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Immunochemical evaluation of two *Toxoplasma gondii* GRA8 sequences to detect acute toxoplasmosis infection

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ABSTRACT

In this work, two *Toxoplasma gondii* GRA8 protein sequences were tested by indirect ELISA and measurement of avidity to differentiate between acute and chronic toxoplasmosis infection. Using the antigen called GRA8B, 79.7% sensitivity and 84.1% specificity was achieved detecting IgG concentrations and a 71.2% sensitivity and a 68.3% specificity detecting IgA concentrations. This study is the first to report IgA detection with GRA8 by ELISA to differentiate stages of infection. Unfortunately the indirect ELISA to detect IgM was not effective in distinguishing stages. Also, this work is the first to report that the GRA8 protein can aid the differentiation between acute and chronic phase infection by measuring IgG antibody avidity, a technique in which we obtained 85.71% and 100% of sensitivity and specificity, respectively. Finally, *in silico* tools were used to explain the differences in our immunochemistry results.

1. Introduction

Toxoplasma gondii is an obligatory protozoan parasite that infects all mammals and birds, producing toxoplasmosis. Worldwide, an estimated one in three people carry this infection. Fortunately, toxoplasmosis is generally asymptomatic and causes a self-limiting disease in humans. But the infection acute phase acquired during gestation can be transmitted to the fetus and may cause miscarriage, permanent neurological damage, visual impairment or other malformations in the newborn [1,2].

Toxoplasmosis during gestation represents a difficult task for the clinician due to its subclinical course in the majority of pregnant women and the unpredictable long-term outcomes of congenital infection. In order to implement suitable therapies in an appropriate time frame and to avoid neonatal malformations or reduced eyesight in newborns, it is essential to establish whether the mother acquired an acute infection [3,4].

Detection of toxoplasma infection and differentiation between the acute and chronic phases are mainly based on serological tests that recognize anti-toxoplasma antibodies in blood. The enzyme-linked immunosorbent assay (ELISA) using antigens obtained from *T. gondii*, is, at present, most commonly employed serological test [5,6]. Moreover, detection of immunoglobulin G, A and M (IgG, IgA and IgM) using *T. gondii* recombinant proteins is already used [6–9].

There are commercial kits for the diagnosis of toxoplasmosis that employ combinations of recombinant antigens and report very high levels of sensitivity and specificity [10,11]. However, considering it is not easy to produce recombinant proteins at the industrial scale, finding a single antigen that can replace several of them could reduce the cost of these kits.

Analysis of the human humoral immune response against *T. gondii* antigens has identified a number of immunoreactive proteins specific to the acute phase. These proteins are normally

Abbreviations: T. gondii, *Toxoplasma gondii*; IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M; Ag, antigen; IFI, indirect immunofluorescence; NIS, negative infection sera; CIS, chronic infection sera; AvI, avidity index; AIS, acute infection sera; PIS, positive infection sera; *E. coli*, *Escherichia coli*; PAGE, polyacrylamide gel electrophoresis; ROC, Receiver Operating Characteristic; SD, standard deviations; CfI, Confidence interval.

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cloned in bacteria and their pattern of immunoreactivity against human sera varies with the immunoglobulin class [6,7,12]. In turn, one of the most promising acute phase-specific antigens is GRA8. This protein is secreted by the *Toxoplasma gondii* dense granule, which allows the survival of the parasite within the host cell. GRA8 is among the antigens (Ags) for which there is consensus about their effectiveness for phase differentiation [13–16]. Moreover, this protein is highly proline-rich (24% overall) and it has some domains in its structure defined by prediction *in silico*: following the first ATG there is a hydrophobic region of 23 amino acids which is signal sequence; another hydrophobic region spanning amino acids 223 to 242 was identified as a transmembrane domain; a predicted cleavage site is located between position 23 and 24; and another three different domains were located on positions 69, 103 and 136 [17,18].

It is important to note that to accurately distinguish different stages of infection, it may be necessary to select protein sequences that react exclusively in the acute phase of infection and show no cross reactivity with proteins from other parasites [19]. If some sequences of *T. gondii* antigens are homologous to sequences of antigens from other parasites, it could react with antibodies already present in the sera of an individual without toxoplasmosis (who has been exposed to other infections), thereby reducing the efficiency of any diagnostic test using these proteins [19]. Even though defining every cross-reactive sequence would be difficult, discarding protein sequences that do not have reactivity against antibodies generated to toxoplasmosis infection reduces the probability of cross-reacting sequences in the Ag. For example, there have been many experimental studies mapping the SAG1 *Toxoplasma gondii* protein that they have found antigenic regions with or without specific toxoplasmosis reactivity [20–22]. In the case of the *T. gondii* proteins SAG2 and MIC1, immunochemical studies have also been used to differentiate the phases of infection. These studies compared different sequences from the same protein and obtained differentiate reactivities [8,9,19].

At the same time, programs that predict antigenic epitopes [23,24] can be very useful in this endeavor, considering that experimental methods to identify epitopes from infectious microorganisms are quite expensive and require long-term trials. In previous work, we found that these programs are acceptably effective [25]. These *in silico* tools can be combined with experimental results, facilitating deep analyses, understanding and hypothesis formulation [22].

In the present study, we evaluated two GRA8 sequences by indirect ELISA, detecting IgG, IgA and IgM to recognize the acute phase of the infection. With the same goal in mind, we measured IgG antibody avidity against one of the sequences. We report the successful cloning of additional GRA8 sequences, which we fruitlessly attempted to express. Furthermore, we analyzed different regions of the protein sequence using *in silico* tools and found differences between expressible regions and non-expressible regions. Finally, we present a hypothesis based on bioinformatic predictions regarding the antigenic relevance of different sequence regions.

2. Materials and methods

2.1. Samples

Serum samples (n = 242) 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 in the health centers between 2009 and 2011. We classified the samples into groups of sera according to the results obtained with the commercial tests: IgG avidity test (VIDAS Toxo IgG Avidity), IgG indirect immunofluorescence (IFI, in-house test), Sabin-Feldman (assay in-house test), IgM and IgA immunosorbent agglutination (ISAGA, bioMérieux), hemagglutination assay (HAI, Toxotest HAI Wiener Lab) and anti-*T. gondii*-IgG ELISA (Sigma-Aldrich, Toxoplasma IgG ELISA kit). 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 people 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 people 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 (AvI) in the avidity assay.

Acute infection sera (AIS): 85 sera from people with recently acquired toxoplasmosis (acute phase). 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.

2.2. Cloning and expression of antigens

The complete coding GRA8 sequence, GRA8T, was divided into five overlapping regions: GRA8A, GRA8B, GRA8C, GRA8D and GRA8E. In each one, there is an overlap of 50 amino acids with the above sequence and of 50 amino acids also with the subsequent sequence (approximately). The primers used for amplification of these sequences by PCR were: GRA8A-FW: 5′-GAATTCATGGCTTTACCATTGCGTG-3′, GRA8A-RV: 5′-AAGCTTCGTTGGCGGGGGATGCTG-3′, GRA8B-FW: 5′-GAATTC GGAATGCCCAAGCCAGAG-3′, GRA8B-RV: 5′-AAGCTTTGGAGTGCCCACTGGATACG-3′, GRA8C-FW: 5′-GAATTCCCGCCAACGGGTT CCCC-3′, GRA8 C-RV: 5′-AAGCTTTGGCACTGGAGGAGCACG-3′, GRA8D-FW: 5′-GAATTCCCCCAGCCGGAGATAC-3′, GRA8D-RV: 5′-AAGCTTTGCCATTGCAGCCACTA CC-3′, GRA8E-FW: 5′-GAATTCGCTCCTCGTGTGCTGG-3′, GRA8E-RV: 5′-AAGCTTATT CTGCGTCGTTACGG-3′, GRA8T-FW: 5′-GAATTCATGGCTTTACCATTGCGTG-3′ and GRA8T-RV: 5′-AAGCTTATTCTGCGTCGTTACGG-3′.

We used 1.5 units of Taq DNA polymerase enzyme (Productos Bio-Lógicos), reaction buffer recommended by the manufacturer; 3 mmol/L $MgCl_2$; 0.25 mmol/l dNTP (mixture in equal amounts of desoxiadenin triphosphate, deoxycytidine triphosphate, desoxiguanidin triphosphate, and deoxythymidin triphosphate); 1 μmol/L of each primer and 0.5 ng DNA template (total genomic DNA purified from *T. gondii*) in a final volume of 50 μL. Thirty-five cycles were performed with the following steps: denaturation at 95 °C for 60 s, hybridization for 60 s and extension at 72 °C for 90 s. The hybridization stage consisted of temperature of 51 °C for GRA8A, 53 °C for GRA8B, 55 °C for GRA8C, 49 °C for GRA8D, 46 °C for GRA8E and 45 °C for GRA8T. Before starting all cycles, the mixture was exposed to 95 °C for 180 s and at the end, to 72 °C for 10 min.

The used *Toxoplasma gondii* strain genotype was type III, accession number in the ToxoDB [26]: AEYH01001155.

GRA8A and GRA8B were cloned into the pET-32a (Novagen), pET-24a (Novagen) and pRSET-B (Invitrogen life technologies) vectors. The GRA8C, GRA8E, GRA8D and GRA8T sequences were cloned into the pET-24a, pET-32a and pMAL-c2X (New England Biolabs) vectors. All sequences were introduced in *Escherichia coli*

BL21 (DE3). GRA8C, GRA8E and GRA8D also were cloned into the pET-32a vector in *E. coli* BL21 Rosetta. All genes were cloned with *Bam*H I (GGATCC) and *Hin*d III (AAGCTT) enzymes. We tried to express sequences in two vectors that do not incorporate fusion protein in antigens (pET-24a and pRSET-B) and two that do incorporate that protein (pET-32a and pMAL-c2X). In turn, the latter have different fusion proteins (Thioredoxin and Maltose Binding Protein, respectively).

The bacteria were cultured at 37 °C until they reached an optic density (OD) between 0.4 and 0.5. Antigen expression was induced for 3 h at 37 °C with 1 mmol/L isopropyl-β-D-thiogalactopyranoside (Promega).

2.3. Purification

The bacterial pellet was suspended in binding buffer (20 mmol/L imidazole, 0.3 mol/L NaCl and 0.05 mol/L NaH_2PO_4), broken by sonication using an ultrasonic disintegrator and centrifuged. Ag was purified from solution with a nickel pseudo-affinity IDA-Sepharose column (Invitrogen, Ni-NTA) (elution solutions: 0.3 mol/L NaCl and 0.05 mol/L NaH_2PO_4, with 50, 100, 250 and 500 mmol/L imidazole).

Protein concentration and purity were evaluated by Bradford assay and 15% polyacrylamide gel electrophoresis (PAGE) under denaturing conditions, following Schägger and von Jagow [27].

2.4. ELISA using GRA8A and GRA8B

Purified recombinant Ags were individually diluted to the following concentrations: 5 μg/ml to detect IgG, 2.5 μg/ml to detect IgA or 10 μg/ml to detect IgM in carbonate-bicarbonate buffer (15 mmol/L Na_2CO_3 and 35 mmol/L $NaHCO_3$, pH 9.6). 0.1 ml of each antigen was added to separate wells of microtiter Maxisorp plates (ELISA-plate, high binding, Greiner bio one). GRA8A and GRA8B were added simultaneously to the same plate. Coated plates were incubated at 4 °C overnight. The following day they were washed three times with PBST (PBS: 137 mmol/L NaCl, 2.7 mol/L KCl, 10 mol/L Na_2HPO_4 and 1.8 mmol/L KH_2PO_4, pH 7.4; with 0.05% tween 20), blocked with 0.2 ml of blocking solution (5% nonfat skim milk in PBS) at 37 °C for 1 h and washed with PBST three times prior to incubation with serum samples. Duplicate serum samples were diluted 1/100 into a PBS-milk (1% nonfat skim milk in PBS). After the addition of 0.1 ml of diluted sample to each well, the plates were incubated at 37 °C for 1 h and washed with PBST three times. Bound human IgG, IgA or IgM was detected using IgG, IgA or IgM anti-human goat antibody conjugated horseradish peroxidase (Abcam). The conjugates were diluted 1/3000 to detect IgG and 1/500 to detect IgA and IgM, into a PBS-milk. After addition of the appropriate conjugate (0.1 ml), the plates were incubated at 37 °C for 1 h and then washed with PBST three times. 0.07 ml of 3,3′,5,5-TetraMethylBenzidine color development reagent (Invitrogen) was added to each well. After 10 min, the color development reaction was stopped by adding 0.05 ml of 0.5 mol/L sulfuric acid and the OD was read at 450 nm in a microtiter plate reader (BioTeK).

2.5. Avidity index assessment using GRA8B

The micro-titer plates were coated with 5 μg/ml of soluble GRA8B in carbonate-bicarbonate buffer at 4 °C overnight (0.1 ml of antigen was added to wells). Plates were washed with PBST three times and blocked with 0.2 ml of blocking solution at 37 °C for 1 h. 0.1 ml of sera diluted 1:6400 in PBS-milk were added in duplicate rows (row A and row B). After incubation at 37 °C for 1 h, they were washed three times with PBST. Then 200 μl of PBS was added to row A and 200 μl of PBS-urea (PBS with 6 mol/L urea) to row B. Plates were incubated at 37 °C for 20 min and then washed with PBST three times. IgG antihuman antibody conjugated with horseradish peroxidase was added at a dilution of 1/17000 in PBS (0.1 ml) and incubated for 1 h. 3,3′,5,5-TetraMethylBenzidine color development reagent (0.07 ml) was added to each well. After 10 min, the color reaction was stopped by adding 0.05 ml of 0.5 mol/L sulfuric acid and color intensity was read at 450 nm. The avidity index was calculated by dividing OD of wells washed with PBS containing urea (U+) by OD of wells washed only with PBS (U−) (AvI = OD (U+)/OD(U−)). This protocol was based on the work of Béla et al. [8].

2.6. Sequence analysis

The following programs were used to study the sequence: FPScan [28], MEMSAT3 [29] and GlobPlot 2.3 [30].

2.7. Antigenicity prediction

We built the three dimensional structure of the protein for structural epitope prediction. It was made mainly with the Modeller9v3 program [31], using BIN1 (PDB: 1mV3) and angiostatin (PDB: 1ki0A) as model proteins. Structural evaluation was done using Verify3D [32], ANOLEA [33] and Ramachandran maps [34].

The antigenic regions in our models were predicted using the AAPPred [23] and DiscoTope [24] programs, which can predict linear and conformational epitopes from sequence and obtained structural model, respectively.

2.8. 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 individual tests to discriminate between AIS and CIS, and between PIS and NIS. To compare trials, Receiver Operating Characteristic (ROC) curves [35] were developed for each discrimination in each separated test. Then, 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 developed using the GraphPad Prism 6 program. The same program was also used to define the optimal threshold value. To discriminate “AIS vs CIS” we used the mean of CIS plus X standard deviations (SD): +2 SD in IgG (1.91 to GRA8A and 1.17 to GRA8B) and +1 SD in IgA (2.57 to GRA8A and 1.03 to GRA8B) and IgM (2.75 to GRA8A and 1.64 to GRA8B). To discriminate “PIS vs NIS” we used the mean of NIS sera +3 SD (1.55 to GRA8A and 1.10 to GRA8B in IgG, 1.21 to GRA8B in IgA and 3.15 to GRA8A in IgM), except for GRA8A IgA (2.48) and GRA8B IgM (1.95), where it was +1 SD. In avidity test we used the mean of AIS +1 SD (0.61). How many times SD was added, it was determined by the program which selects “X” SD for maximum sensitivity and specificity. In AIS vs. CIS, sensitivity was defined as the proportion of AIS that had a positive reaction (AIS+/AIS · 100) and specificity was defined as the proportion of CIS that had a negative reaction (CIS-/CIS · 100). In PIS vs. NIS, sensitivity was defined as the proportion of the PIS that had a positive reaction (PIS+/PIS · 100) and specificity was defined as the proportion of NIS that had a negative reaction (NIS−/NIS · 100).

2.9. Statistical analyses

Confidence intervals (CfIs) were calculated for average OD of each sera group, with 90% confidence using GraphPad Prism 6. When the intervals did not overlap there was statistical significance that these parameters (OD averages) were different with 90% confidence.

3. Results

3.1. Cloning, expression and purification

The GRA8 sequences were successfully cloned. The PCR-amplified nucleotide sequences corresponded to the sequence of accession number AF150729 in the NCBI [36], between nucleotides +139 to +939 for GRA8T (amino acids 1 to 267), +139 to +423 for GRA8A (amino acids 1 to 95), +280 to +573 for GRA8B (amino acids 48 to 145), +415 to +714 for GRA8C (amino acids 93 to 192), +583 to +840 for GRAD (amino acids 149 to 234) and +646 to +939 for GRA8E (amino acids 170 to 267).

Successful cloning was always confirmed by sequencing of the gene inserts inside vectors, which were extracted from each constructed final strain.

Of the 6 successfully constructed clones for GRA8A and GRA8B, only the genes from the pET-32a vector were successfully expressed.

None of the 15 successfully constructed clones for GRA8C, GRA8D, GRA8E and GRA8T could be expressed.

The primary structure of GRA8A and GRA8B, considering the extra amino acids added by the expression vector, extended from 275 and 278, respectively. Fig. 1 shows polyacrylamide gels of antigens before and after being purified.

3 ml of purified antigen (with concentrations between 0.5 and 1 mg/ml) were obtained with 100 ml of induced culture of recombinant *E. coli*. Only these elutions (with those concentrations) were used for immunoassay (for example: we used only 100–2 from three elutions in Fig. 1B).

3.2. Immunoassays

3.2.1. ELISA to detect IgG

Indirect ELISA was performed to detect specific IgG antibodies against *T. gondii* using GRA8A and GRA8B antigens. 69 AIS, 69 CIS and 73 NIS were tested. The average ODs, average OD ranges with 90% confidence and bar graphs of all sera ODs for GRA8A and GRA8B are shown in Fig. 2. We could say that ODs different were obtained with statistically significant for each group of sera with both Ags. GRA8B had the biggest difference between AIS and CIS signals, as calculated using the difference from the average OD. GRA8B had less NIS and CIS signal than GRA8A. While the OD value of AIS with GRA8A was higher than GRA8B.

Through the development of ROC curves, the ability to discriminate between “AIS vs. CIS” and “PIS vs. NIS” was evaluated. Table 1 shows various parameters of comparison for each set of determinations. GRA8B always had the greatest area under it's ROC curve. For both Ags, the discrimination between AIS and CIS were of greater value than between PIS and NIS.

3.2.2. ELISA to detect IgA

Indirect ELISA to detect specific IgA antibodies against *T. gondii* was performed using GRA8A and GRA8B antigens. 59 AIS, 61 CIS and 69 NIS were tested. Table 2 details OD averages and their 90% confidence intervals. Using only the GRA8B antigen, CIS average and AIS average CfIs were not overlapped with statistical significance. GRA8A average signals were higher than the GRA8B average signals (with 90% confidence).

We evaluated the ability to discriminate between: “AIS and CIS” and “NIS and PIS” (Table 1). The area under the “AIS vs. CIS” ROC curve was greater than the area under the “PIS vs NIS” ROC curve. Also the areas under GRA8B curves were greatest.

3.2.3. ELISA to detect IgM

Indirect ELISA to detect specific IgM antibodies against *T. gondii* was performed using the GRA8A and GRA8B antigens. 45 AIS, 27 CIS and 51 NIS were tested. The average ODs and their confidence intervals are shown in Table 2. All CfIs overlapped for both antigens. GRA8A mean values were higher than GRA8B mean values, with significance statistical. Then we evaluated the ability to discriminate between: “AIS and CIS” and “PIS and NIS” (Table 1). GRA8B ROC curves had the greatest areas.

3.2.4. Avidity assay

We evaluated GRA8B performance to discriminate between 14 AIS and 14 CIS, measuring IgG antibody AvI. The use of urea in the assays always decreased signal. Table 3 shows the average AvI and average AvI intervals with 90% confidence for each group of sera. CfIs did not overlap, so it was possible to determine that the antibodies of sera from each group had different AvI, with statistical significance. The area under the ROC curve was 0.918, sensitivity was 85.71% and specificity was 100% (Table 1).

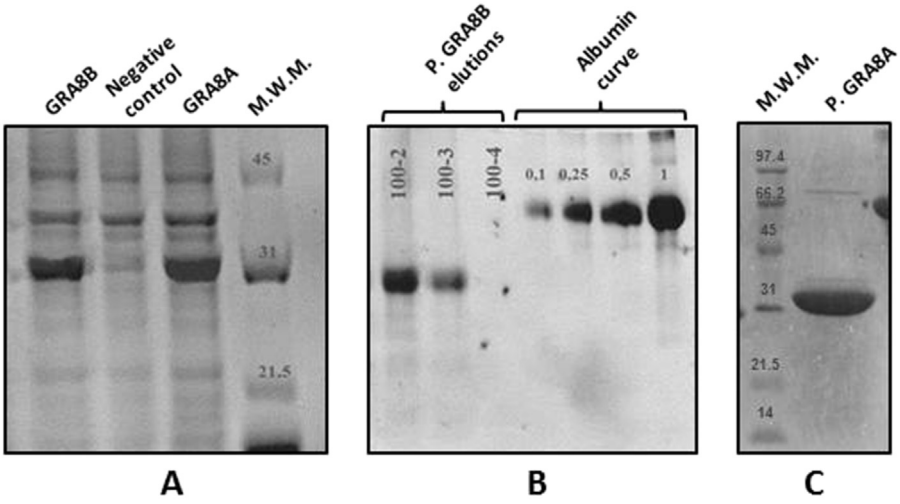

Fig. 1. Polyacrylamide gel with antigens before and after being purified. Three polyacrylamide gels are shown. Fig. A shows GRA8A, GRA8B, *E. coli* without inducing any antigen and molecular weight marker (MWM) (KDa). Fig. B has different purified GRA8B elutions (100 mmoL/L imidazole; 2nd, 3rd and 4th elution) and an albumin curve (0.1, 0.25, 0.5 and 1 mg/ml). Fig. C shows purified GRA8A and molecular weight marker.

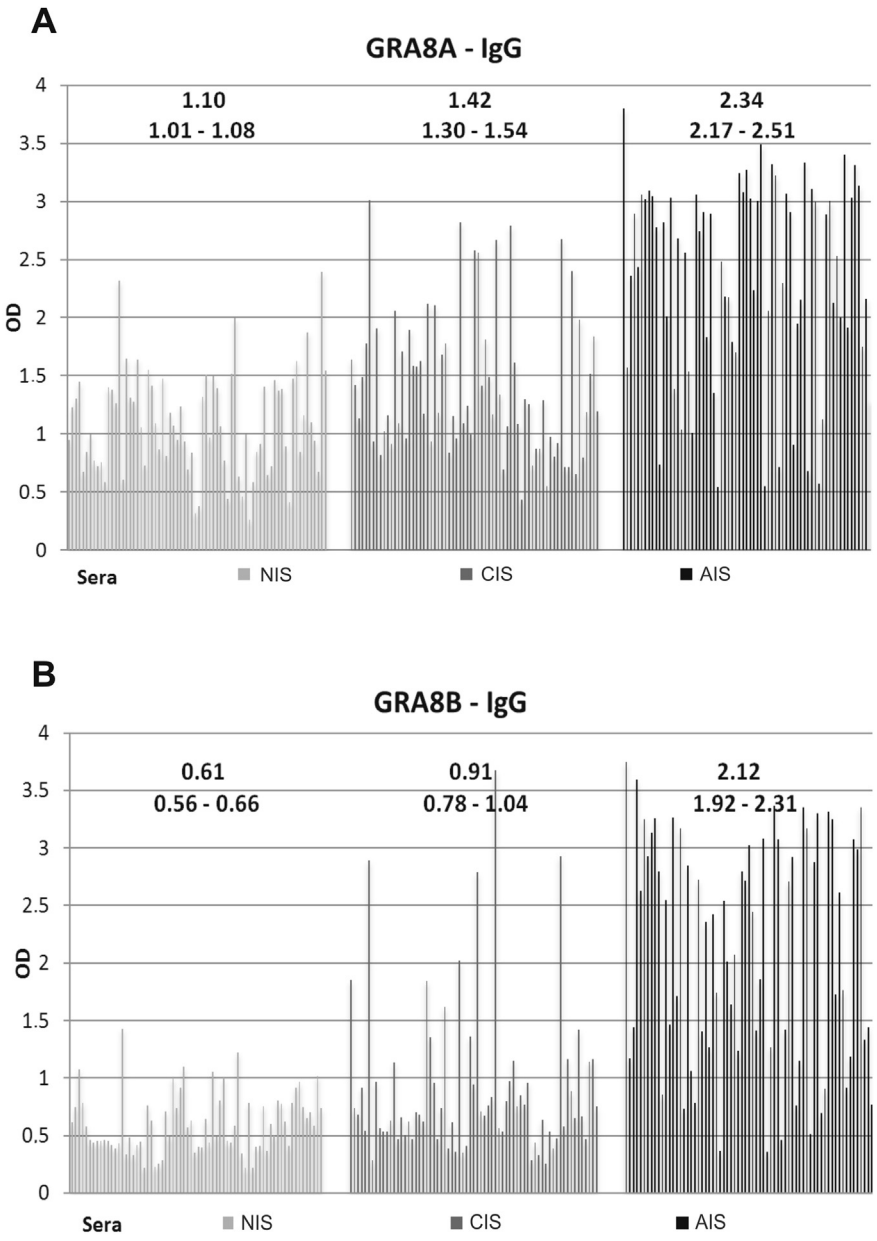

Fig. 2. Optical density in indirect ELISA to detect specific IgG using GRA8 antigens. Optical density for all sera tested by indirect ELISA to detect specific IgG using GRA8A (A) or GRA8B (B) antigens. OD averages and their ranges with 90% confidence are indicated above each sera group. Pale gray, dark gray and black bars correspond to NIS, CIS and AIS groups, respectively.

3.3. Bioinformatics

3.3.1. Sequence comparison

We compared and analyzed the regions that were successfully expressed (amino acid positions 1 to 145; GRA8A and GRA8B sequences) with those that were not (amino acid positions 146 to 267; GRA8C, GRA8D and GRA8E) with help of *in silico* tools. We used FPScan program and determinated GRA8 has an abnormally

Table 1
Parameters of assay results.

	Assay Proteins	IgG ELISA		IgA ELISA		IgM ELISA		Avidity
		GRA8A	GRA8B	GRA8A	GRA8B	GRA8A	GRA8B	GRA8B
AIS vs CIS	Sensitivity	72.5	79.7	74.6	71.2	57.8	65.2	85.7
	Specificity	82.6	84.1	45	68.3	59.3	59.3	100
	Area	0.797	0.849	0.604	0.733	0.533	0.595	0.918
PIS vs NIS	Sensitivity	58	52.2	57.1	36.1	88.9	56.2	–
	Specificity	90.3	97.1	62.3	94.2	27.1	70.6	–
	Area	0.759	0.790	0.596	0.631	0.554	0.585	–

Parameters of discrimination in avidity and indirect ELISA to IgG, IgA and IgM specific antibodies. Area: area under the ROC curve. All values are expressed in %, except areas under the curves.

Table 2
Optical densities in ELISA to detect IgA and IgM.

Assay	Sera	GRA8A		GRA8B	
		Average OD	Ranges of average OD	Average OD	Ranges of average OD
ELISA to IgA	AIS	2.70	2.83–2.56	1.43	1.59–1.27
	CIS	2.48	2.60–2.36	0.94	1.01–0.87
	NIS	2.40	2.50–2.30	0.93	1.00–0.86
ELISA to IgM	AIS	2.60	2.74–2.45	1.79	2.00–1.58
	CIS	2.66	2.87–2.46	1.52	1.73–1.30
	NIS	2.70	2.86–2.55	1.88	2.08–1.69

Average optical densities and their 90% confidence intervals of AIS, CIS and NIS in ELISA to detect IgA and IgM specific antibodies, using the GRA8A and GRA8B antigens.

Table 3
Average avidity indexes and their confidence intervals.

Sera	Average AvI	Ranges of average AvI
AIS	0.54	0.39–0.69
CIS	0.87	0.79–0.85

Average avidity indexes and their 90% confidence intervals of AIS and CIS, using GRA8B antigen.

high proportion of prolines (23.97% of prolines in the sequence) and these prolines are concentrated in a region that extends from amino acid 40 to 161, comprising nearly all of the GRA8B region (proportion of prolines in the GRA8B region is 43.44%). Also we observed there are 2 sequences composed of threonine repeats that extend for 5 and 6 amino acids with positions starting at 164 and 214, respectively. The first sequence is located in GRA8C and GRA8D, and the second in GRA8D and GRA8E. Employing the MEMSAT3 program, transmembrane region was located between amino acid positions 225 and 241. This sequence is located within GRA8D and GRA8E. Finally through GlobPlot 2.3, intrinsic disorder sequences were found throughout the entire GRA8 protein.

3.3.2. *Epitope prediction*

The following amino acids belonging to GRA8 antigenic determinants were found using antibody epitope prediction programs:

AAPpred: 44 to 78, 86 to 91 and 190 to 196.
Discotope: 34 to 38, 41, 50, 57, 61, 62, 68 to 71, 73 to 79, 81 to 83, 88 to 98, 104 to 132, 135, 142 to 144, 150, 157, 158, 191 to 195 y 197 to 200.

Fig. 3 illustrates predicted epitope amino acids and the location of GRA8A and GRA8B sequences on a representative diagram of GRA8 primary structure. In the same figure, 2 defined regions (I and II) of concentrated antigenic amino acids are shown. Region I would be present in both GRA8A and GRA8B. Region II would be exclusive to GRA8B. In the rest of the protein, there would be fewer antigenic amino acids.

4. Discussion

The GRA8 complete coding DNA was divided into 5 overlapping regions: GRA8A, GRA8B, GRA8C, GRA8D and GRA8E. These five sequences and the entire coding DNA sequence, GRA8T, were cloned and evaluated to find the most suitable antigenic regions to differentiate phases of toxoplasmosis infection. All sequences were successfully cloned in different vectors and bacteria, but only the GRA8A and GRA8B sequences could be effectively expressed.

Carey et al. [17] defined a transmembrane region between the amino acid positions 223–242 using bioinformatics, which approximately coincides with our predictions. Babaie et al. [18] reported that this region strongly reduces the expression of the whole protein in *E. coli*. This could explain why GRA8T, GRA8D and GRA8E sequences spanning the transmembrane region, could not be expressed. Though this explanation does not include GRA8C. However, reviewing the sequences that have been expressed in previous studies, we found no reports of independent expression of the central or carboxy-terminal region of GRA8 [4,6,7,13–16,37]. We were able to define the expressible sequence more precisely by our work schedule.

Regarding immunoassays, the areas under the GRA8B ROC curves were consistently larger than the areas under the GRA8A curves (Table 1), although GRA8A always had higher ODs (with 90% confidence; Fig. 2, Table 2). However, when evaluating IgG differentiation using GRA8A was also promising (Fig. 2). Given the high reactivity of GRA8A (more than once NIS had an OD double that of GRA8B; Fig. 2, Table 2), this antigen may have unspecific epitopes in the amino-terminal region [19].

Indirect ELISA detecting IgG antibodies to GRA8B had a sensitivity of 79.7% and a specificity of 84.1% in the differentiation between the acute and chronic phases (Table 1). Some studies reported work with the GRA8 antigen to detect IgG. Aubert et al. [6], Li et al. [15] and Hiszczyjska-Sawicka et al. [14] cloned sequences

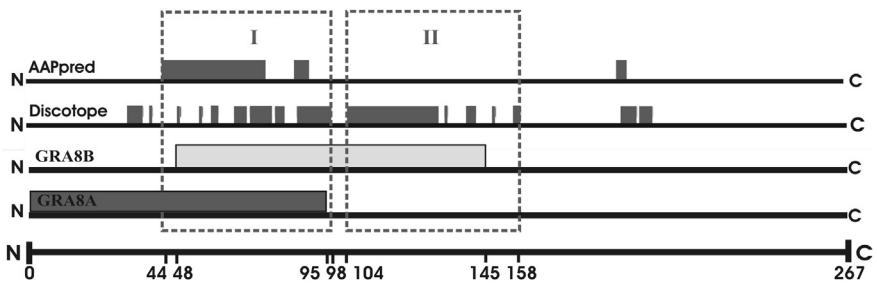

Fig. 3. GRA8 epitope amino acid location. Diagram of the complete GRA8 amino acid sequence. Predicted epitope amino acids (AAPpred and Discotope) and the location of GRA8A and GRA8B expressed sequence are illustrated in different shade of gray. Two defined regions are also shown: I and II.

most similar to GRA8B. Our results were less promising than those reported for Li et al., similar to Aubert et al. and superior those of Hiszczyjska-Sawicka et al. However, before Li et al. obtained significant results, this same research group tested a region of GRA8 that covered almost the entire length of Ag and obtained lower quality results [4]. Pfrepper et al. evaluated the largest GRA8 region by cloning a sequence that measured 1137 nucleotides (instead of 801), including a repeated carboxy-terminal region. These results were less significant than ours stage differentiation [7].

The indirect ELISA to detect IgA antibodies achieved a sensitivity of 71.2% and specificity of 68.3% (Table 1), using GRA8B to discriminate between AIS and CIS. This study is the first to report IgA detection with GRA8 by ELISA to differentiate stages.

The indirect ELISA to detect IgM was not effective in distinguishing stages of infection, even with GRA8B, whose sensitivity and specificity were 65.2% and 59.3%, respectively (Table 1). Other authors have used IgM to analyze GRA8 [6,7,13,16,37]. Of these researchers, Aubert et al. [6] obtained the results more similar to ours, and also worked with the sequence most similar to GRA8B. Lu et al. [16] and Suzuki et al. [37] obtained the best results acute phase detection, but their study used ELISA double sandwich. This procedure has some advantages over indirect ELISA, including the reduction of false positives from natural antibodies or rheumatoid factors, and the reduction of false negative results generated by competitive inhibition with specific IgG antibodies [37,38].

Of all the indirect ELISA assays, the IgG detection had the greatest areas under the ROC curves (Table 1).

Avidity assays performed better than any other type of immunoassay studied here obtaining 85.71% sensitivity and 100% specificity (section 3.2.4.). Pfrepper et al. [7] provide the only previously published information on the avidity of IgG antibodies against GRA8, while they collected sera from two people throughout the course of infection, they did not observe an increase in antibody avidity.

Finally, we compared the sequences that could be expressed to those that couldn't. Several differing characteristics were found between the two regions, including intrinsic disorder (section 3.3.1.). In *Escherichia coli*, the regions with high intrinsic disorder are prone to proteolysis [39] and the target region to proteases generally are carboxi-terminal regions [40,41], which explains out inability to obtain the sequences of the GRA8 carboxy-terminal half region (including GRA8C), a situation not only reported in the present work [13,18].

Furthermore, we identified the locations of antigenic amino acids, fortunately these were concentrated in the region we were able to express (section 3.3.2.), which decreased the importance of expressing remaining sequence. Furthermore, only GRA8B contained both of the two antigenic regions identified (I and II), explaining why this sequence more effectively differentiated the phases of infection than GRA8A.

5. Conclusion

In this paper two sequences of GRA8 protein were assayed by immunochemistry. These study offers promising results in the differentiation of infection stage by indirect ELISA, specifically using GRA8B to detect IgG and IgA and using GRA8A to detect IgG. Furthermore, very good results were obtained by measuring IgG antibody avidity with GRA8B. This work is the first to report the IgA detection by ELISA and the utility of measuring avidity with GRA8 to detect the acute phase of toxoplasmosis.

Finally, the un-expressible sequences had several distinctive characteristics. Notably, high intrinsic disorder may explain the inability to obtain the sequences of the carboxy-terminal half of the protein. Furthermore, antigenic amino acid prediction coincided with our experimental results, with regards to GRA8B versus GRA8A antigenicity. By the same method, the predicted antigenicity of the expressible sequences was greater than for the non-expressible.

Compliance with ethical standards

All of the studies reported here were performed in compliance with current applicable laws and regulations local.

Ethical approval

All procedures performed in studies involving human participants 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 human sera and the privacy rights of human subjects always were observed.

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