

HIGH DIAGNOSTIC EFFICIENCY OF IGM-ELISA WITH THE USE OF MULTIPLE ANTIGEN PEPTIDES (MAP1) FROM *T. gondii* ESA (SAG-1, GRA-1 AND GRA-7), IN ACUTE TOXOPLASMOSIS

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SUMMARY

The main serological marker for the diagnosis of recent toxoplasmosis is the specific IgM antibody, along with IgG antibodies of low avidity. However, in some patients these antibodies may persist long after the acute/recent phase, contributing to misdiagnosis in suspected cases of toxoplasmosis. In the present study, the diagnostic efficiency of ELISA was evaluated, with the use of peptides derived from *T. gondii* ESA antigens, named SAG-1, GRA-1 and GRA-7. In the assay referred to, we studied each of these peptides individually, as well as in four different combinations, as Multiple Antigen Peptides (MAP), aiming to establish a reliable profile for the acute/recent toxoplasmosis with only one patient serum sample. The diagnostic performance of the assay using MAP1, with the combination of SAG-1, GRA-1 and GRA-7 peptides, demonstrated better discrimination of the acute/recent phase from non acute/recent phase of toxoplasmosis. Our results show that IgM antibodies to MAP1 may be useful as a serological marker, enhancing the diagnostic efficiency of the assay for acute/recent phase of toxoplasmosis.

KEYWORDS: Toxoplasmosis; Serology; MAPs; ESA.

INTRODUCTION

Toxoplasmosis is a prevailing infection among humans and other warm-blooded animals, caused by an intracellular protozoa, *Toxoplasma gondii*. There is a significant danger to the fetuses of mothers who acquire the infection during pregnancy, and it has been established as a cause of life-threatening disease in immunocompromised individuals. The diagnosis of *T. gondii* infection is of considerable importance, because there are specific anti-*T. gondii* therapies. Serological diagnosis of acute Toxoplasmosis has traditionally been made by detection of specific IgM antibodies or by the demonstration of a significant increase in specific IgG antibody levels, or both. Because specific IgM antibodies can persist for several months, or even years in some individuals, following acute infection, interpretation of serological tests might be troublesome when acute Toxoplasmosis is suspected⁹. The diagnosis of chronic stage of infection or of past exposure to *T. gondii* is made by detection of specific IgG antibodies and the absence of the acute-phase markers IgM and IgA²². The currently available commercial tests to detect IgG, IgM and IgA antibodies rely mostly on preparations of crude parasite antigens, i.e. with tachyzoites harvested from infected mice or cell cultures. However, the use of whole-tachyzoites antigens can result in false-positive reactions thereby reducing the clinical value based on serological data^{9,11,13,28}. Tachyzoite antigens are obtained differently in respect to their source and process for their collection and purification. These facts imply that the reactivity among antigens may vary greatly,

contributing to inaccurate provision of diagnostic efficiency by assay systems. Several reports^{1,2,4,6,7,10,16,23,27,29} have emphasized the value of the detection of specific antibodies raised to excreted-secreted antigens (ESA) of *T. gondii* for the diagnosis of acute toxoplasmosis. The *T. gondii* ESA constitutes 90% of the circulating antigens in infected humans, thus is one of the first targets of the host immune response⁶.

Recombinant antigens and synthetic peptides have been prepared from ESA antigens, however, the exact composition and association of recombinant antigens and peptides to be used in immunoassays to detect toxoplasma antibodies is still an open question. The identification of human immunodominant B-cell epitopes within the *T. gondii* antigens can help to find those antigens involved in the specific B-cell response, and they will be useful for immunoassays to detect anti-toxoplasma antibodies^{15,25,28}. TAM³² described in 1988 a novel immunological approach, consisting of some immunodominant sequences of peptides associated by means of a small peptidyl core matrix (multiple Antigen Peptide - MAP). This association presents several advantages such as: the antigen composition of the test being precisely known, being able to use more than one defined antigens; immunogenicity, antigenicity and possibility for easy standardization of the method. So, multiple antigen peptides may be an alternative source of antigens that are characteristic of the acute or chronic stages of the infection, serving as a tool to discriminate the acute/recent toxoplasmosis from chronic stages. The design of MAP chosen for study by above authors consisted of a core

matrix with heptalysine containing three dendritic arms of *T. gondii* ESA peptides in length. The ESA peptides chosen were granule protein 1 (GRA-1) correlated with chronic toxoplasmosis, granule protein 7 (GRA-7) detected in acute toxoplasmosis^{16,22} and surface antigen 1 (SAG-1) present only in the tachyzoite stage and marker of acute toxoplasmosis^{2,7,26}. In the present study, we evaluated the diagnostic efficiency of ELISA with the use of SAG1, GRA1 and GRA7 peptides derived from *T. gondii* ESA, individually and in combined forms as MAP, for the detection of IgG, IgM and IgA antibodies, aiming to establish a profile of acute/recent toxoplasmosis, with a single serum sample.

MATERIAL AND METHODS

Serum samples. A total of 300 serum samples was obtained from individuals during routine screening for Toxoplasmosis and analyzed by a commercially available kit for ELISA (VIDAS BioMérieux-Brazil) and classic serological methods (indirect immunofluorescence assay (IFA) (BioMérieux-Brazil) and indirect hemagglutination assay (HAI) (BioMérieux-Brazil) to detect IgM and IgG antibodies, anti-*T. gondii* and avidity of specific IgG antibodies. Sera were selected as previously described by FERREIRA & CAMARGO⁹; MONTOYA²⁴ and PIETKIEWICZ *et al.*²⁸, based on clinical and serological data. Five groups were studied according to the clinical and serological findings as follows. **Group I** comprised 25 patients, presenting clinical and serological symptoms compatible with acute or acute/recent toxoplasmosis. They exhibited specific IgM antibodies (titer ≥ 64), IgG antibodies (titer ≤ 128) by IFA and HAI, and low avidity of *T. gondii*-specific IgG. Serum samples were positive for *T. gondii*-specific IgG and IgM by automated ELISA assay. **Group II** had 25 patients, without clinical symptoms or serological profiles of non-acute/recent toxoplasmosis. They showed specific IgM antibodies (titer ≤ 64), IgG antibodies (titer ≥ 28) by IFA and HAI, and high avidity of *T. gondii*-specific IgG. Serum samples were positive for *T. gondii*-specific IgG and IgM by automated ELISA assay. **Group III** included 100 individuals, exhibiting specific IgG antibodies (titer ≥ 256) by IFA and HAI, negative results for *T. gondii*-specific IgM by IFA and HAI, and high avidity of *T. gondii*-specific IgG. Serum samples were positive for IgG and negative for IgM *T. gondii*-specific by ELISA automated assay. No patient presented clinical symptoms, but, serological data was suggestive of past toxoplasmosis. **Group IV** consisted of 100 clinically healthy controls without present or previous serological data suggesting toxoplasmosis, i.e. neither IgM nor IgG antibodies were detected by IFA, HAI or ELISA automated assay. **Group V** had 50 patients with unrelated infections, but who had defined clinical and serological diagnosis of rubella (10), mononucleosis (10), cytomegalovirus (10), malaria (10) and Chagas disease (10). The ethical committee of the Institute of Tropical Medicine, University of São Paulo approved the project.

ESA. *T. gondii* *T. gondii* tachyzoites, RH strain, were obtained from the peritoneal cavity of Balb/c mice infected two days earlier and inoculated into culture of human fibroblast cells for three hours at 37 °C. The culture supernatants were centrifuged at 1000g for 10 minutes and filtered through 0.22- μ m-pore-size membrane (Millipore Corporation, Bedford, Massachusetts). The ESA was stored at -70 °C with 100 U/mL aprotinin (Sigma Chemical Co, St. Louis, Missouri).

Viability of tachyzoites and protein concentration. After washing three times with PBS pH 7.2, the culture of human fibroblasts was inoculated with 1×10^8 tachyzoites and incubated at 37 °C. Five-milliliter

aliquots of culture were taken at various intervals from 1, 2, 3, 4, 5, 6, 12 and 24 hours post-infection. The number and viability of tachyzoites were evaluated in these aliquots by the Tripán blue method and the protein concentration was determined using the Bio-RAD DC Protein Assay (France).

PAGE. The ESA was electrophoresed in polyacrylamide slab gel using a discontinuous sodium dodecyl sulfate (SDS) buffer system as described by LAEMMLI²⁰, with an 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 RainbowTM, Amersham Life Sciences, UK) were used to plot the calibration curve. Proteins were transferred from SDS slab gel to a polyvinylidene difluoride membrane (Immobilon PVDF, Millipore, USA).

Synthesis of SAG-1, GRA-1 and GRA-7 peptides. The SAG-1, GRA-1 and GRA-7 peptides were synthesized from PVDF membrane obtained after *T. gondii* ESA proteins electrophoresed as above on Tentagel S resin (Rapp Polymere GmbH, Tübingen, Germany) by using a Rainin Symphony Multiplex synthesizer (Protein Technology, Tucson, Arizona) with standard 9-7 fluorenylmethoxycarbonyl (Fmoc) chemistry. Standard double couplings were performed by using a fourfold excess of Fmoc-protected amino acids active *in situ* with equimolar amounts of N-hydroxybenzotriazole and 2-(1 H-benzotriazole-1-yl)-1,3,3-tertamethyluronium tetrakisfluoroborate twice for 20 minutes each time. The Fmoc protecting group was removed by using a mild base treatment with 2% piperidine 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in dimethyl sulfoxide-70% dimethylformamide with *in situ* activation. The peptide sequence chosen for SAG-1 is based in KATO *et al.*¹⁹; for GRA-1 in BEGHETTO *et al.*³; and for GRA-7 in SOUSA *et al.*³¹.

MAP: Combination of SAG-1, GRA-1 and GRA-7 peptides. After the synthesis completion, the peptide was cleaved from the resin by incubation for 2.5 hours with 90% trifluoroacetic acid-5% thioanisole-3% ethanedithiol-2% anisole. The peptide was precipitated from the mixture by using *t*-butyl methyl ether. After centrifugation, the pellet was washed three times with *t*-butyl methyl ether and dried overnight in a vacuum (TAM³²). The purity of the crude peptide was checked by reverse-phase high-performance liquid chromatography, lyophilized, and stored at 4 °C until use.

ELISA with individual peptides (SAG-1, GRA-1 and GRA-7). Microtiter plate (Immuno Plate Maxisorp; Nunc) wells were coated overnight at room temperature with 50 μ L of each of the peptides SAG-1, GRA-1 or GRA-7 diluted in 0.05 M carbonate buffer (pH 9.6) at the optimal concentration of 10 μ g/mL determined by block titration. After coating, the wells were washed three times with PBS/0.25% Tween 20 (PBS-T) and blocked with 200 mL of 5% skim milk in PBS-T (blocking solution) for two hours at 37°C. The plates were then washed as described above and 100 μ L of test or control serum were applied to each well. To test for IgG, the sera were diluted 1:100 in blocking solution. In the case of IgM or IgA detection, the sera were diluted at the optimal 1:25 in blocking solution, as determined by previous block titration. The plates were incubated for two hours at room temperature and washed as described above. Goat anti-human IgG, IgM, or IgA peroxidase-labeled antibodies (Sigma Chemical Co., St Louis, Missouri) diluted 1:200 were used as the secondary antibody. After being incubated for one hour at room temperature and washings,

immune complexes were developed with ortho-phenylenediamine as the chromogenic substrate. Ate reactivity was measured at 490 nm in an automatic enzyme-linked immunosorbent assay (ELISA) reader (Reader 230S- Anthos). Each serum was assayed in duplicate by ELISA. For each peptide the cut-off level was determined according to the mean value of optical density (plus three standard deviations) of the negative controls.

ELISA with MAPs. Each microtiter plate well (Immuno Plate Maxisorp; Nunc) was coated overnight at room temperature with 100 µL of the MAP1, MAP2, MAP3 or MAP4 diluted in 0.05 M carbonate buffer (pH 9.6) at the optimal concentration of 5 µg/mL found by block titration. After coating, the wells were washed three times with PBS/0.25% Tween 20 (PBS-T) and blocked with 200 mL of 5% skim milk in PBS-T (blocking solution) for two hours at 37 °C. The plates were then washed as described above and 100 µL of test or control serum were applied to each well. For the detection of IgG to MAP1, MAP2, MAP3 or MAP4, sera were diluted 1:200 in blocking solution. To detect IgM and IgA to MAP1, MAP2, MAP3 or MAP4, sera were diluted at 1:50 in blocking solution, as determined by block titration. The plates were incubated for two hours at room temperature and washed as described above. Goat anti-human IgG, IgM, or IgA peroxidase-labeled antibodies (Sigma Chemical Co., St Louis, Missouri) diluted 1:200 were used as the secondary antibody. After being incubated for one hour at room temperature and washings performed, immune complexes were developed with ortho-phenylenediamine as the chromogenic substrate. The reactivity was measured at 490 nm in an automatic enzyme-linked immunosorbent assay (ELISA) reader (Reader 230S- Anthos). Each serum was assayed in duplicate by ELISA. For each MAP the cut-off level was determined according to the mean value of optical density (plus three standard deviations) of the negative controls.

Diagnostic performance of ELISA using different peptides. The diagnostic parameters were defined in terms of sensitivity, specificity and efficiency, according to FERREIRA & AVILA (2001)⁸. The sensitivity was determined independently for groups I(25), II(25) and III(100): (ELISA positive)/(Positive by conventional reference data). The specificity was calculated with the sum of results obtained from control groups IV(100) and V(50): (ELISA negative)/(Negative by reference data). For the diagnostic efficiency, in turn, the calculation was as follows: (ELISA positive + ELISA negative)/(Positive by reference data + Negative by reference data). The reference data corresponds to the aforementioned conventional clinical and serological findings.

RESULTS

A total of 300 serum samples belonging to five groups of patients was studied by ELISA, using each of three peptides independently and also in their four different combinations.

Reactivities of IgG, IgM and IgA antibodies with SAG-1, GRA-1 and GRA-7 peptides. As shown in Table 1, IgG, IgM and IgA antibodies' reaction to SAG-1, GRA-1 and GRA-7 were found in patients from group I, II and III. In terms of sensitivity, the IgG-ELISA using individually three different peptides varied from 98% to 68%. In the IgM-ELISA with the use of same peptides, the sensitivity varied from 88% to 5%. In the IgA-ELISA antibodies to these three peptides were observed in patients from group I and II, ranging from 92% to 12%. However in the group III, IgA antibodies were detected by the use of these antigens. In groups IV

Table 1
Sensitivity of IgG-, IgM- and IgA-ELISA with the use of SAG-1, GRA-1 and GRA-7 peptides and with their four different combinations as MAP1, 2, 3 and 4 for the diagnosis of human toxoplasmosis

Antibodies to Peptide (S)	Group I % Sensitivity	Group II % Sensitivity	Group III % Sensitivity
IgG to:			
SAG-1	88 (22/25)	84 (21/25)	98 (98/100)
GRA-1	76(19/25)	68(17/25)	97(97/100)
GRA-7	84 (21/25)	76 (19/25)	94 (94/100)
MAP1	100 (25/25)	100 (25/25)	100 (100/100)
MAP2	0 (0/25)	0 (0/25)	0 (0/25)
MAP3	0 (0/25)	0 (0/25)	0 (0/25)
MAP4	0 (0/25)	0 (0/25)	0 (0/25)
IgM to:			
SAG-1	88 (22/25)	16 (4/25)	5 (5/100)
GRA-1	52 (13/25)	44 (11/25)	13 (13/100)
GRA-7	88 (22/25)	24 (6/25)	6 (6/100)
MAP1	100 (25/25)	0 (0/25)	0 (0/100)
MAP2	0 (0/25)	-	-
MAP3	0 (0/25)	-	-
MAP4	0 (0/25)	-	-
IgA to:			
SAG-1	92 (23/25)	12 (3/25)	0 (0/100)
GRA-1	84 (21/25)	48 (12/25)	0 (0/100)
GRA-7	84 (21/25)	16 (4/25)	0 (0/100)
MAP1	96 (24/25)	92 (23/25)	0 (0/100)
MAP2	0 (0/25)	-	-
MAP3	0 (0/25)	-	-
MAP4	0 (0/25)	-	-

and V, specific IgG, IgM or IgA antibodies to SAG-1, GRA-1 or GRA-7 peptides were not observed, indicating the absence of cross-reactivity by the assay. It is worth mentioning the cut-off values determined for the detection of specific antibodies. In the detection of IgG antibodies to different peptide antigens as isolate or associate forms, considering five patient groups, cut-offs varied from 0.258 to 0.421, while for the detection of IgM antibodies, cut-offs ranged from 0.145 to 0.188, and for IgA antibodies, from 0.111 to 0.127. The overall data demonstrates that reactivities of IgG, IgM and IgA antibodies with SAG-1 were higher in patients from group I of acute/recent toxoplasmosis than other groups. However in group II and III, only IgG antibodies were highly reactive with the same peptide.

Reactivities of IgG, IgM and IgA antibodies with the combination of peptides MAP1, MAP2, MAP3 and MAP4 by ELISA. All sera from patients with acute/recent toxoplasmosis (group I), anti-MAP1 (SAG-1, GRA-1 and GRA-7) were detected. Figure 1 shows that IgG antibodies from all 25 (100%) patients reacted with MAP1, and their positive values were far from the stipulated value of cut-off (0.258). In other words, the mean value of the 25 sera was 3.2, with standard deviation (SD) of 0.014. In addition, these 25 (100%) sera reacted with MAP1 by IgM-ELISA. The mean value found for these sera was 3.8 (SD 0.012). In respect to IgA-ELISA, 96% (24/25) sera were positive and their mean value was 3.5 (SD 0.013). All 25 sera from patients with non-acute/recent toxoplasmosis (group II) as shown in Figure 1, had IgG antibodies and

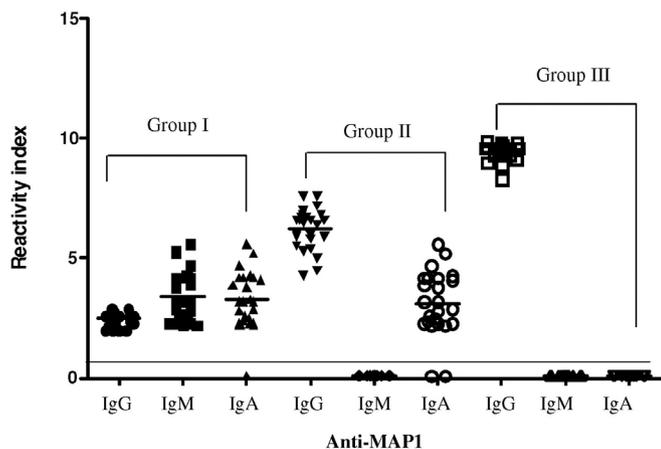


Fig. 1 - Reactivity of IgG, IgM and IgA antibodies with MAP1 (SAG-1, GRA-1 and GRA-7) peptides, in the study of serum samples from group I, including patients with acute/recent toxoplasmosis, group II with non-acute/recent toxoplasmosis and group III with chronic toxoplasmosis, by ELISA.

their values were far above the cut-off level. The mean value of the 25 sera was 6.8 (SD 0.016). In this group, these 25 sera did not react with MAP1 by IgM-ELISA. The mean value of the 25 sera was 0.4 (SD 0.018). On the other hand, 23/25 sera (92%) showed IgA reactivity against MAP1. The mean value of the 23 sera was 2.6 (SD 0.014). In all 100 sera from patients with chronic toxoplasmosis (group III), IgG antibodies reacted strongly with MAP1, and none of the positive results were close to the cut-off value. Their mean value was 9.2 (SD 0.016). In contrast, this group had no IgM and IgA antibodies reacting with MAP1. In control groups IV and V, there were no anti-MAP1 IgG, IgM and IgA, i.e., no cross-reactivity was observed in these groups (Fig. 2). As for the IgG-ELISA using MAP2, MAP3 and MAP4, antibodies were not detected in any sera from groups I, II and III. The IgM-ELISA and IgA-ELISA did not detect antibodies to same antigens in any sera from group I. On the other hand, in sera from groups II and III, IgM and IgA antibodies to MAP2, MAP3 and MAP4 were found. In respect to groups IV and V, IgG, IgM and IgA antibodies were not seen, showing no cross-reactivity in any of the serum samples. Thus, it can be stressed that a maximum

efficiency was observed by IgM-ELISA utilizing MAP1 antigen for the diagnosis of acute/recent toxoplasmosis.

DISCUSSION

A century has elapsed since the early studies of Sabin and Feldman about the diagnosis of Toxoplasmosis. Traditional manual laboratory methods, adequate for small routines, have been replaced with automated or semi-automated procedures involving little manipulation and risk of human error. These systems can be used for large routine volumes at a reasonable price. In addition, continuous scientific and technical evolution has led to decisive progress in diagnosis through the search of gold standard tests that will provide safe information about the real health status of the patient. For years, the serological profile defined by experienced investigators has been accepted in laboratory diagnostic routine. However, current laboratory practice using highly sensitive automated equipment has led to problems with the detection of parasite specific IgG and IgM antibodies^{12,33}. Several authors have shown that IgM to *T. gondii* can be detected for a long period after the primary infection and false positivities with IgG to *T. gondii* have been reported which are non-significant and erroneously interpreted as positive results indicating acute infection and protection¹⁹. Research and development have continued to advance and new parameters have been developed for the diagnosis of toxoplasmosis in its different phases, such as the avidity test of IgG antibodies and detection of the parasite by a molecular method such as polymerase chain reaction (PCR). The IgG avidity determination is an important serological marker that can be used to distinguish between acute/recent and non-acute phase, from a single serum sample. However, low IgG avidity results may persist for as long as one year after the primary infection because of immunosuppressive conditions or anti-toxoplasma pharmacotherapy that may affect the kinetics of the IgG avidity maturation^{5,11,13,20,23}. The PCR has been successfully used in immunocompromised patients, ocular toxoplasmosis and congenital toxoplasmosis. However, some problems can be observed in laboratory practice, such as false negative results after treatment with anti-toxoplasma drugs, low and focal distribution of parasites in the tissues or the presence of non-viable parasites in autolyzed tissue. Excreted and secreted antigen (ESA) of *T. gondii* has been used in detection of specific antibodies for the diagnosis of acute toxoplasmosis^{1,2,3,4,6,7,15,27}. As previously mentioned, circulating antigens in infected humans are constituted by *T. gondii* ESA, and are one of the first targets of the host immune response²⁶. In addition, the ESA plays a role in the persistent stimulation of cell-mediated immunity in chronically infected healthy subjects. Several fractions from *T. gondii* ESA isolated or in combination have been used in the diagnosis of toxoplasmosis. Among the fractions of *T. gondii* ESA, surface antigen 1 (SAG-1) has been extensively studied. SAG-1 is the most immunodominant and stage-specific antigen present in tachyzoite stage and not in bradyzoite stage. SAG-1 is one of the first antigens recognized by IgM antibodies during acute/recent phase. However, as confirmed here with our previous² data and that of others^{7,26}, specific anti-SAG-1 antibodies can be detected in chronic toxoplasmosis and non-acute/recent phase. Other important ESA fractions used in various studies for the diagnosis of toxoplasmosis originate from granule-dense organelles (GRA-7). This antigen produces a strong antibody response in the acute phase of infection and is found in the parasitophorous vacuole and the cytoplasm of the tachyzoite-infected host cell, leading to immune recognition due to constant rupture of the

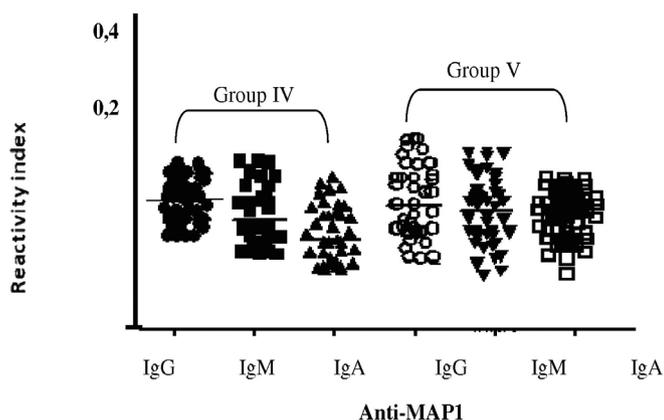


Fig. 2 - Reactivity of IgG, IgM and IgA with MAP1 (SAG-1, GRA-1, GRA-7) peptides, by ELISA, in the study of serum samples of group IV, consisting of clinically healthy individuals, and group V, with unrelated pathologies.

cyst cells¹⁶. The granule-dense 1 antigen (GRA-1) reacts strongly with serum samples from pregnant women infected with *T. gondii*³ and, as we found here, with sera from chronic and acute/recent toxoplasmosis, as in reported¹⁶ data. The present study was undertaken since the exact composition and conformation of *T. gondii* ESA antigens to be used to detect IgG and IgM antibodies in an immunoassay remained an open question. The assay performance varies significantly due to the influence of factors such as the number of serological targets to be sequestered within internal organelles, as well as the method utilized for the parasite antigen preparation. Advances in molecular biology permitted the obtainment of purified antigens, being an attractive alternative to improving the sensitivity and specificity of serological tests, in particular, for the detection of IgM antibodies. In this context, the multimeric nature of the MAP constructs provides reproducible results and increased surface binding. MAPs may also improve the early detection of antibodies of low affinity, such as those of the IgM isotype, during the early phase of infections. Significant advances by many laboratories have led to applications of MAPs for serodiagnosis. MAP showed a sensitivity increase of >10⁸-fold compared with peptide antigen in an enzyme-linked immunosorbent assay (ELISA)²⁸. These results indicate that MAPs may be antigens of choice for solid phase based immunoassays and they also are a promising tool for serodiagnosis in naturally immunized or infected individuals²⁸. In a recent study, NOYA *et al.*²⁷ compared various antigenic preparations with MAP for the diagnosis of different infections and reported high sensitivity and specificity of assays at low cost.

In this work, we evaluated the diagnostic efficiency of IgG, IgM- and IgA-ELISA, using a combination of SAG-1, GRA-1 and GRA-7 constructed by the MAP system, to establish a profile of acute toxoplasmosis in single serum samples. In other words, we searched for a serologic marker among IgG, IgM and IgA antibodies by the use of MAP1 peptides, and conferring on ELISA maximum diagnostic efficiency. We already mentioned that the SAG-1, GRA-1 and GRA-7 in individual analysis and the sensitivity of the IgG-, IgM- and IgA-ELISA was low, due to the false negative results observed in the study of group of patients I, II and III (Table 1). In addition, positivity observed in these same groups of patients, by means of the above referred to assays, did not permit discrimination of acute/recent toxoplasmosis from other phases of the infection (Table 1). The IgG-ELISA using independently the peptides GRA-1 and GRA-7, although showing good sensitivities for chronic toxoplasmosis, cannot be helpful as confirmatory assays, as referred to JACOBS *et al.* (1999)¹⁷ and BEGHETTO *et al.* (2001)³. Maximum specificity was observed in the control groups (IV and V) including clinically healthy individuals and others with unrelated pathologies. After the study of each fraction, we dealt with different peptide combinations: MAP1 (SAG-1, GRA-7 and GRA-1); MAP2 (SAG-1 and GRA-1); MAP3 (SAG-1 and GRA-7) and MAP4 (GRA-1 and GRA-7) to see their ability to react with IgG, IgM and IgA *T. gondii* by ELISA. In the initial evaluation of the different peptide combinations by the assay, we observed that when SAG-1 was present, as in the MAP1, the sensitivity and specificity in the detection of IgG, IgM and IgA antibodies in different toxoplasmosis phases was higher compared with those without SAG-1, as MAP4 (Table 1). Our results are consistent with the results of other investigators who have found that SAG-1 improves the sensitivity and specificity of serological assays^{19,25}. In the study of other peptide combinations such as, MAP2, 3 and 4, the assay presented low diagnostic performance, and the acute/recent, non-acute/recent and

chronic phase could not be distinguished as in the assay using MAP1. These results indicate that different proportions of SAG-1 peptides found in the ESA *T. gondii* will influence greatly the diagnosis features of the assay, in the routine diagnosis of acute/recent toxoplasmosis.

We analyzed the diagnostic efficiency of ELISA using MAP1 peptides for the detecting of IgG, IgM and IgA antibodies in three different groups and we found an increase of the assay sensitivity, mainly in the detection of IgM antibodies. Therefore, the sensitivity and the specificity of IgM-ELISA for acute/recent toxoplasmosis were maximum (100%) and, consequently, its diagnostic efficiency. In contrast, the non-acute/recent toxoplasmosis group, in spite of presenting some residual IgM antibodies detectable by a commercial kit, IgM antibodies to MAP1 were demonstrated. NOYA *et al.*²⁷ investigated the use of MAP in an assay for the diagnosis of unrelated pathologies and they found similar specificity and sensitivity as we did with MAP1. In study of PIETKIEWICZ *et al.*²⁸ using recombinant (SAG-1, GRA-1 and GRA-7) antigens, the acute/recent and non-acute/recent phase of toxoplasmosis could not be discriminated. Our results indicate that MAP1 may be the antigen of choice for the use in solid phase based immunoassays and these are promising tools for the serodiagnosis of acute/recent toxoplasmosis. The combination of SAG-1, GRA-1 and GRA-7 peptides, derived from ESA *T. gondii*, as MAP1 system will be helpful in the laboratory practice to distinguish acute/recent from non-acute/recent toxoplasmosis, with a single serum sample.

RESUMO

Alta eficiência diagnóstica do IgM ELISA com o uso de múltiplos antígenos peptídicos (MAP1) de *T. gondii* ESA (SAG-1, GRA-1 e GRA-7) na toxoplasmose aguda

O principal marcador sorológico para o diagnóstico da toxoplasmose aguda ou recente são os anticorpos IgM específicos, a par de anticorpos IgG de baixa avidéz. Entretanto em alguns pacientes, estes anticorpos podem persistir, ultrapassando o período da fase aguda/recente, contribuindo para erro diagnóstico em casos suspeitos de toxoplasmose. No presente estudo, a eficiência diagnóstica de ELISA foi avaliada, com o uso de frações ou peptídeos originados dos antígenos ESA de *T. gondii*, denominados de SAG-1, GRA-1 e GRA-7. No referido ensaio, estudamos cada uma destas frações isoladamente, como também em quatro diferentes combinações, ou múltiplos peptídeos antigênicos (MAP), visando estabelecer um perfil confiável para a toxoplasmose aguda/recente em amostra única de soro. A melhor eficiência diagnóstica do ensaio foi encontrada com o uso da combinação de peptídeos SAG-1, GRA-1 e GRA-7, denominada MAP1. A detecção de anticorpos IgG e IgM anti-MAP-1 apresentou melhores características diagnósticas para a diferenciação entre a fase aguda/recente da fase não aguda/recente na toxoplasmose. Nossos resultados mostram que anticorpos IgM anti-MAP-1 poderão prestar auxílio como um marcador sorológico, aumentando a eficiência diagnóstica do ensaio para a fase aguda/recente da toxoplasmose.

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Received: 3 November 2009

Accepted: 18 February 2010