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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*