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Effectiveness of two sequences of *Toxoplasma gondii* SAG2 protein to differentiate toxoplasmosis infection stages by measuring IgG, IgA and IgM antibodies

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Here, we evaluated two *Toxoplasma gondii* SAG2 antigen sequences which differ in the presence of the amino-and carboxy-terminal sequences in one of them. They were tested with several techniques to differentiate between acute and chronic phase of toxoplasmosis: avidity assay and indirect ELISA for the detection of anti-*Toxoplasma* IgG, IgA and IgM antibodies. Significant differences were found between them. The best results were obtained with the sequence denominated SAG2c, which yielded sensitivity of 73.8% and specificity of 80.3% in the detection of IgG by ELISA. Measurement of IgA yielded 67.2% and 81.8% of sensitivity and specificity, respectively. The avidity assay yielded 100% sensitivity and 81.82% specificity. Finally, the molecular modeling and epitope predictions of the protein are developed to find the antigenic region locations, specific and nonspecific, on three-dimensional structure. This paper is the first report of promising results of SAG2 used for detecting IgA antibodies to differentiate toxoplasmosis infection stages.

INTRODUCTION

Toxoplasmosis, a human infection caused by *Toxoplasma gondii*, is generally asymptomatic and induces a self-limited disease. However, the acute phase of infection acquired during gestation can be transmitted to the fetus and may cause miscarriage, as well as permanent neurological damage, visual impairment and/or other malformations to the newborn (Peng *et al.*, 2011; Robert-Gangneux & Dardé, 2012).

Toxoplasmosis during gestation represents a difficult task for the clinician due to its subclinical course in most pregnant women and the unpredictable long-term outcome of congenital infection. To implement suitable therapies in good time and to avoid neonatal malformations, it is essential to determine if the woman acquired the acute infection during pregnancy (Villena *et al.*, 1998; Li *et al.*, 2000).

The enzyme-linked immunosorbent assay (ELISA) using antigens (Ags) purified from *T. gondii* is the most commonly employed technique as commercial kit for the diagnosis of toxoplasmosis (Aubert *et al.*, 2000; Pietkiewicz *et al.*, 2004). Moreover, uses of ELISAs with *T. gondii* recombinant proteins have already been reported for the detection of immunoglobulin G, A and M (IgG, IgA and IgM) (Parmley *et al.*, 1992; Pfrepper *et al.*, 2005; Béla *et al.*, 2008). In addition, recombinant antigens have been used in IgG avidity test (Béla *et al.*, 2008) or western blot (Parmley *et al.*, 1992). The use of recombinant proteins instead of purified proteins from parasite offers several advantages: there is precise knowledge of

antigen composition, more than one defined antigen can be used and the method obtainment can be easily standardized (Pietkiewicz *et al.*, 2004; Selseleh *et al.*, 2012).

The analysis by serodiagnosis of the human humoral immune response against *T. gondii* antigens has identified a number of acute phase specific immunoreactive proteins: the surface antigens SAG2 (Béla *et al.*, 2008) and SAG1 (Nigro *et al.*, 2003; Araújo and Ferreira *et al.*, 2010); the dense granule antigens GRA1 (Araújo & Ferreira *et al.*, 2010), GRA2 (Golkar *et al.*, 2007), GRA3 (Beghetto *et al.*, 2003), GRA4 (Li *et al.*, 2000), GRA6 (Hiszczyjska-Sawicka *et al.*, 2005), GRA7 (Araújo & Ferreira *et al.*, 2010) and GRA8 (Aubert *et al.*, 2000); the rhoptry antigens ROP1 and ROP2 (Aubert *et al.*, 2000); and the antigens BSR4 (Chen *et al.*, 2012), MAG1 (Pfrepper *et al.*, 2005), P25 (Li *et al.*, 2000), SUB1 (Hruzik *et al.*, 2011), TgERP (Hill *et al.*, 2011) and P68 (Aubert *et al.*, 2000). These recombinant proteins expressed in bacteria have been used to detect antibodies against the parasite in serum, but their pattern of immunoreactivity with human sera varies with the immunoglobulin class (Robert-Gangneux & Dardé, 2012).

SAG2 is one of the most important and widely studied antigens to differentiate stages of toxoplasmosis infection. Many authors reported several results using different sequences from this recombinant antigen (Prince *et al.*, 1990; Parmley *et al.*, 1992; Li *et al.*, 2000; Hiszczyjska-Sawicka *et al.*, 2005; Béla *et al.*, 2008; Lau and Fong, 2008).

One of the most important effects in the use of different sequences of the antigen for diagnostic assays is the inclusion or not of antigenic epitopes, which will define if the antigen binds to a higher or lower number of antibodies. Epitopes can be located in the structure of the protein by experimental antigenic mapping (Kato *et al.*, 2007; Cardona *et al.*, 2009; Maksimov *et al.*, 2012) or by prediction computer programs (Davydov & Tonevitsky, 2009; Jens *et al.*, 2012). Considering that experimental methods to identify epitopes are quite expensive and require long-term trials, the use of *in silico* tools becomes an attractive option. In a previous work, we found that these programs have acceptable effectiveness (Costa *et al.*, 2013). There are other programs that help to study different molecular aspects, some of them allowing protein modeling, which are broadly used nowadays (Crawford *et al.*, 2010; Tankin *et al.*, 2011; Bai *et al.*, 2012; Saouros *et al.*, 2012). They are employed to discover the spatial organization of a protein, which may provide relevant data on its function and active sites. All these *in silico* tools can be combined with experimental results, thus facilitating deep analyses, better understanding and hypothesis formulation (Kato *et al.*, 2007; Tonkin *et al.*, 2011).

Here, we evaluated results of diagnostic assays of two SAG2 sequences, SAG2L and SAG2c, which differ in the presence of the amino-and carboxy-terminal sequences in one of them. The immunoassays focused on the distinction between acute and chronic phase of toxoplasmosis. Both sequences were evaluated to detect specific IgG, IgA and IgM by indirect ELISA. SAG2c was also evaluated by measuring IgG avidity. Finally, the molecular modeling and epitope predictions of the protein are developed to explain the different experimental results.

MATERIALS AND METHODS

Samples

Serum samples (242 from all studied groups) were obtained from three Argentine health centers: Laboratorio de Toxoplasmosis del Hospital Alemán, Laboratorio Central de la Provincia de Santa Fe and Centro de Salud de la Universidad Nacional del Litoral. Sera were collected from adults (that were attended for different reasons in the health centers) between 2009 and 2011. We classified the samples into groups of sera according to the results obtained with the commercial routine tests: IgG avidity test (VIDAS Toxo IgG Avidity, bioMérieux, Marcy l'Etoile, France), IgG indirect immunofluorescence (IFI, in-house test), Sabin-Feldman (assay in-house test), IgM and IgA immunosorbent agglutination (ISAGA,

bioMérieux, Marcy l'Etoile, France), hemagglutination assay (HAI, Toxotest HAI, Wiener Lab, Rosario, Argentina) and anti-*T. gondii*-IgG ELISA (Toxoplasma IgG ELISA kit, Sigma-Aldrich, St. Louis, U.S.A.). All employed sera were subjected to 3 to 6 assays to confirm by several commercial tests that serological group the individual belonged.

Four serological groups were formed:

Negative infection sera (NIS): 73 sera from individuals without toxoplasmosis. These were evaluated with IgM ISAGA and also in at least two of the following techniques: IgG ELISA, IFI, Sabin-Feldman assay and/or HAI. Sera were negative in these techniques.

Chronic infection sera (CIS): 84 sera from individuals with chronic phase of toxoplasmosis infection. These were tested in at least two of the following techniques: IgG ELISA, IFI, Sabin-Feldman assay and/or HAI. Sera were positive in these techniques. But sera were negative in IgM ISAGA and had a high avidity index (AI) in the avidity assay.

Acute infection sera (AIS): 85 sera from individuals with recently acquired (acute phase) toxoplasmosis. These were evaluated in at least two of the following techniques: IgG ELISA, IFI, Sabin-Feldman assay and/or HAI. Sera were positive in these techniques. Sera were also positive in IgM and IgA ISAGA and they had low avidity in avidity assay.

Positive infection sera (PIS): 169 sera from individuals with toxoplasmosis. This group consisted of combined AIS and CIS.

All procedures performed in these studies were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained for experimentation with patient sera and the privacy rights of human subjects always were observed.

Cloning and expression of antigens

Primers used for amplification of sequences encoding SAG2L and SAG2c by PCR were: SAG2L-FW 5_GGATCCAATCTTTGTCTTGTCGGAACT_3, SAG2L-RV 5_GAATTCTTACACAAACGTGATCAACAAA_3, SAG2c-FW 5_GGATCCACCACCGAGACGCCAGC_3 and SAG2c-RV 5_GAATTCTTGCCCGTGAGAGACACAG_3.

Escherichia coli BL21 (DE3) was transformed with the construct pET32a+ (Novagen, Darmstadt, German) and SAG2c or SAG2L. These cells were grown at 37ºC until they reached an optical density (OD) between 0.4 and 0.5. The expression of antigens was induced for 3 hours at 37ºC with 1 mmol/L isopropyl-β-D-thiogalactopyranoside (Promega, Madison, U.S.A.).

Purification

The SAG2c and SAG2L purifications were performed as described by Béla *et al.* (2008); but SAG2L had the following modifications. The broken bacteria pellet was washed five times with 1% triton. Inclusion bodies were dissolved in binding buffer with 1% sodium dodecyl sulfate and 4 mol/L urea. Antigen was purified from this solution using nickel pseudo-affinity IDA-Sepharose column (Ni-NTA, Invitrogen, Carlsbad, U.S.A.) and elution solutions with 4 mol/L urea.

The purity and concentration of the proteins were evaluated by 15% polyacrylamide gel electrophoresis (PAGE) following Schägger *et al.* (1987). Quantification was performed by the software Quantity One (Bio-Rad Inc., Hercules, U.S.A.).

ELISA using SAG2c and SAG2L

Indirect ELISA was performed as described by Hiszczyjska-Sawicka *et al.* (2005) with the following modifications. Purified recombinant Ags were diluted to the concentration of 2.5 mg/ml (to detect IgG and IgA) or 15 mg/ml (to detect IgM) in carbonate-bicarbonate buffer. 0.1 ml of each antigen was added to separate wells of microtiter Maxisorp plates (ELISA-plate, Greiner bio one, Frickenhausen, Germany). SAG2c and SAG2L were added simultaneously in the same plate. Duplicate serum samples were diluted 1/200 (to detect IgG), 1/25 (to detect IgA) or 1/10 (to detect IgM) into a PBS-milk (1% fetal calf serum in PBS). Bound human IgG, IgA or IgM was detected by using IgG, IgA or IgM anti-human goat antibody

conjugated horseradish peroxidase (Abcam, Cambridge, England). The conjugates were diluted 1/3000, 1/500 or 1/2000, respectively.

Avidity index assessment

The avidity assay was performed as described by Béla *et al.* (2008) with the following modifications. The micro-titer plates were coated with 0.1 ml of 2.5 µg/ml of soluble antigen in carbonate-bicarbonate buffer. Sera diluted in PBS-milk (1/3200) were added in duplicate rows (row A and row B). After incubation at 37ºC for 1 h., 200 µl of PBS was added to row A and 200 µl of PBS-urea (PBS with 6 mol/L urea) to row B. It was incubated at 37ºC for 20 min. Then, IgG antihuman antibody conjugated with horseradish peroxidase at the dilution of 1/20000 was added. Avidity index was calculated as the result of absorbance (Abs) of wells washed with PBS containing urea (U+), divided by the Abs of wells washed with PBS (U-) (AI = Abs(U+)/Abs(U-)).

Molecular modeling

The SAG2L sequence was used for molecular modeling. To construct three-dimensional structures, templates were searched by Swiss-Model Workspace-Template Identification (http://swissmodel.expasy.org/workspace/index.php?func=tools_target identification1). The template selected for the construction of final three-dimensional models, was the crystal structure of SporoSAG (PDB: 2wnkA; sequence identity: 29%), which presents the conserved SAG superfamily motif. Other selected model patterns were SAG1 and BSR4, with lower sequence identities. Different structures were built using Modeller9v3 program (Eswar *et al.*, 2006). Model selection was performed with the ANOLEA program (Melo & Feytmans, 1997). Then, corrections of alignments, reconstructions of loops and minimizations of energy were performed with a systematic analysis of the results using Verify3D (Lüthy *et al.*, 1992), ANOLEA, Ramachandran maps (Ramachandran *et al.*, 1963), LOBO program (Tosatto *et al.*, 2002) and UCSF Chimera 1.6.2 (Pettersen *et al.*, 2004).

Evaluation of the surface-exposed regions

The Swiss-PDB Viewer program 4.10 (Guex *et al.*, 1997) was used to find regions of the primary structure of the protein that were exposed on surface in the predicted three-dimensional model. The results were compared with those obtained in the antigenic determinant predictions, because for an epitope to bind an antibody, it should be exposed on the surface.

Antigenicity prediction

The antigenic regions in our models were predicted using the AAPPred (Davydov & Tonevitsky *et al.*, 2009) and DiscoTope (Jens *et al.*, 2012) programs, which can predict linear and conformational epitopes from the sequence and the structural model, respectively.

Parameters for evaluating the techniques

The signals in techniques obtained from each group of samples (AIS, CIS, NIS and PIS) were used to assess the ability of tests to discriminate between AIS and CIS, and between PIS and NIS. To compare the trials, Receiver Operating Characteristic (ROC) curves (Metz *et al.*, 1978) were developed for each discrimination in each separate test. The areas under the different ROC curves (where a value of 1 indicates a perfect classification of the sera and 0.5 equals a random determination) were compared using the GraphPad Prism 6 program. The same program was also used to define the optimal threshold value to obtain maximum sensitivity and specificity. In AIS vs CIS, sensitivity was the proportion of AIS that offered positive reaction (AIS+/AIS . 100) and specificity, the proportion of CIS that offered negative reaction (CIS-/CIS . 100). In PIS vs NIS, sensitivity was the proportion of the PIS that offered positive reaction (PIS+/ PIS . 100) and specificity, the percentage of NIS that offered negative reaction (NIS-/ NIS . 100).

Statistical analyses

Confidence intervals (CIs) were calculated for average OD of each sera group and for the areas under the ROC curves, with 90%

confidence using GraphPad Prism 6. When the intervals did not overlap, differences between these parameters (OD averages and areas under the ROC curves) were statistically significant with 90% confidence.

RESULTS

Cloning, expression and purification

SAG2c and SAG2L were successfully cloned and expressed. The nucleotide sequences amplified by PCR were 100% homologous to the sequence of accession number FJ825705 in the NCBI (http://www.ncbi.nlm.nih.gov/), between nucleotides +81 and +519 for SAG2c and between nucleotides +1 and +564 for SAG2L.

SAG2c was found to be very soluble, remaining mostly in the supernatant fraction, whereas SAG2L was mainly found in the precipitate, forming inclusion bodies. However, both proteins were obtained in useful quantities with no observable contamination on PAGE, to be employed in immunoassays.

Immunoassays

ELISA for IgG detection:

We performed indirect ELISAs to detect specific IgG antibodies against SAG2c and SAG2L, with 56 AIS, 61 CIS and 61 NIS. The average OD, ranges of average OD with 90% confidence and bar graph are shown in Figures 1 and 2. SAG2c signals allowed better discrimination between groups. With SAG2L assay was not possible to determine statistically that serological groups had different mean ODs. Moreover SAG2L had greater signals than SAG2c with CIS and NIS, as opposed to AIS group (with 90% confidence). These data indicate greater nonspecific reactivity using SAG2L than SAG2c.

The ability of both antigens to discriminate "AIS vs CIS" and "PIS vs NIS" was evaluated by developing ROC curves. Table 1 shows various parameters of comparison. The value of area under the curve was always higher for SAG2c than for SAG2L, with statistical significance. SAG2L offered similar discrimination values at random.

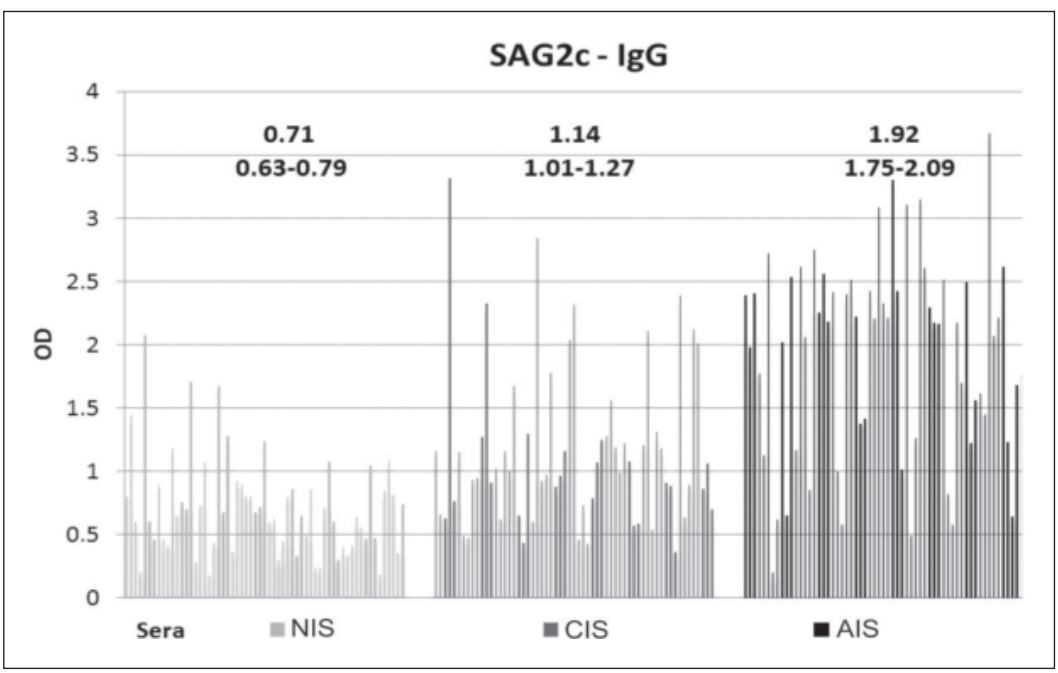

Figure 1. Optical density for all sera tested by indirect ELISA, for detecting specific IgG and using SAG2c antigen. OD averages and their ranges with 90% confidence are indicated above each sera group. Pale gray, dark gray and black bars correspond to NIS, CIS and AIS groups, respectively.

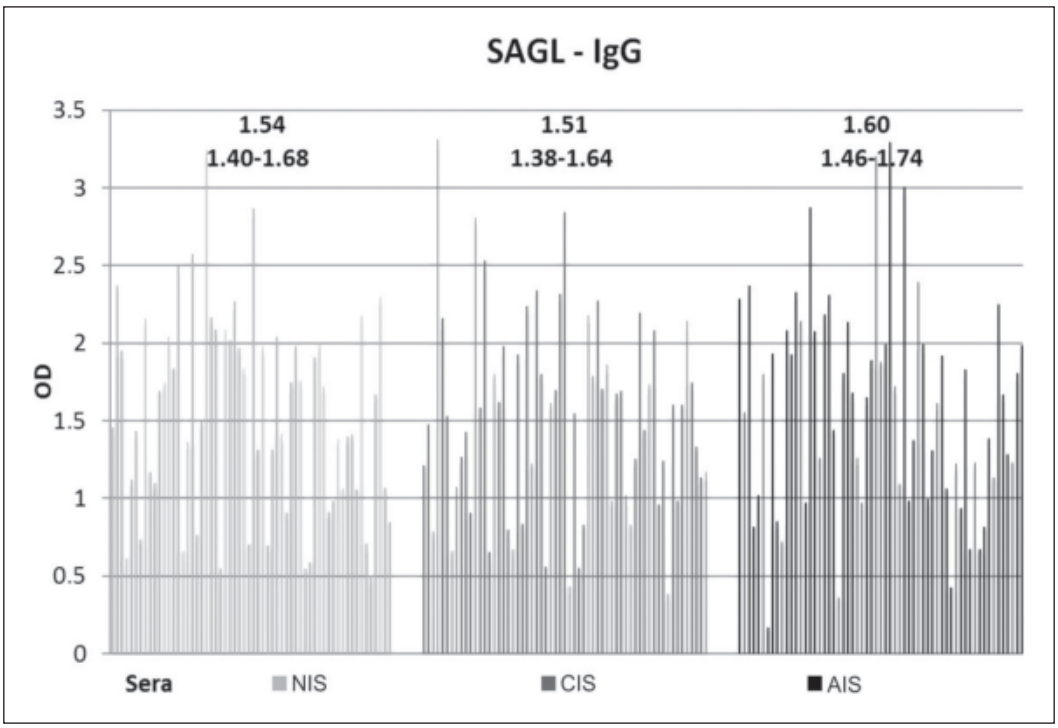

Figure 2. Optical density for all sera tested with indirect ELISA to detect specific IgG using SAG2L antigen. OD averages and their ranges with 90% confidence are indicated above each sera group. Pale gray, dark gray and black bars correspond to NIS, CIS and AIS groups, respectively.

Table 1. Parameters of discrimination between different groups in indirect ELISA performed to detect IgG, IgA and IgM specific antibodies, separately. Area: area under the ROC curve. All values are expressed in %, except areas under the curves and threshold values

ELISA	Isotype Proteins	IgG		IgA		IgM	
		SAG2c	SAG2L	SAG2c	SAG2L	SAG2c	SAG2L
AIS vs. CIS	Threshold value	1.30	1.80	1.77	2.71	0.55	1.47
	Sensitivity	73.8	44.3	67.2	62.3	81.8	52.3
	Specificity	80.3	72.1	81.8	47.3	42.9	61.4
	Area IC	0.778 0.701–0.85	0.545 0.459–0.631	0.751 0.675–0.827	0.528 0.439–0.617	0.597 0.495–0.699	0.512 0.409–0.062
PIS vs. NIS	Threshold value	1.30	1.80	1.56	1.84	0.70	1.58
	Sensitivity	73.8	44.3	54.3	8.,9	53.5	59.1
	Specificity	86.9	66.9	84.1	8.7	55.0	55.0
	Area IC	0.820 0.769–0.871	0.502 0.427–0.578	0.688 0.624–0.753	0.502 0.430–0.574	0.521 0.441–0.602	0.554 0.476–0.633

ELISA for IgA detection:
As specific IgA antibodies where found to be useful to discriminate between acute and chronic infections, SAG2c and SAG2L antigens were evaluated by indirect ELISA to detect this specific immunoglobulin isotype against *T. gondii*. This test included 61 AIS, 54 CIS and 69 NIS. The OD averages and ranges of OD averages assessed with 90% confidence are shown in Table 2. The analysis of SAG2L ODs from the three groups did not show statistically significant differences. However, SAG2c exhibited a difference in mean signal between AIS and CIS with 90% confidence. Likewise, OD means of all groups using SAG2L were higher than using SAG2c, confirming nonspecific reactions with the use of SAG2L. This would suggest the existence of nonspecific epitopes in SAG2L.

The ability to discriminate different groups was evaluated through ROC curves (Table 1). SAG2c always had higher average values of areas than the complete protein, with statistical significance. SAG2L again offered similar discriminations at random.

ELISA for IgM detection:
Finally, indirect ELISAs were performed to detect specific IgM antibodies against *T. gondii* antigens using SAG2c and SAG2L, with 44 AIS, 44 CIS and 60 NIS. Table 2 shows OD averages and ranges of OD averages. The analysis of the CI did not allow us to determine if the ODs from groups were different within each Ag, but we confirmed that SAG2L signals were greater than SAG2c signals. The CI of the areas under the ROC curves for the four tests were overlapped (Table 1). The largest area was obtained with SAG2c in the differentiation of "AIS versus CIS".

Avidity assay:
We evaluated SAG2c performance to discriminate between 9 AIS and 11 CIS, by measuring antibody AI. Figure 3 shows the bar graph, the average AI and average AI intervals with a 90% confidence. Antibodies of sera from each group had different AI with statistical significance. The area under the ROC curve obtained to differentiate AIS from CIS was 0.889 (CI: 0.759-1), sensitivity was 100% and specificity was 81.8%.

SAG2 Molecular Modeling
The predicted three-dimensional structure of SAG2 was a globular domain with two appendages separated from the central structure (Figure 4). Each of these appendages corresponded to the amino-and carboxy-terminal ends. SAG2c sequence would have this structure without the extra appendages, whereas SAG2L would not have this structure. Indeed, since the latter sequence was obtained only with a chaotropic compound, which would have probably altered its natural structure, its tridimensional structure is probably different from that predicted in this work. It should be taken into account that the prediction of potential epitopes of the protein needs the structure originally expressed by the parasite.

Table 2. Average optical densities and range of values for a 90% confidence, with AIS, CIS and NIS obtained with IgA and IgM ELISA using the antigens SAG2L and SAG2c

Sera	ELISA for IgA detection				ELISA for IgM detection			
	SAG2c		SAG2L		SAG2c		SAG2L	
	Average OD	Ranges of average OD	Average OD	Ranges of average OD	Average OD	Ranges of average OD	Average OD	Ranges of average OD
AIS	2.23	2.03–2.43	2.59	2.44–2.75	0.83	0.75–0.92	1.51	1.40–1.62
CIS	1.40	1.25–1.55	2.62	2.45–2.79	0.73	0.64–0.82	1.53	1.38–1.68
NIS	1.27	1.15–1.40	2.63	2.77–2.49	0.77	0.69–0.86	1.59	1.49–1.70

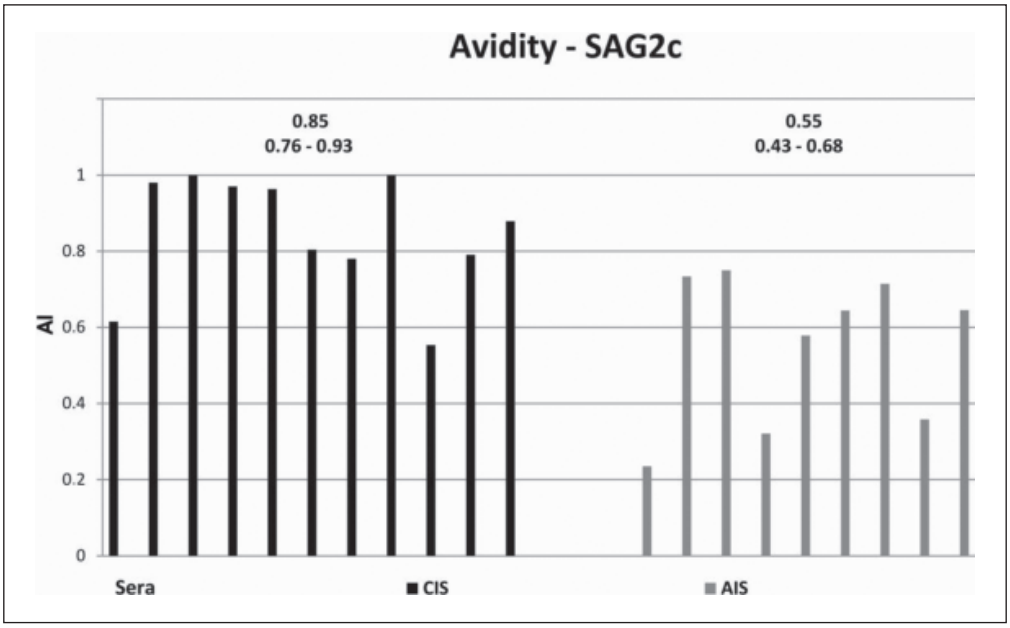

Figure 3. Avidity Index values obtained with 9 AIS (gray bars) and 11 CIS (black bars). Values above the bars are mean AI and confidence intervals for 90%.

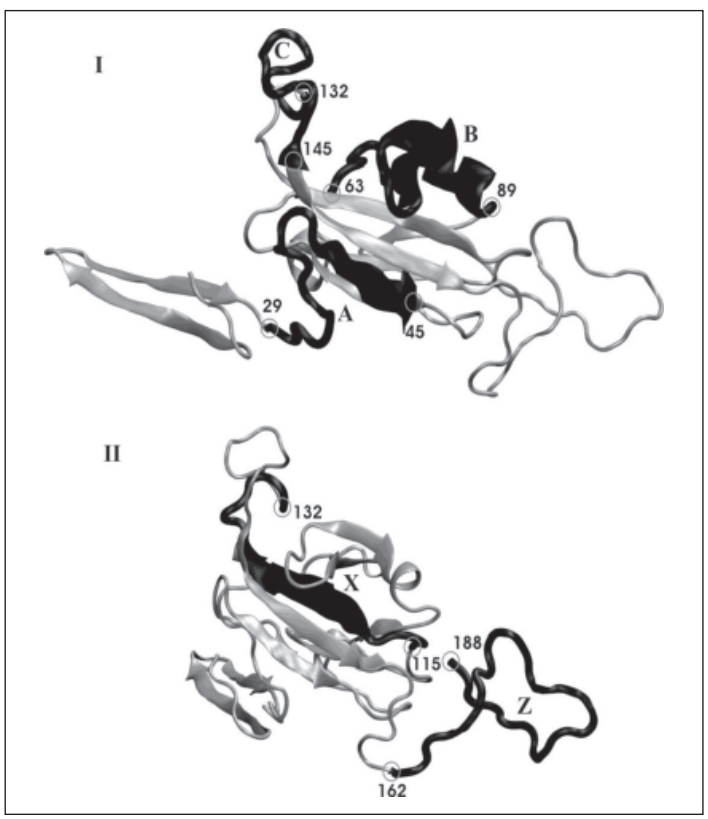

Figure 4. SAG2 predicted three-dimensional structure (gray), showing the locations of the estimated antigenic regions A, B, C, X and Z (black).

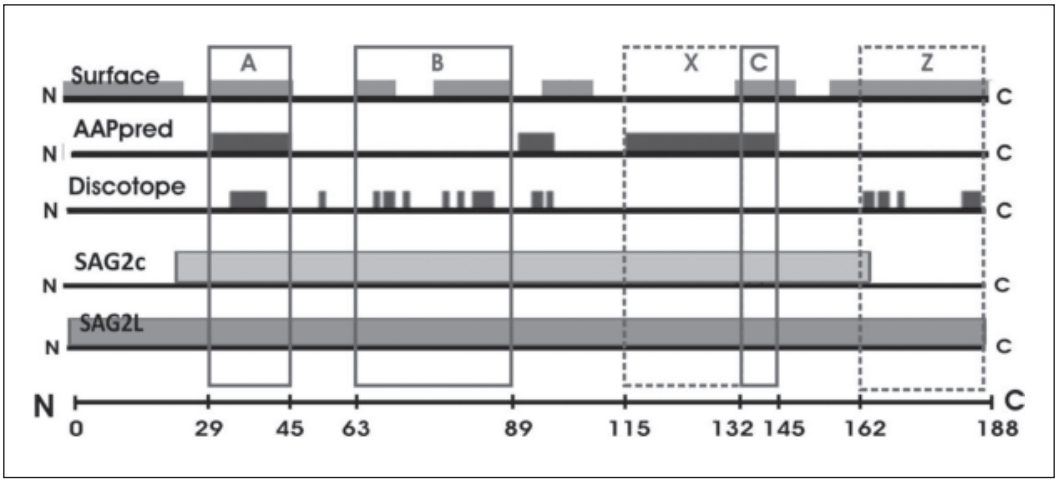

Figure 5. Diagram of the SAG2 complete amino acid sequences, which are shown with different shades of gray: the protein sequences exposed on the surface (Surface), prediction of amino acids belonging to epitopes (AAPpred and Discotope) and location of expressed sequence SAG2c and SAG2L. Five defined regions are also shown: A, B, C, X and Z.

Regions exposed on surface and epitope prediction

Figure 5 shows the different sequences determinated in this work on a representative diagram of SAG2 primary structure. There are amino acids exposed on the surface (Surface) and amino acids belonging to SAG2 antigenic determinants (AAPpred and Discotope). Furthermore, others five defined regions are shown (A, B, C, X and Z).

We found a high non-specific reactivity in SAG2L. The NIS and CIS had double OD with SAG2L with respect to SAG2c, for detection of the three antibody classes. Therefore, it was assumed that SAG2L would have nonspecific epitopes. Depending on the available sequences to bind antibodies in SAG2c and those available only for SAG2L, the antigenic regions were classified into two groups: specific antigenic sequences (A, B and C) and nonspecific antigenic sequences (X and Z). A, B and C regions are sequences that besides being indicated as antigenic by the epitope prediction programs, they were exposed on the surface of the SAG2 predicted structure. The X and Z regions were identified as antigenic, but were not found in SAG2c, because they do not belong to the sequence (Z) or are not found on the surface (X). The SAG2L antigen, which probably had its conformational structure altered (by chaotropic compound used for purification), would have the X region exposed on the surface. Figure 4 also shows the estimated antigenic region locations, specific and nonspecific, on the predicted three-dimensional structure.

DISCUSSION

Here, we evaluated SAG2c and SAG2L, whose sequences differ precisely in the inclusion or not of the amino-and carboxy-terminal regions. These sequences are the appendages separated from globular central structure of the protein.

The first difference found between both sequences was the generation of inclusion bodies by SAG2L. Prince *et al.* (2007) and Béla *et al.* (2008) worked with sequences involving the carboxy-terminal region (but not the amino terminal region), and did not report solubilization problems of the protein. Furthermore, Fong and Lau (2008) did not report difficulties in the use of the whole protein with both terminal regions, although they used the yeast *Pichia Pastoris* for Ag expression. Although we were not able to confirm that the amino- or carboxy-terminal

region independently turns SAG2 insoluble, we found that the combination of both sequences triggers protein precipitation and formation of inclusion bodies. If these two sequences are removed, 41 amino acids in total (22.04% of SAG2L sequence), the protein becomes soluble; and as we confirmed in this work, the protein obtained soluble (SAG2c) had better differentiation of toxoplasmosis infection phases than protein obtained initially precipitated (SAG2L).

We attempted to remove urea from SAG2L before the start of the immunoassays, by dialysis and purification through extraction from the polyacrylamide gel, to allow a better folding of SAG2L, but results were not successful in either case. Good amounts of SAG2L were obtained by dialysis, but the protein precipitated immediately after the end of the process, whereas a very low yield of the Ag was obtained by extraction from PAGE (data not shown). However, these Ag solutions were diluted 100 to 200 times (depending of protein concentration in the stock solution) for the coating of flat-bottom plates. It is very important differentiate the ELISA assays, where SAG2L signals were higher than SAG2c signals in the three groups of sera (in which SAG2L was used in a solution of 4 mol/L urea but was diluted to 0.04 mol/L in the assay); regarding avidity assay with SAG2c, in which the final concentration was 6 mol/L urea in the test wells and the signals from urea samples dropped considerably. Therefore, since the avidity assay evidenced urea effect through a decrease in sample signals, we assumed that high SAG2L signals (in ELISAs) were due to differences in exposed sequences with respect to SAG2c. These high signals in diagnostic assays employing SAG2L are "noise" in the signal that prevents the use of SAG2L as an antigen in the tests, unlike SAG2c.

The analysis of indirect ELISAs to detect IgG and IgA showed that SAG2c always generated an area under the ROC curve different and greater than SAG2L (with 90% confidence). SAG2L always had a performance very similar to random differentiation. On the other hand, in the detection of IgM to both Ags, the CI of the areas under the curves always included the value of 0.5. The detection of IgM practically did not discriminate between different groups with either of the two Ags.

In the detection of total IgG antibodies by indirect ELISA, a sensitivity of 73.8% and a specificity of 80.3% were reached using SAG2c for discrimination between CIS and AIS. The comparison of our results with other works that evaluated similar sequences to SAG2c showed higher values (sensitivity and specificity) than those reported by Hiszczyjska-Sawicka *et al.* (2005), but lower than those reported by Li *et al.* (2000) and Parmley *et al.* (1992). However, those two studies used smaller sample sizes than those used here (10 AIS and 10 CIS in Li *et al.*, 31 AIS and 31 CIS in Parmley *et al.*). Regarding the results for SAG2L, we were unable to differentiate infection stages, which is consistent with the work of Lau and Fong (2008), who used the same extent of Ag. This would be because SAG2L exposes nonspecific epitopes. Prince *et al.* (1990) and Béla *et al.* (2008) compared two different SAG2 sequences simultaneously, and detected IgG, but covering different regions from the ones covered in our work. The discriminations obtained by those authors were lower than those reported here. Furthermore, none achieved significant changes in immunoassays when they modified the Ag sequence extensions.

The analysis of IgA by indirect ELISA with SAG2c to differentiate AIS from CIS yielded a sensitivity of 67.21% and a specificity of 81.82%. These values are much higher than those obtained by Parmley *et al.* (1992; sensitivity of 15.38% and a specificity of 100%), the only investigators who reported the evaluation of this isotype against SAG2 to differentiate the acute phase from the chronic phase.

The detection of IgM with SAG2c yielded a sensitivity of 81.8% and a specificity of 42.9%, discriminating between AIS and CIS by indirect ELISA. Lau and Fong (2008) and Parmley *et al.* (1992) were the only authors that evaluated SAG2 (SAG2L and SAG2c sequences, respectively) against IgM to discriminate the acute phase from the chronic

phase. Their results agree with those obtained in this work. The wrong discrimination may be due to two disadvantages of indirect ELISA in the detection of IgM antibodies: a high number of false positives from natural antibodies or rheumatoid factors, and a high number of false negative results generated by competitive inhibition with specific IgG antibodies (Robinson *et al.*, 2004).

The avidity assay was developed with the best SAG2 antigen, SAG2c. The discrimination between AIS and CIS yielded a sensitivity of 100% and a specificity of 81.8%. The discrimination obtained in our study by measuring IgG avidity was higher than that of Béla *et al.* (2008), the only investigators who reported the evaluation of avidity with SAG2. Measurement of avidity in our work yielded a higher area under ROC curve (0.889) than that obtained with indirect ELISA for detecting IgG (0.778). Therefore, the avidity assay showed the best ability to differentiate AIS from CIS in this work. However, it should be tested with a larger number of sera. Note that the largest area under the ROC curve for all trials discriminating between PIS and NIS was obtained using SAG2c to detect IgG (0.82).

SAG2L did not yield good results in any test, although this was not due to a lack of reactivity; indeed, the ODs obtained with SAG2L were higher than those obtained with SAG2c, with statistical significance. The mean ODs of NIS and CIS groups were twice as high using SAG2L as using SAG2c, to detect the three antibody isotypes. Such difference in reactivity was probably due to the exclusive presentation of nonspecific epitopes in SAG2L. Epitope predictor programs found two regions (X and Z) that would not be present in SAG2c; hence, these sequences might bind antibodies with cross-reactivity to other Ag previously presented to the immune system of the patients, which explains the high reactivity of SAG2L against NIS and CIS.

The predicted specific C antigenic region is consistent with the unique epitope determined experimentally via crystallization of an antibody-antigen complex by Cunha-Junior *et al.* (2010). A more complete experimental antigenic mapping should be performed to confirm the antigenicity of A and B regions. Determining the nonspecific reactivity of X and Z regions is not so important as to justify the investment of time and material resources necessary for the experimental procedures. However, our hypothesis of its existence should be taken into account in any study attempting to select and clone SAG2 reduced regions.

CONCLUSIONS

In this paper, significant differences were found in obtaining the recombinant protein and its immunochemical assays, when the extent of the protein was reduced in only 41 amino acids. With respect to reactivity variations between SAG2L and SAG2c, we proposed the existence of specific and nonspecific SAG2 epitopes and we located them with *in silico* tools.

We also obtained promising results to differentiate the acute and chronic phases of toxoplasmosis, in the detection of IgG by indirect ELISA with SAG2c.

This is the first report of promising results of SAG2 used for detecting IgA antibodies to differentiate toxoplasmosis infection stages. Finally, we obtained a better differentiation than values reported in the literature, by measuring IgG antibody avidity.

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REFERENCES

Araújo, P.R.B. & Ferreira, A.W. (2010). 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. *Revista del Instituto de Medicina Tropical* **52**: 63-68.

Aubert, D., Maine, G.T., Villena, I., Hunt, J.C., Howard, L., Sheu, M., Brojanac, S., Chovan, L.E., Nowlan, S.F. & Pinon, J.M. (2000). Recombinant antigens to detect *Toxoplasma gondii*-specific immunoglobulin G and immunoglobulin M in human sera by enzyme immunoassay. *Journal of Clinical Microbiology* **38**: 1144-1150.

Bai, Y., He, S., Zhao, G., Chen, L., Shi, N., Zhou, H., Cong, H., Zhao, Q. & Zhu, X.Q. (2012). *Toxoplasma gondii*: Bioinformatics analysis, cloning and expression of a novel protein TgIMP1. *Experimental Parasitology* **132**: 458-464.

Beghetto, E., Buffolano, W., Spadoni, A., Del Pezzo, M., Di Cristina, M., Minenkova, O., Petersen, E., Felici, F. & Gargano, N. (2003). Use of an Immunoglobulin G Avidity Assay Based on Recombinant Antigens for Diagnosis of Primary *Toxoplasma gondii* Infection during Pregnancy. *Journal of Clinical Microbiology* **41**: 5414-5418.

Béla, S.R., Oliveira Silva, D.A., Cunha-Júnior, J.P., Pirovani, C.P., Chaves-Borges, F.A. & de Carvalho, F.R. (2008). Use of SAG2A recombinant *Toxoplasma gondii* surface antigen as a diagnostic marker for human acute toxoplasmosis: analysis of titers and avidity of IgG and IgG1 antibodies. *Diagnostic Microbiology and Infectious Disease* **62**: 245-254.

Cardona, N., de-la-Torre, A., Siachoque, H., Patarroyo, M.A. & Gomez-Marin, J.E. (2009). *Toxoplasma gondii*: P30 peptides recognition pattern in human toxoplasmosis *Experimental Parasitology* **123**: 199-202.

Chen, X.Z., Guo, K., Chen, Y., Liu, L.L., Shen, J.L., Jiao, Y.M., Fang, Q. & Sun, X. (2012). Recombinant expression and immunologic identification of bradyzoite-specific antigen BSR4 of *Toxoplasma gondii*. *Chinese Journal of Parasitology & Parasitic Disease* **30**: 170-173.

Costa, J.G., Faccendini, P., Sferco, S., Lagier, C. & Marcipar, I. (2013). Evaluation and comparison of the ability of online available prediction programs to predict true linear B-cell epitopes. *Protein and Peptide Letters* **20**: 724-730.

Crawford, J., Tonkin, M.L., Grujic, O. & Boulanger, M.J. (2010). Structural characterization of apical membrane antigen 1 (AMA1) from *Toxoplasma gondii*. *Journal of Biological Chemistry* **285**: 15644-15652.

Cunha-Junior, J.P., Silva, D.A.O., Silva, N.M., Souza, M.A., Souza, G.R.L. Prudencio, C.R., Pirovani, C.P., Cascardo, J.C.M., Barbosa, B.F., Goulart, L.R. & Mineo, J.R. (2010). A4D12 monoclonal antibody recognizes a new linear epitope from SAG2A *Toxoplasma gondii* tachyzoites, identified by phage display bioselection. *Immunobiology* **215**: 26-37.

Davydov, Y.I. & Tonevitsky, A.G. (2009). Prediction of Linear B-Cell Epitopes. *Molecular Biology* **1**: 150-158.

Eswar, N., Marti-Renom, M.A., Webb, B., Madhusudhan, M.S., Eramian, D., Shen, M., Pieper, U. & Sali, A. (2006). Comparative Protein Structure Modeling With MODELLER. in: Current Protocols in Bioinformatics, Wiley, J. (editor). Supplement

Golkar, M., Rafati, S., Abdel-Latif, M.S., Brenier-Pincharta, M.P., Fricker-Hidalgoe, H., Simab, B.K., Babaieb, J., Pellouxa, H., Cesbron-Delauwa, M.F. & Merciera, C. (2007). The dense granule protein GRA2, a new marker for the serodiagnosis of acute *Toxoplasma* infection: comparison of sera collected in both France and Iran from pregnant women. *Diagnostic Microbiology and Infectious Disease* **58**: 419-426.

Guex, N. & Peitsch, M.C. (1997). Swiss-model and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophesis* **18**: 2714-2723.

Hill, D., Coss, C., Dubey, J.P., Wroblewski, K., Sautter, M., Hosten, T., Muñoz-Zanzi, C., Mui, E., Withers, S., Boyer, K., Hermes, G., Coyne, J., Jagdis, F., Burnett, A., McLeod, P., Morton, H., Robinson, D. & McLeod, R. (2011). Identification of a sporozoite-specific antigen from *Toxoplasma gondii*. *Journal of Parasitology* **2**: 328-337.

Hiszczyjska-Sawicka, E., Kur, J., Pietkiewicz, H., Holec, L., Gasior, A. & Myjak, P. (2005). Efficient production of the *Toxoplasma*

gondii GRA6, P35 and SAG2 recombinant antigens and their applications in the serodiagnosis of toxoplasmosis. *Acta Parasitológica* **50**: 249-254.

Hruzik, A., Asif, A.R. & Gro, U. (2011). Identification of *Toxoplasma gondii* SUB1 Antigen as a Marker for Acute Infection by Use of an Innovative Evaluation. *Method Journal of Clinic Microbiology* **49**: 2419–2420.

Jens, V.K., Claus, L., Ole, L. & Morten, N. (2012). Reliable B Cell Epitope Predictions: Impacts of Method Development and Improved Benchmarking *Plos Computational Biology* **8**: 1-10.

Kato, M., Claveria, F.G., Maki, Y., Sanda, K., Tanaka, T., Omata, Y., Nagasaw, H. & Suzuki, N. (2007). Reactivity of Synthetic SAG1 (p30) Peptide Sequences with RH, S273 and Beverley Strain-Induced Anti- *Toxoplasma gondii* Antibodies. *Pathobiology* **74**: 50-56.

Lau, Y.L. & Fong, M.Y. (2008). *Toxoplasma gondii*: Serological characterization and immunogenicity of recombinant surface antigen 2 (SAG2) expressed in the yeast *Pichia pastoris*. *Experimental Parasitology* **119**: 373-378.

Li, S., Galvan, G., Araujo, F.G., Suzuki, Y., Remington, J.S. & Parmley, S. (2000). Serodiagnosis of recently acquired *Toxoplasma gondii* infection using an Enzime-linked immunosorbent assay with a combination of recombinanat antigens. *Clinical and Vaccine Immunology* **7**: 781-787.

Lüthy, R., Bowie, J.U. & Eisenberg, D. (1992). Assessment of protein models with three-dimensional profiles. *Nature* **356**: 83-85.

Maksimov, P., Zerweck, J., Maksimov, A., Hotop, A., Grob, U., Pleyer, U., Spekker, K., Däubener, W., Werdermann, S., Niederstrasser, O., Petri, E., Mertens, M., Ulrich, R.G., Conraths, F.J. & Scharesa, G. (2012). Peptide Microarray Analysis of In Silico-Predicted Epitopes for Serological Diagnosis of *Toxoplasma gondii*. Infection in Humans *Clinical and Vaccine Immunology* **17**: 865-874.

Melo, F. & Feytmans, E. (1997). Novel knowledge-based mean force potential at atomic level. *Journal of Molecular Biology* **267**: 207-222.

Metz, C.E. (1978). Basic Principles of ROC Analysis. *Seminars in Nuclear Medicine* **8**: 283-298.

Nigro, M., Gutierrez, A., Hoffer, A.M., Clemente, M., Kaufer, F., Carral, L., Martin, V., Guarnera, E.A. & Angel, S.O. (2003). Evaluation of *Toxoplasma gondii* recombinant proteins for the diagnosis of recently acquired toxoplasmosis by an immunoglobulin G analysis. *Diagnostic Microbiology and Infectious Disease*. **47**: 609-613.

Parmley, S.F., Sgarlato, G.D., Mark, J., Prince, J.B. & Remington, J.S. (1992). Expression, Characterization, and Serologic Reactivity of Recombinant Surface Antigen P22 of *Toxoplasma gondii*. *Journal of Clinical Microbiology* **30**: 1127-1133.

Peng, H.-J., Chen, X.-G. & Lindsay, D.S. (2011). A review: competence, compromise, and concomitance–reaction of the host cell to *Toxoplasma gondii* infection and development. *Journal of Parasitology* **97**: 620-628.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C. & Ferrin, T.E. (2004). UCSF Chimera: a visualization system for exploratory research and analysis. *Journal Computational Chemistry* **25**: 1605-1612.

Pfrepper, K.I., Enders, G., Gohl, M., Krczal, D., Hlobil, H., Wassenberg, D. & Soutschek. (2005). Seroreactivity to and avidity for recombinant antigens in toxoplasmosis. *Clinical and Vaccine Immunology* **12**: 977-982.

Pietkiewicz, H., Hiszczyjska-Sawicka, E., Kur, J., Petersen, E., Nielsen, H.V., Stankiewicz, M., Andrzejewska, I. & Myjak, P. (2004). Usefulness of *Toxoplasma gondii*-specific recombinant antigens in serodiagnosis of human toxoplasmosis. *Journal of Clinical Microbiolology* **42**: 1779-1781.

Prince, J.B., Auer, K.L., Huskinson, J., Parmley, S.F., Araujo, F.G. & Remington, J.S. (1990). Cloning, expression, and cDNA sequence of surface antigen P22 from *Toxoplasma gondii*. *Molecular and Biochemical Parasitology* **43**: 97-106.

Ramachandran, G.N., Ramakrishnan, C. & Sasisekharan, V. (1963). Stereochemistry of polypeptide chain configurations. *Journal of Molecular Biology* **7**: 95–99.

Robert-Gangneux, F. & Dardé, M.-L. (2012). Epidemiology of and diagnostic strategies for toxoplasmosis. *Clinical Microbiology Reviews* **25**: 264-296.

Robinson, S.A., Smith, J.E. & Millner, P.A. (2004). *Toxoplasma gondii* major surface antigen (SAG1): *in vitro* analysis of host cell binding. *Parasitol.* **128**: 391-396.

Saouros, S., Dou, Z., Henry, M., Marchant, J., Carruthers, V.B. & Matthews, S. (2012). Microneme protein 5 regulates the activity of *Toxoplasma* subtilisin 1 by mimicking a subtilisin prodomain. *Journal of Biological Chemistry* **287**: 36029-36040.

Schägger, H. & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry* **166**: 368-379.

Selseleh, M., Keshavarz, H., Mohebali, M., Shojaee, S., Modarressi, M.H., Eshragian, M.R. & Selseleh, M. (2012). Production and evaluation of *Toxoplasma gondii* recombinant surface antigen 1 (SAG1) for serodiagnosis of acute and chronic toxoplasma infection in human sera. *Iranian Journal of Parasitology* **7**: 1-9.

Tonkin, M.L., Roques, M., Lamarque, M.H., Pugnière, M., Douguet, D., Crawford, J., Lebrun, M. & Boulanger, M.J. (2011). Host cell invasion by apicomplexan parasites: insigts from the co-structure of AMA1 with RON2 peptide. *Science* **333**: 463-467.

Tosatto, S.C.E., Bindewald, E., Hesser, J. & Männer, R. (2002). A Divide and Conquer Approach to Fast Loop Modeling. *Protein Engineering* **15**: 279-286.

Villena, I., Aubert, D., Leroux, B., Dupouy, D., Talmud, M., Chemla, C., Trenque, T., Schmit, G., Quereux, C., Guenounou, M., Pluot, M., Bonhomme, A. & Pinon, J.M. (1998). Pyrimethamine-sulfadoxine treatment of congenital toxoplasmosis: follow-up of 78 cases between 1980 and 1997. Reims Toxoplasmosis Group. *Scandinavian Journal of Infectious Diseases* **30**: 295-300.