

Research Article

A Recombinant Antigen-Based Enzyme Immunoassay Using Recombinant ROP2 (ELISA-Rrop2) for the Diagnosis of Congenital Toxoplasmosis

Jaqueline Dario Capobiango^{1*}, Sthefany Pagliari², Aline Kuhn Sbruzzi Pasquali², Beatriz Ninob², Fernanda Pinto Ferreira³, Thaís Cabral Monica², Italmar Teodorico Navarro³, João Luis Garcia³, Regina Mitsuka-Breganó⁴, and Edna Maria Vissoci Reiche⁵

¹Department of Pediatrics and Pediatric Surgery, State University of Londrina, Brazil

²Post-graduate Program in Animal Sciences, State University of Londrina, Brazil

³Department of Preventive Veterinary Medicine, State University of Londrina, Brazil

⁴Department of Pathological Sciences, State University of Londrina, Brazil

⁵Department of Pathology, State University of Londrina, Brazil

*Corresponding author

Jaqueline Dario Capobiango, Department of Pediatrics and Pediatric Surgery, The Science Center for Health, Universidade Estadual de Londrina, Londrina, Paraná, Brazil, Av. Robert Koch, 60, Vila Operária, CEP 86038-440, Tel: 55-43-3371-2353; Fax: 55-43-3375-1000; Email: jaquedc@uel.br

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Abstract

The diagnosis of congenital toxoplasmosis is difficult because the majority of infected children have negative results for anti-*Toxoplasma gondii* IgM. We evaluated an enzyme immunoassay (ELISA) with the recombinant ROP2 antigen (ELISA-rROP2) of *T. gondii* for detecting IgG antibodies. The tachyzoite RH strain was used for DNA extraction and incorporation into an *Escherichia coli* (*E. coli*) vector for rROP2 protein expression. Polystyrene microplates were sensitized with the rROP2 protein, and serum samples from 27 children with suspected congenital toxoplasmosis were tested. Nine (33.3%) children had a confirmed diagnosis of congenital toxoplasmosis, and in 18 cases (66.7%), the condition was ruled out. The test showed a sensitivity of 44.4% [95% confidence interval (CI) 13.7 - 78.8], a specificity of 55.6% (95% CI 30.8 - 78.5), a positive predictive value of 33.3% (95% CI 10.0 - 65.1), and a negative predictive value of 66.7% (95% CI 38.4 - 88.2).

Conclusion: Further studies should be performed with ELISA-rROP2 method for the detection of anti-*T. gondii* IgG for diagnosis of congenital toxoplasmosis.

INTRODUCTION

The diagnosis of congenital toxoplasmosis is difficult because many children do not present symptoms at birth and the sensitivity of anti-*Toxoplasma gondii* (*T. gondii*) IgM has been reported to be between 50% and 75% in children who have the disease [1-3].

In addition, it is difficult to interpret the results obtained with serological tests, such as indirect immunofluorescence (IIF), indirect enzyme-linked immunoassays (ELISA), and chemiluminescence microparticle immunoassays for (CMIA) for the detection of anti-*T. gondii* IgG, and with capture ELISA for the detection of anti-*T. gondii* IgM. Thus, the diagnosis is defined by repeated serological tests as well as the evaluation of sequential samples obtained at weekly time intervals, both of which lead to a delay in the diagnosis of congenital infection. Moreover, there is the need to use various methods to search for the different classes of antibodies against the parasite. In this context, the use of specific antigens derived from *T. gondii* can be an important tool for the diagnosis of congenital infection [4].

T. gondii belongs to the Apicomplexa family of protozoa, which includes *Plasmodium* sp., *Cryptosporidium* sp., *Neospora caninum*, and *Eimeria* sp. All of these species have an apical complex, which includes secretory organelles, micronemes, rhoptries (ROP) and dense granules (GRA) that are involved in the invasion of the host cell by *T. gondii* [5].

Rhoptries 2, ROP4 and ROP7 are the main proteins in the ROP family and are highly specific to *T. gondii*. Both ROP2 and ROP4 are expressed in tachyzoites, bradyzoites and sporozoites; therefore, they are present in various stages of infection [5].

The objective of this study was to evaluate the diagnostic potential of using an ELISA with the recombinant ROP2 antigen (ELISA-rROP2) of *T. gondii*, for the detection of IgG antibodies in serum from children with suspected congenital toxoplasmosis.

MATERIALS AND METHODS

We obtained serum samples from children suspected to have congenital toxoplasmosis from a serum bank at the

"Health Surveillance Program for Gestational and Congenital Toxoplasmosis" in the Londrina macroregion, Paraná State. The Health Department of Londrina and the Clinical Analysis Laboratory of the University Hospital of Londrina State University provided a total of 128 samples from children with suspected congenital toxoplasmosis treated in the city of Londrina from January 2011 to December 2013. The samples were stored in a freezer at -20°C until use. This study was approved by Ethical Committee of the State University of Londrina, and parental informed consent was obtained in all cases prior to the study.

It was possible to diagnose congenital *T. gondii* infection in 27 children based on medical records and on the following clinical criteria [3,6-8]: 1) sero positivity for anti-*T. gondii* IgM before six months of age; 2) persistent sero positivity for anti-*T. gondii* IgG after 12 months of life and during the follow-up, regardless of the presence of signs or symptoms of the disease; and 3) the presence of signs and/or symptoms suggestive of congenital toxoplasmosis, such as chorioretinitis, calcification of the brain and/or hydrocephalus in patients whose mothers were sero positive for anti-*T. gondii* IgG, and after exclusion of other possible infections, such as syphilis, cytomegalovirus, and rubella.

Serological screening was performed to detect anti-*T. gondii* IgG by IIF (Biolab-Merieux, Immunoblot®, Jacarépaguá, Rio de Janeiro, Brazil). The patients with evidence of anti-*T. gondii* IgG $\geq 1:16$ by IIF were considered to have been exposed to the parasite, and those with results $< 1:16$ were considered to be susceptible [9]. IgG antibodies and anti-*T. gondii* IgM antibodies were determined by CMIA (Architect®, System Abbott, Wiesbaden, Germany), and the titers were expressed in IU/mL (for IgG) and as positive or negative (for IgM).

Swiss Webster mice were inoculated intraperitoneally with a suspension of live tachyzoites of the RH strain of *T. gondii*. Subsequently, the samples from the peritoneal wash were centrifuged, and the sediment was standardized at 10^9 tachyzoites/mL by counting in a Neubauer chamber [10]. The RH strain tachyzoites were used for DNA extraction and incorporation into an *Escherichia coli* (*E. coli*) vector for rROP2 protein expression as described in previous studies [11,12]. Briefly, 1,103 base pairs (bp) from the segment of nucleotides 1,022 to 2,125, fragment 196-561 [13,14] of the *ROP2* gene (GenBank Accession No. Z36906), were expressed in *E. coli*. The resulting rROP2 protein was evaluated by polyacrylamide gel electrophoresis with dodecyl sodium sulfate (SDS-PAGE) and was found to have a molecular weight of 47 kDa. The protein concentration was determined by the Lowry method [15], and samples were diluted to 2.5 $\mu\text{g}/\text{mL}$.

Polystyrene microplates were sensitized to the rROP2 protein [11]. The protein was added to 0.1 M carbonate/bicarbonate buffer (pH 9.6) at a concentration of 2.5 $\mu\text{g}/\text{mL}$ of protein, and 100 μL of the mixture were added into each well of the microplates and subsequently incubated at 4°C overnight. The microplates were washed with phosphate-buffered saline with Tween (PBS-T, 0.05% Tween 20, pH 7.4). Subsequently, the wells were blocked with 200 μL of blocking buffer (PBS-T) with 8% powdered skim milk for 1 h. After washing the microplates, aliquots of serum were diluted 1:200 in a wash buffer containing 5% powdered skim milk. A 100 μL aliquot of the positive and

negative controls, as well as the serum samples, was added to the microplate wells, which were incubated at 37°C for 1 h. After further washing the microplates, 100 μL of combined human anti-IgG antibody labeled with peroxidase were used to detect the presence of antibodies bound to the antigen adsorbed on the microplate. After further washing, the reaction was revealed by the addition of 100 μL of a chromogenic substrate (40 mg ortho-phenylenediamine, Sigma, St Louis, MO, USA) in 100 mL of 0.1 M pH 6.0 citrate phosphate buffer and 40 μL of H_2O_2 . After 15 min, the reaction was stopped by the addition of 50 μL of 1 N HCl. The optical density (OD) was determined using a spectrophotometer (Spectra II Microplate Reader, Tecan (Tecan Group, Mannedorf, Switzerland) at a wavelength of 490 nm. The values in the negative controls were used to calculate the cut-off, which was set as the average DO obtained in negative controls plus two standard deviations (SD) [12,16]. We considered that serum samples with $\text{DO} \geq 1.0$ for the ratio of the DO sample/DO cut-off to be positive for rROP2 IgG [16].

The specificity of the ELISA-rROP2 method was determined using 21 serum samples from individuals sero negative for toxoplasmosis (negative for anti-*T. gondii* IgG and IgM) and who showed sero reactivity for other pathogens or antigens, such as *Treponema pallidum* (n=2), *Trypanosoma cruzi* (n=4), *Leishmania* spp. (n=1), *Paracoccidioides brasiliensis* (n=2), and human immunodeficiency virus type 1 (HIV-1, n=4). We also evaluated serum samples from patients with reactivity to serological markers of autoimmunity, such as antinuclear antibodies (n=4) and anti-DNA double strand antibodies (n=4).

Databases were created using Microsoft Excel 2007 (Microsoft Corporation, Redmond, USA) and in Epi Program Info 3.4 (CDC, Atlanta, USA). The statistical analysis was performed using the GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA) and SPSS 15.0 (IBM SPSS Software, Inc., Armonk, USA) software. Categorical variables were expressed as absolute numbers (n) and percentages (%) and were analyzed by the Chi-square or Fisher's exact test, when appropriate. Continuous variables were expressed as medians and inter quartile ranges (IQR) of 25%-75%. For the comparison of the levels of antibodies among groups of patients, we used the Mann-Whitney test. The 95% confidence intervals (CIs) were also determined. Values of $p < 0.05$ were considered statistically significant.

RESULTS

We included serum samples from 27 children, 9 (33.3%) with congenital toxoplasmosis and 18 (66.7%) for whom a diagnosis of congenital toxoplasmosis had been ruled out. The age of the children at the time of sample collection ranged from 2 days to 22 months, with a median of 5 months, and an IQR (25 and 75%) of 1 month and 12 months, respectively.

Seven children (77.8%) showed clinical manifestations of congenital toxoplasmosis. The diagnosis was made based on the presence of anti-*T. gondii* IgM in four (44.5%) of the nine children diagnosed with congenital infection. Positivity for the ELISA-rROP2 anti-*T. gondii* IgG was observed in 4 (44.4%) samples from children with congenital toxoplasmosis and in 8 (44.4%) samples from uninfected children ($p = 1.000$). The test therefore showed a sensitivity of 44.4% (95% CI 13.7 - 78.8), a specificity of 55.6%

(95% CI 30.8 - 78.5), a positive predictive value of 33.3% (95% CI 10.0 - 65.1), and a negative predictive value of 66.7% (95% CI 38.4 - 88.2).

Among the 21 samples from patients with other diseases, all of them showed negative results by the ELISA-rROP2 method. One patient with congenital toxoplasmosis had negative results by IIF and CMIA, but the ELISA-rROP2 was positive. In the evaluation of sequential samples from this child, it was possible to detect the persistence of negative results by IIF and CMIA as well as the persistence of reactivity for the ELISA rROP2 method.

The titers of anti-*T. gondii* IgG antibodies obtained using the ELISA rROP2 did not show significant difference between the infected and uninfected children ($p = 0.5203$) (Figure 1). However, when we compared the values of anti-*T. gondii* IgG obtained with IIF and CMIA between the two groups, we observed that there were significant differences among infected and uninfected children ($p < 0.0001$ and $p = 0.0002$, respectively) (Figures 2 and 3).

DISCUSSION

The detection of IgG antibodies against rROP2 of *T. gondii* in serum samples from patients with acquired toxoplasmosis showed a sensitivity of 90% [17,18]. This method has been used for the diagnosis of recent infection in pregnant women, with a sensitivity ranging from 87% to 97.6% [13,19].

Altcheh et al., analyzed the value of recombinant antigens ROP2, GRA4 and GRA7 in 23 infants with congenital toxoplasmosis and in 36 uninfected infants, and found greater positivity to rROP2 in the infected patients (91% vs 67%, $p = 0.05$) [4]. In the present study, both groups showed an equal frequency of sero positivity by the ELISA-rROP2 method. However, the specificity

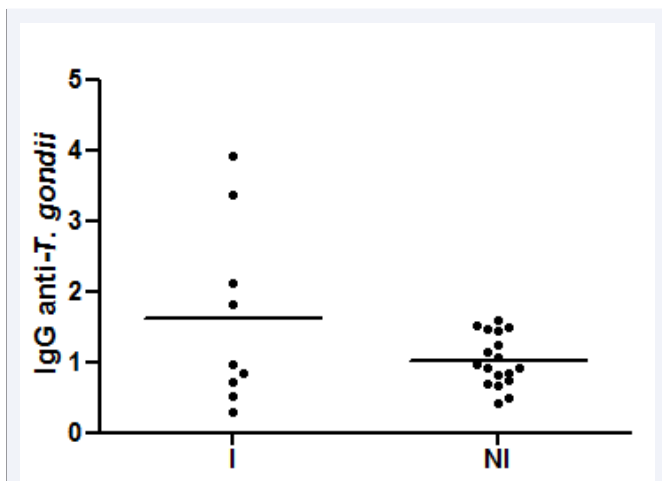


Figure 1 Levels of IgG antibodies anti-Toxoplasma gondii obtained with enzyme immunoassay with recombinant Roptria 2 antigen (ELISA-rROP2) in samples of children with congenital toxoplasmosis (I) and without congenital toxoplasmosis (NI). The values are expressed in index of ELISA ≥ 1.0 obtained with the mean optical density (OD) of the sample/the mean OD of the negative controls plus two standard deviations. The bars represent the median of titles obtained for each group of children evaluated (Mann-Whitney test, $p = 0.5203$).

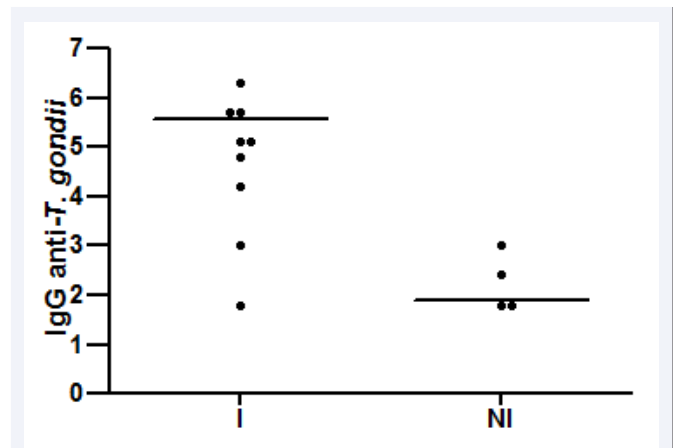


Figure 2 IgG anti-Toxoplasma gondii antibody titers obtained with indirect immunofluorescence (IIF) in samples from children with congenital toxoplasmosis (I) and without congenital toxoplasmosis (NI). The semi-quantitative values of IgG are expressed in logarithm of 10 (\log_{10}); 14 samples showed values above the limit of the axis. The bars represent the median of titers obtained for each group of children evaluated (Mann-Whitney test, $p < 0.0001$).

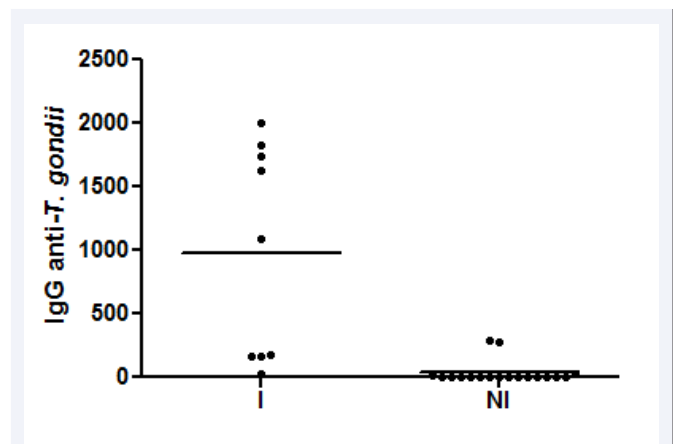


Figure 3 IgG anti-Toxoplasma gondii antibody titers of obtained with chemiluminescence microparticles immunoassay (CMIA) in samples of children with congenital toxoplasmosis (I) and without congenital toxoplasmosis (NI). The values are expressed in IU/mL. The bars represent the median of titers obtained for each group of children evaluated (Mann-Whitney test, $p = 0.0002$).

for the ELISA-rROP2 method in our study was higher than the 33.0% reported by Altcheh et al. [4].

Martin et al. demonstrated 100.0% specificity for an ELISA-rROP2 method in patients sero negative for toxoplasmosis but with no reactivity indicating other diseases [13]. In the present study, the results are consistent with these previous results regarding samples from patients with other diseases; however, among samples from 14 uninfected children with negative results in the IIF, three (21.4%) showed positive results by CMIA and six (42.9%) showed positivity by the ELISA-rROP2. These results may be explained by individual differences in the response to different antigens of *T. gondii*, which could lead to the non-recognition of the antigens presented in commercial

tests. Another explanation could be that the infection occurred late during pregnancy, so there was insufficient time for the production of antibodies by the child [20].

The low sensitivity found for the ELISA-rROP2 method may also be explained by the poor immune response to a single antigen because there may not be recognition of epitopes present in this antigen. This has been reported in adults, and it was suggested that targeting a combination of recombinant antigens may lead to better results [16,17,21]. However, even in children determined to be sero negative by conventional methods, the ELISA-rROP2 test showed positivity, which indicate a false positive result. One explanation for this finding may be the persistence of maternal IgG anti-*T. gondii* antibodies, which would recognize the rROP2 protein used in the ELISA.

The false negative results obtained among children with congenital toxoplasmosis could be explained by maternal treatment and/or early treatment of the child, reducing the response to antigens of *T. gondii*. This phenomenon has previously been described for commercial tests [3,22,23].

As infected children showed reactivity to ELISA-rROP2 only after two months of life, it is possible that this method cannot be used for the early diagnosis of infections that occur close to childbirth. Van Gelder et al. also reported that anti-rROP2 IgG antibodies may not be detected in adults in the very early stages of infection [17]. On the other hand, other authors have described that the ROP2 antigen can be expressed in various stages of infection [13,24]. Buffolano et al. reported that using other recombinant antigens in combination (SAG1, MIC, MIC2, MIC4, and AMA1) in an ELISA improved the diagnosis of congenital toxoplasmosis [24].

Altcheh et al., compared the kinetics of anti-*T. gondii* IgG antibodies in samples from uninfected infants and observed a significant difference in the age of the children with negative sero conversion of IgG using commercial tests and an ELISA-rROP2 method (5.8 months vs 3.7 months, respectively) [4]. Therefore, further studies are needed to define the kinetics of these antibodies in children and their relationship with the timing of fetal infection.

The anti-*T. gondii* IgG antibody titers detected by IIF and CMIA were higher in samples from children with congenital toxoplasmosis than in those without congenital infection. Thus, conventional IIF and CMIA methods remain the best choice for the serological monitoring of children with suspected congenital infection.

There are some limitations of the present study that must be considered, such as the evaluation of a small number of samples from infected children; moreover, we did not evaluate the IgM antibodies against the rROP2 antigen in both infected and uninfected children, which could increase the sensitivity of this method for the diagnosis of congenital infection.

CONCLUSION

Future work should benefit greatly by using our data that provide support for research ELISA rROP2 method for the detection of anti-*T. gondii* IgG antibodies, beyond that, other coating concentrations and different blocking solutions might

improve the assay. Furthermore, studies of recombinant antigens of *T. gondii*, including ROP2, and simultaneous comparison of the levels of antibodies against various antigens in the serum of the mother and the child must be carried out for a better evaluation of the effectiveness of ELISA with recombinant proteins for the diagnosis of congenital toxoplasmosis.

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