significant.

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![Fig. 1. Kinetics of IgM and IgA antibodies to *Toxoplasma gondii* determined by capture ELISA using soluble tachyzoite antigens (STAg) in sera from patients with toxoplasmosis at different time points of illness onset.](image)

The kinetics of the humoral immune response to *T. gondii* in patients with acute toxoplasmosis was initially evaluated by measuring IgM and IgA antibodies to STAg by capture ELISA in different time points. The highest IgM level was found in the early acute phase (0–3 months) of infection, with significant decrease at 3–6 and 6–9 months of illness onset (P < 0.05) (Fig. 1). Mean IgM levels were similar from 6 to 12 months of infection, with 100% of positive samples up to 9 months and even maintaining 88% of positivity from 9 to 12 months of illness onset (Fig. 1). These findings were confirmed when using a commercial test (ELFA, VIDAS® Toxo IgM), that provided 100% positive IgM samples up to 6 months of infection, 92% from 6 to 9 months, and 83% from 9 to 12 months of illness onset (data not shown).

A gradual reduction in IgA levels to STAg was also observed during the months analyzed, although with lower mean levels as compared to IgM (Fig. 1). IgA levels were higher in the initial phases (0–6 months) of infection than the subsequent time points (P < 0.05). Mean IgA levels maintained above the cutoff value during the follow-up, but only 78% of positive IgA samples were detected in the first 3 months of infection, with gradual decrease for 53%, 52% and 38% in the 2nd, 3rd, and 4th time points analyzed, respectively.

### 3. Results

#### 3.1. Kinetics of IgM and IgA antibodies to STAg

The kinetics of the humoral immune response to *T. gondii* in patients with acute toxoplasmosis was initially evaluated by measuring IgM and IgA antibodies to STAg by capture ELISA in different time points. The highest IgM level was found in the early acute phase (0–3 months) of infection, with significant decrease at 3–6 and 6–9 months of illness onset (P < 0.05) (Fig. 1). Mean IgM levels were similar from 6 to 12 months of infection, with 100% of positive samples up to 9 months and even maintaining 88% of positivity from 9 to 12 months of illness onset (Fig. 1). These findings were confirmed when using a commercial test (ELFA, VIDAS® Toxo IgM), that provided 100% positive IgM samples up to 6 months of infection, 92% from 6 to 9 months, and 83% from 9 to 12 months of illness onset (data not shown).

A gradual reduction in IgA levels to STAg was also observed during the months analyzed, although with lower mean levels as compared to IgM (Fig. 1). IgA levels were higher in the initial phases (0–6 months) of infection than the subsequent time points (P < 0.05). Mean IgA levels maintained above the cutoff value during the follow-up, but only 78% of positive IgA samples were detected in the first 3 months of infection, with gradual decrease for 53%, 52% and 38% in the 2nd, 3rd, and 4th time points analyzed, respectively.

#### 3.2. Kinetics of IgG and subclasses (IgG1 and IgG3) antibodies to SAG2A and STAg

In order to analyze the kinetics of IgG and subclasses, an indirect ELISA was carried out using recombinant SAG2A in comparison with STAg (Fig. 2). The comparative analysis between IgG antibodies to SAG2A and STAg displayed a similar reactivity profile that was increasing up to 6–9 months of infection, although a higher reactivity was observed to SAG2A than STAg in each time point analyzed (P < 0.001) (Fig. 2A). The kinetics of IgG1 antibodies to SAG2A and
STAg showed an increase in the reactivity from 0 to 6 months of infection, with a slight decrease or maintenance of their levels up to 12 months of illness onset, but with no significant difference between the antigens in each time point (Fig. 2B).

The response profile of IgG3 antibodies to SAG2A showed high levels in the initial phases (0–6 months) of infection, followed by a continuous decrease over the time (Fig. 2C). In contrast, when ELISA was developed with STAg as antigen, IgG3 levels were low in the first 3 months of infection, followed by a slight increase at 3–6 months and reduction in the subsequent time points. Interestingly, IgG3 levels to SAG2A were significantly higher than to STAg in the first 3 months of infection (*p* < 0.0001).

To underline the differential profile of the IgG3 response to SAG2A and STAg, the ratio between the levels of IgG3 and IgG1 antibodies was also analyzed over the time (Fig. 2D). The IgG3/IgG1 ratio to SAG2A was higher than 1.0 in the first 3 months of infection and showed a marked decrease during the follow-up, while the IgG3/IgG1 ratio to STAg was lower than 1.0 and constant over the time. Also, the IgG3/IgG1 ratio to SAG2A was significantly higher than to STAg only in the first 3 months of infection (*p* < 0.0009). Serum samples were also analyzed for the detection of IgG2 and IgG4 antibodies in indirect ELISA, but they were non-reactive to SAG2A and STAg (data not shown).

### 3.3. Antibody isotype positivity to STAg and SAG2A

Differences among the percentages of antibody isotype positivity for SAG2A and STAg in each time point are demonstrated in Fig. 3. Regarding the IgG antibody (Fig. 3A), positivity for STAg was increasing, with 85% in the first 3 months, 97.5% at 3–6 months and 100% from 6 to 12 months of infection. Similar results were obtained when using a commercial test (ELFA, VIDAS® Toxo IgG II), with 88% of positive IgG sera up to 3 months of infection and 100% from 3 to 12 months of illness onset (data not shown). IgG positivity for SAG2A was also increasing, ranging from 78% to 92%, but slightly lower than for STAg, although with no significant difference in any time point analyzed.

The IgG1 positivity for STAg was higher from 3 to 12 months of infection (>95%) as compared to the first 3 months (80%) (*p* < 0.05). On the other hand, IgG1 positivity for SAG2A was lower than for STAg in all time points (66–85%), even though statistical significance was achieved only at 6–9 months of infection (*p* < 0.05).

As shown in Fig. 3C, IgG3 positivity for STAg was lower in the first 3 months of infection (58%) as compared to subsequent times (75–88%), with significant difference only at 3–6 months of infection (*p* < 0.05). In contrast, IgG3 positivity for SAG2A was high from 0 to 9 months (>92%), with significant decrease at 9–12 months (67%) of infection. When comparing the antigens in each time point, IgG3 positivity was significantly higher for SAG2A than STAg at 0–3 months and 6–9 months of infection (*p* < 0.05).

The positive serum samples with high IgG3/IgG1 ratio (>1.0) were also analyzed during the follow-up (Fig. 3D). It was observed higher positivity for SAG2A in the first 3 months of infection when compared to the subsequent time points (*p* < 0.05). The comparative analysis between SAG2A and STAg showed that the positivity of IgG3/IgG1 ratio > 1.0 for SAG2A was higher than STAg in all time points (*p* < 0.05). No significant difference was observed for STAg in any time points analyzed.

### 3.4. Association between IgG3/IgG1 ratio to SAG2A and classical serological markers

The association between the serological results based on SAG2A reactivity (IgG3/IgG1 ratio) and classical serological markers to characterize acute phase of toxoplasmosis (IgM and IgA antibodies to STAg) was evaluated in each time point (Figs. 4 and 5). Serological results showing a high IgG3/IgG1 ratio (>1.0) and positive IgM levels (EI ≥ 1.2) were predominantly found in the first 3 months of
infection (74%, Fig. 4A), with significant reduction in the subsequent time points (50%, 37.5% and 32%; Fig. 4B–D, respectively) \( (P < 0.05) \). On the other hand, the association between a low IgG3/IgG1 ratio \(<1.0\) and positive IgM levels was mainly noted from 3 to 12 months \( (50\%, 62.5\%, \text{and} \ 56\%; \ \text{Fig.} \ 4B–D, \ \text{respectively}) \) in comparison with the first 3 months of infection \( (24\%, \ \text{Fig.} \ 4A) \ (P < 0.05) \).

As shown in Fig. 5, the association between IgG3/IgG1 ratio \( >1.0\) and positive IgA levels \( (\text{EI} \geq 1.2) \) was mostly verified from 0 to 3 months of infection \( (58.3\%, \ \text{Fig.} \ 5A) \), with significant decrease in the subsequent times \( (25\%, 29.7\%, \ \text{and} \ 16\%; \ \text{Fig.} \ 5B–D) \ (P < 0.05) \). In contrast, serological results with low IgG3/IgG1 ratio \(<1.0\) and negative IgA levels \( (\text{EI} < 1.2) \) were predominantly found from 6 to 12 months \( (37\% \ \text{and} \ 44\%; \ \text{Fig.} \ 5C \ \text{and} \ D) \) in comparison with 0–6 months of infection \( (9\% \ \text{and} \ 22\%; \ \text{Fig.} \ 5A \ \text{and} \ B) \ (P < 0.05) \).

3.5. Proposed schedule for the diagnosis of toxoplasmosis

From the analysis of our results, we proposed a schedule for the serodiagnosis of toxoplasmosis in order to complement the conventional techniques \( (\text{Fig.} \ 6) \). Routinely, the serum samples from patients with suspect of toxoplasmosis are tested for screening assays, including the indirect ELISA for IgG and capture ELISA for IgM and IgA antibodies to \( T. \ gondii \) using STAg as antigen. Serum samples that are nonreacting in these assays are considered negative for \( T. \ gondii \) infection, while those with positivity only for IgG antibodies are related to chronic phase. The three possible serological results that could indicate the status of serum samples as belonging to the acute phase are the concomitant presence of IgG, IgM, and IgA antibodies, or the presence of IgG and IgM, or the presence of IgM and IgA, in the absence of IgG antibodies.

In order to better define this diagnosis status, we proposed as complementary assays the evaluation of IgG subclasses, especially IgG1 and IgG3 to SAG2A by indirect ELISA, characterizing two possible outcomes: early acute and convalescent phases. The early acute phase, in our study, was established by the association of high IgG3/IgG1 ratio \( (>1.0) \) with positive IgM and/or IgA antibodies. Thus, when using as first test the in house capture IgM-ELISA \( (100\% \ \text{sensitivity} \ \text{and} \ 3\% \ \text{specificity}) \) or capture IgA-ELISA \( (78\% \ \text{sensitivity} \ \text{and} \ 52\% \ \text{specificity}) \) and as second test the IgG3/IgG1 ratio assay \( (76\% \ \text{sensitivity} \ \text{and} \ 56\% \ \text{specificity}) \), the combination of these serial tests resulted in significant increase of specificity for IgG3/IgG1 ratio \( (>1.0) \) with positive IgM \( (58\%) \) or IgA \( (79\%) \) to include a sample within 0–3 months of infection. On the other hand, the convalescent phase was characterized by low IgG3/IgG1 ratio \(<1.0\) associated with the positive IgM and/or negative IgA to \( T. \ gondii \). The performance of assay combinations evaluated by serial tests showed a significant increase of specificity, especially regarding the IgG3/IgG1 ratio \(<1.0\) and negative IgA \( (83\%) \) to include a sample within 6–12 months of infection.
Fig. 4. Association between IgG3/IgG1 ratio to recombinant SAG2A antigen and IgM antibodies to soluble antigen (STAg) from *Toxoplasma gondii* determined by ELISA in 130 serum samples from 19 patients that were grouped within each time (A–D) of infection. The IgG3/IgG1 ratio $>1.0$ and ELISA index (EI) $\geq 1.2$ for IgM antibodies are represented by dashed lines and indicate positive serum samples. Double-positive, double-negative or single-positive percentages are indicated in each corresponding corner. Different letters (a and b) indicate statistically significant differences between each time of infection in the corresponding corner (Chi-square test, *P* < 0.05).

Fig. 5. Association between IgG3/IgG1 ratio to recombinant SAG2A antigen and IgA antibodies to soluble antigen (STAg) from *Toxoplasma gondii* determined by ELISA in 130 serum samples from 19 patients that were grouped within each time (A–D) of infection. The IgG3/IgG1 ratio $>1.0$ and ELISA index (EI) $\geq 1.2$ for IgA antibodies are represented by dashed lines and indicate positive serum samples. Double-positive, double-negative or single-positive percentages are indicated in each corresponding corner. Different letters (a and b) indicate statistically significant differences between each time of infection in the corresponding corner (Chi-square test, *P* < 0.05).
4. Discussion

Several reports have demonstrated the use of *T. gondii* recombinant proteins as potential source of antigens for serodiagnosis of toxoplasmosis instead of conventional extracts of *T. gondii* tachyzoites [10,11,14,16,25–28]. The recombinant antigens have been used in the development of new tests capable to discriminate acute from chronic phase of *T. gondii* infection [29,30]. Considering the potential damages in fetus associated with congenital toxoplasmosis, the development of new serologic tests that are able to discriminate recent from past infections, including the early acute phase and convalescent phase, will be clinically relevant, especially during pregnancy [10].

Potential markers for acute phase of toxoplasmosis were already described using the recombinant MAG1 [16] and GRA2 antigens [14,31]. Recently, our previous study reported the use of recombinant SAG2A in ELISA, demonstrating increased positivity for SAG2A in acute phase sera, and indicating that SAG2A could be a potential diagnostic marker for distinguishing the infection phases [15]. In the present study, we evaluated the kinetics of antibodies against *T. gondii* in sequential serum samples from patients with toxoplasmosis, utilizing the recombinant SAG2A in comparison with STAg.

First, we characterized the profile of IgM and IgA to STAg in serum samples grouped in four different time points. IgM levels were high in the initial phases, decreased over the time, but maintained above the cutoff value, with the majority of patients being considered positive up to 12 months of infection. This result indicates that high levels of IgM antibodies were produced in early stage of *T. gondii* infection and the persistence of IgM antibodies confirms that this isotype, by itself, has not high accuracy as a marker of acute phase [9,10], particularly for predicting the time of infection onset in pregnant women [32].

Regarding the kinetics of IgA antibodies, IgA levels were lower when compared with those of IgM and showed a gradual decrease over the time. Also, IgA seropositivity was relatively low (78%) compared to IgM seropositivity (100%) in the first 3 months of infection. IgA antibodies have been considered interesting markers of acute toxoplasmosis because the kinetics of this isotype is earlier than of IgM antibodies [33]. However, the main problem in the detection of IgA isotype at routine laboratory is the low sensitivity of the assays, since the detection of IgA to *T. gondii* showed limited diagnostic value, even using the capture ELISA [34]. Such fact is particularly important in neonatal screening for congenital toxoplasmosis, since previous reports showed a poor performance of IgA tests in newborn sera, especially when their mothers seroconverted in early pregnancy [35].

In the present study, the follow up of IgG antibodies showed a similar kinetics for both antigens, although with higher IgG levels to SAG2A than STAg. These findings reinforce our previous reports that showed higher IgG reactivity to SAG2A than STAg in acute phase sera [15]. However, IgG seropositivity to both antigens was similar, with slightly lower positive rate in samples from 0 to 3
months of infection, compatible with an early acute phase, whose data were confirmed by a commercial test (ELFA, VIDAS® Toxo IgG II). Previous studies have evaluated the kinetics of IgG antibodies to *T. gondii* recombinant proteins, such as ROP2, GRA1 or GRA6 Nt, in infants with congenital toxoplasmosis [36] or pregnant women [37], showing a highly variable IgG profile. Thus, SAG2-related proteins may be potential tools in kinetics studies for IgG antibodies to *T. gondii*.

Differences in biological properties of human IgG subclasses have encouraged the investigation of IgG isotype responses to a variety of infectious agents [15,29,38,39]. The sequential determination of IgG subclasses was evaluated in primary and reactivating toxoplasmosis, and IgG1 was the predominant isotype at the different stages of infection [40]. In our study, the kinetics of IgG1 to SAG2A and STAg was similar in patients with toxoplasmosis up to 12 months of infection, although higher IgG1 reactivity to SAG2A was observed in sera from acute to chronic phase in our previous report [15]. These divergences can be due to differences in the study designs, particularly related to the time of illness onset, since the data herein shown represent a longitudinal study, whereas our previous data were obtained from a prevalence study, with two well defined serologically groups [15].

The kinetics of IgG3 to STAg was increasing and similar to that observed for IgG1 antibodies, contrasting to the kinetics of IgG3 toward SAG2A, which showed high levels from 0 to 6 months of infection, with continuous decreasing over the time. IgG3 seropositivity to SAG2A was higher than to STAg in the initial phases of infection. It is noteworthy that IgG3 reactivity to *T. gondii* protein of 22 kDa (SAG2) was also detected in serum samples from patients in the acute phase [39]. Interestingly, the active fetal synthesis of the IgG3 isotype has been related to clinical problems, while the IgG1 synthesis was associated with the protection from damage [6]. Also, these isotypes were related with the respective cytokine phenotypes, suggesting nonprotective and protective roles in congenital toxoplasmosis [6]. For IgG3, an explanation for its nonprotective role can be related to high affinity of this isotype for the inhibitory signal-linked Fc-γRI [immunoreceptor tyrosine-based inhibition motif (ITIM)], which downregulates immune functions [41]. Thus, SAG2A could represent a key parasite molecule to induce early IgG3 synthesis, contributing to evasion mechanisms of the parasite of the immune response.

To reinforce these findings, IgG3/IgG1 ratios were analyzed and we observed higher IgG3/IgG1 ratio to SAG2A than STAg in the first 3 months of infection. In addition, a high percentage of serum samples with IgG3/IgG1 ratio > 1.0 to SAG2A than STAg was detected in all time points, particularly from 0 to 3 months of infection. These findings reinforce the usefulness of this parameter as an additional tool to be considered together with other serological markers to establish the diagnosis status for toxoplasmosis. Thus, the association between high IgG3/IgG1 ratio (>1.0) to SAG2A and positive IgM to STAg was predominantly found in sera from patients from 0 to 3 months of infection, indicating this combination of tests as useful tools to increase the specificity and to better characterize the early acute phase. On the other hand, the association of low IgG3/IgG1 ratio (<1.0) with positive IgM was predominantly detected from 3 to 12 months of infection, thus excluding the first 3 months of infection and indicating a convalescent phase of toxoplasmosis. Regarding the IgA isotype, we observed that the association of high IgG3/IgG1 ratio to SAG2A with positive IgA to STAg was predominantly detected from 0 to 3 months of infection, characterizing the early acute phase. In contrast, the low IgG3/IgG1 ratio associated with negative IgA was mostly found from 6 to 12 months of infection, suggesting the convalescent phase of toxoplasmosis.

In conclusion, the present study constitutes the first effort to clarify the kinetics of IgG antibodies and subclasses against the recombinant SAG2A protein from *T. gondii* in sequential samples from patients with toxoplasmosis. Our results demonstrate a differential kinetics of IgG3 antibodies to SAG2A comparatively to STAg and the association of the IgG3/IgG1 ratio to SAG2A with classical serological markers of acute phase could be potential tools to distinguish early acute from convalescent phases of *T. gondii* infection.

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References

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