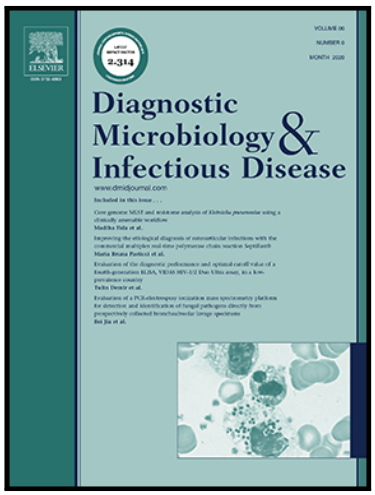

Evaluation of Toxoplasma gondii recombinant antigens for early diagnosis of congenital toxoplasmosis

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Highlights

- Recombinant antigens can be successfully used in the serodiagnosis of congenital toxoplasmosis in humans.
- Gra4-Gra7 chimeric antigen was successfully expressed and purified for diagnostic purposes
- Gra4-Gra7 chimeric antigen, together with Mic1 and Gra8, may be useful in the early detection of congenital toxoplasmosis.

Short Report

Evaluation of *Toxoplasma gondii* recombinant antigens for early diagnosis of congenital toxoplasmosis

Running title: Diagnosis of congenital toxoplasmosis

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ABSTRACT

The performance of *Toxoplasma* rGra8, rMic1, and the chimeric rGra4–Gra7 antigens for early congenital toxoplasmosis (CT) diagnosis was evaluated. Sera from CT patients showed high IgG reactivity to rMic1, rGra8, and rGra4–Gra7. The seroreactivity of samples from uninfected infants was lost within 2 months of age.

Keywords*: toxoplasmosis;* diagnosis; congenital infection; recombinant antigen; serology

Toxoplasmosis is a zoonotic infection caused by the protozoan parasite *Toxoplasma gondii*. Toxoplasmosis is widely distributed in humans, with an estimated 25% – 30 % of the world's population being infected with *T. gondii* [1]. Although infection is usually asymptomatic in immunocompetent adults, it can become clinically significant in immunosuppressed individuals and cases of congenital toxoplasmosis (CT) [1]. CT results from primary maternal *T. gondii* infection during pregnancy. The majority of infected infants are asymptomatic; it is estimated about 85% of CT cases have subclinical infection [2]. However, if CT is not treated during the first year of life, chorioretinitis can develop several years later [3]. Serologic diagnosis in newborns is complicated by the presence of maternal IgG in uninfected (UI) infants, which decline between 6 and 12 months of age [4]. The presence of anti-*T. gondii* IgM in infants is an indication of CT, but highly sensitive systems are required for its detection [5,6]. As such, there is a need for more accessible serologic tests for the early diagnosis of CT.

One way to simplify CT diagnosis is to use *T. gondii* recombinant antigens (rAgs) that detect specific IgG antibodies mainly in the acute phase. However, there have been few studies evaluating the applicability of these rAgs to the diagnosis of CT [7-9]. Meanwhile the specificity of these antigens was good, the detection sensitivity of IgG for these markers in CT was low.

In order to expand the panel of recombinant antigens (rAgs) with the potential for early diagnosis of CT, we expressed and purified rGra8, rMic1 and a chimera rGra4-rGra7 (Fig S1). PCR amplification was carried out to obtain rMic1 using cDNAs as template. The complete Mic1 (TGME49_291890) open reading frame was amplified using an upstream sense primer (Mic1-F, 5′-CACCGGGCCAGAAGCATATGGAGAAG-3′) containing additional bases (CACC) for directional cloning and a downstream antisense primer (Mic1-R, 5′-TCAAGCAGAGACGGCCGTAGG-3′). The PCR product was cloned into the pET200 Directional TOPO plasmid according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Gene sequences corresponding to rGra8 (Toxodb geneID: TGME49_254720) and rGra4-

Gra7 (TGME49_310780-TGME49_203310, respectively) were synthesized by GenScript (Piscataway, NJ, USA). They were cloned in pET-28a+ plasmid. Sera from pregnant women with acute or chronic infection or who were seronegative were used for the standardization of IgG-ELISA as described [10]. Briefly, MaxiSorp multiwell plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with rAgs at a final concentration of 5 µg/ml in coating buffer. The mean optical density (OD) of duplicate wells was taken as the final value. A serum sample was considered positive if the final OD value was higher than the cutoff, which was calculated as the mean OD of a negative sample +3 standard deviations (SD). The optimal concentration of each rAg and dilutions of serum samples and conjugate secondary antibodies were determined by the chessboard/checkboard titration method [11]. We observed that the acute-phase markers rGra4, rGra7, rGra4-Gra7 and rGra8 showed high seroreactivity in acute infection as compared to chronic infection (Fig. S2). rMic1 had a less evident acute marker seroreactive profile (Fig. S2).

We next evaluated the potential utility of the new rAgs for early diagnosis of CT. CT was established by demonstration of the presence of specific IgM in the first weeks or months of life, or persistence of positive IgG after 7 months of life. Almost all children with CT arrived at the Hospital with symptoms (Table S1). UI children were born from a mother who seroconverted during pregnancy, but their anti-*T. gondii* IgG antibodies decay during the first year of age (Table S2). Sixty serum samples from 12 CT infants, with a mean age of 2.6 ± 2.4 months, and 51 serum samples from 12 UI infants with a mean age of 1.9 ± 2.3 months were evaluated. Maternal sera obtained on the same day as the first serum sample from the infants were also included. It should be noted that as sample collection was not synchronized and the number of samples collected for each age varied. The rAG-ELISA was performed in a single-blind study without knowing the diagnosis of children. CT serum samples reactivity's against rGra8, rGra4-Gra7 and rMic1 were higher than UI serum samples throughout the follow-up (Fig. 1, Fig. S3). Moreover, nearly all of the UI samples were negative within 2 months of age against rAgs (Table

1, Table S3, Fig. S3). Anti-rAg antibody levels showed a correlation between mothers and infants and between CT and UI, at least in serum samples from the first months of age (FigS3). However, anti-rAgs IgG response rapidly decreased in UI sera with high titers of antibody from the mother. In contrast, sera from some infants with CT presented a trigger of reactivity within first months, especially for rGra4-Gra7 and rMic1 (Fig. S3). There was no differentiation in the profiles for the two CT asymptomatic cases compared to the symptomatic ones.

Our model of early CT detection is based on the fact that the humoral IgG response against a particular rAg is triggered during acute infection but rapidly declines, and is negative in UI patients. In this context, the utility of rGra8 and rGra4-Gra7 for CT diagnosis was expected, as they were reported as an acute phase marker in acute acquired toxoplasmosis [10,12]. In the present work, nearly all of the CT patients showed reactivity to rGra8 and rGra4–Gra7 throughout the first year after birth while most UI infants became negative within the first 2 months of age. The identification of rMic1 as an early CT marker was surprising. rMic1 presented a good diagnostic value, but was not specific to the acute phase [13]. In any case, an analysis with a greater number of samples at the first or second month of age is necessary to determine that rMic1 and rGra8 present better or similar value for the early diagnosis of CT than rGra4, rGra7, rSAG1 and/or rMic3.

An important aspect of this study was our analysis of rAgs using the sera of infants and their mothers, especially in the case of UI infants. When the maternal serum had high IgG titers against a rAg, seropositivity could be expected in UI infants, at least within the first month after birth. For a rAg to be useful for the early detection of CT, the IgG level against it must show a rapid decrease in UI infants. This was observed for rGra8, rMic1, and rGra4–Gra7.

In conclusion, we identified new rAgs (rGra8, rMic1, and the chimeric protein rGra4–Gra7) that can serve as diagnose markers of early CT. The fact that they are recombinant proteins facilitates the development and standardization of inexpensive diagnostic systems (for

example, ones based on a lateral flow model incorporating several markers) that are accessible to low-income populations or remote communities without access to referral centers.

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SOA (Researcher), MC (Researcher), AMA (Researcher) and EMR (Fellow) are members of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). A. Ganuza (Technician) is member of Consejo de Investigaciones Científicas (CIC). SOA (Full Professor) and MC (Assistant Professor) are also members of Universidad Nacional de San Martin.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics statements

The studies involving human participants were reviewed and approved by Hospital de Niños Ricardo Gutierrez. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

No potentially identifiable human images or data is presented in this study.

Study protocol was reviewed by Research & Teaching Committee and the Bioethics Committee of the “Ricardo Gutiérrez” Children´s Hospital, and the Secretariat Committee for Research Involving Human Subjects (DI-2014-221). It is highlighted that the remnants of blood samples

142 employed on this work were obtained in the framework of the usual clinical and biochemical
143 controls during children follow-up, preserved at -20°C with coding and adequate protection of
144 personal data.
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Author Contribution

EMR generated almost all of the recombinant proteins and performed most of the assays, analyzed the data and reviewed and edited the draft manuscript. GM obtained the serum samples, analyzed the data and reviewed and edited the draft manuscript. GB and SM obtained the serum samples and performed the conventional serologic analysis. AG generated rMic1 rAg and

performed some analyses. AMA contributed to the bioinformatic analysis and antigen selection. MC, JA, and SOA formulated research goals and aims, supervised the study, contributed to data analysis, and wrote the manuscript.

Legends

Fig. 1. Kinetic profile of the IgG humoral reactivity average against rAgs in infants with CT or UI infants. Serum samples were analyzed for rAgs by IgG-ELISA (see Tables S1 and S2). Follow-up samples from 12 infants with CT (black) and 12 UI infants with maternal anti-*T. gondii* antibodies (red) were analyzed. Data represent mean ± standard deviation of the mean. Cutoff value was determined from the average of 11 negative sera by cELISA from UI group +3 standard deviations. O.D. obtained by each serum sample was relativized with respect to the cutoff determined in the different assays.

Table 1. Serum samples seropositivity within 2 months of age[1]

	cELISA +	rGra8 +	rGra4-Gra7 +	rMic1 +
UI	12/12	0/13	3/12	1/13
CT	12/12	9/11*	10/12	10/12

[1]Positive data beyond two months was included as positive within two months of age.
*One serum sample (1791) was ruled out of the analysis because the first serum sample collected was at 13 months of age, and the rGra8 IgG ELISA resulted negative, preventing knowing the age of the first negative sample.

Author Contribution

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