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## Original Article

# Design and Optimization of an IgG Avidity ELISA Based on *Toxoplasma gondii* Lysate Antigen for Differentiating Acute and Chronic Infections in Pregnant Women

**Mostafa Tork [1,2], Shahabeddin Sarvi [1,3], Hossein Asgarian-Omran [4,5], Mitra Sadeghi [1,3], Bahare Basirpour [1,2], Eisa Nazar [6,7], *Seyed Abdollah Hosseini [1,3], *Ahmad Daryani [1,8], Sargis A. Aghayan [8,9]**

1. *Toxoplasmosis Research Center, Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran*
2. *Student Research Committee, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*
3. *Department of Parasitology and Mycology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*
4. *Department of Immunology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*
5. *Gastrointestinal Cancer Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*
6. *Orthopedic Research Center, Mazandaran University of Medical Sciences, Sari, Iran*
7. *Psychiatry and Behavioral Sciences Research Center, Addiction Institute, Mazandaran University of Medical Sciences, Sari, Iran*
8. *Laboratory of Molecular Parasitology, Scientific Center of Zoology and Hydroecology, NASRA, 7P. Sevak st. Yerevan 0014, Armenia*
9. *Laboratory of Zoology, Research Institute of Biology, Yerevan State University, Yerevan, 0025, Republic of Armenia*

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***Correspondence**
**Emails:**
daryanii@yahoo.com,
hosseini4030@gmail.com

***Abstract***

***Background:*** Toxoplasmosis is a significant global health concern. Definitive diagnosis of toxoplasmosis is a serious challenge due to false positive serological results. This study focuses on optimization and standardization of an in-house IgG avidity ELISA for *Toxoplasma gondii* using freshly prepared *Toxoplasma* lysate antigen (TLA).

***Methods:*** Samples were collected during routine pregnancy screening. Following tachyzoite purification, TLA concentration was determined by the Bradford assay at 20 µg/mL. The indirect ELISA assay was then performed with a urea treatment step (6 M). Using 75 serum samples classified as acute or chronic toxoplasmosis, the IgG avidity test was performed by ELISA in two rows of microplates with 6 M urea and PBS, and then the avidity index was calculated.

***Results:*** After calculating the cutoff values, the sensitivity and specificity of the in-house *Toxoplasma* IgG avidity kit were 92% (95% CI: 74–99%) and 100% (95% CI: 86–100%), respectively, with an area under the ROC curve (AUC) of 0.977 (95% CI: 0.951–1.000).

***Conclusion:*** The results of the IgG avidity test designed in this study along with standardizing the method and interpreting the results showed the promising performance of this assay to differentiate the acute from the chronic stages of toxoplasmosis.

## Introduction

*Toxoplasma gondii* is a notable parasite of the phylum Apicomplexa. This protozoan parasite has the unique ability to infect a broad spectrum of warm-blooded vertebrates, including humans and other animals. *T. gondii* has a complex life cycle and can cause significant health issues, particularly in immunocompromised individuals and pregnant women (1-3). Humans can become infected with *T. gondii* through various routes (4). In general, *T. gondii* pathogenicity is usually asymptomatic in immunocompetent individuals. However, the consequences of toxoplasmosis infection can lead to significant damage in fetuses and individuals with immunodeficiency [$HIV^+$/AIDS, cancer, or organ transplant patients] (5, 6). If diagnosed late or incorrectly, it can cause irreparable damage and may result in severe fetal complications (7, 8). Different conventional approaches, such as imaging, immunological, and molecular techniques have been employed to diagnose toxoplasmosis, with immunological methods being the most widely used (9).

Nowadays, routine toxoplasmosis screening approaches in laboratories are the examination of anti-*T. gondii* IgM and IgG antibodies levels by ELISA technique. A true IgG+ serology result indicates previous infection, but is not applicable in determining the period of infection. Also, IgM+ values can indicate acute toxoplasmosis. Although this antibody with a lifespan of 9-12 months can even remain stable for years after an acute infection [false positive] and therefore it is not equivalent to parasitemia and is not a suitable indicator to start treatment (10, 11).

A complementary method to help distinguish between recent and past infections is the measurement of IgG avidity, which was first described by Hedman et al in Finland during the 1980s (12). Avidity refers to the aggregation power of polyclonal IgG molecules to bind to multiple epitopes of proteins. Following an infection, the functional binding affinity of IgG gradually intensifies (13).

After infection by *T. gondii*, IgG antibodies gradually reach the high-avidity region, which indicates chronic infection (13). This method not only confirms the presence of anti-*Toxoplasma* antibodies but also estimates the approximate timing (last 3 to 4 months) of the mother's infection (14-16).

The availability of fresh RH-strain tachyzoites, together with the simplicity and cost-effectiveness of the method, encouraged us to investigate the use of *Toxoplasma gondii* lysate antigen (TLA) for an avidity assay. Therefore, this study aimed to optimize and standardize an in-house IgG avidity ELISA using freshly prepared TLA derived from purified RH-strain tachyzoites. The assay was analytically validated by determining the optimal antigen coating concentration, urea treatment conditions, and cut-off value, and by comparing its performance with a commercial reference kit.

## Materials and Methods

### *Ethical approval*

This study followed all the ethical issues and was registered with the code: (IR.MAZUMS.AEC.1401.026) in the Ethics Committee of Mazandaran University of Medical Sciences.

### *Sample size and participants*

Samples were collected during routine pregnancy screening for toxoplasmosis (Jan 2022 to Sep 2023) from Tehran Province, Iran, and were categorized based on ELISA kit results (NovaLisa®: Avidity *T. gondii* IgG, Germany, catalog No. 0483). Overall, 100 serum samples were obtained, including:

- 50 samples with IgM-; IgG+; and high avidity (AI > 60), classified as chronic infection
- 25 samples with IgM+; IgG+; and low avidity (AI < 45), classified as acute infection

- 25 samples from individuals with autoimmune disorders (RF/ANA+) who were negative for *T. gondii* IgG and IgM, included to assess potential cross-reactivity

### *Inoculation of T. gondii RH strain tachyzoites in mice*

Ten female BALB/c mice (8–10 weeks old, 18–20 g) were obtained from the Animal Research Center of Mazandaran University of Medical Sciences. Mice were intraperitoneally inoculated with fresh *T. gondii* RH strain tachyzoites ($2 \times 10^4$). After 3–4 days, symptomatic mice were euthanized, and peritoneal fluid was collected using sterile PBS containing Pen-Strep and aspirated through a 20-gauge needle 10–20 times (17).

### *Purification of tachyzoites*

The removed peritoneum aspirate containing tachyzoites was transferred to a sterile tube and underwent centrifugation at 65 × g for 5 min. The resulting supernatant was isolated and centrifuged again at 440 × g for 20 min. Following the removal of the supernatant, normal saline was introduced to the precipitate, and the mixture was centrifuged at 260 × g for 10 min. The final step involved discarding the supernatant, performing another centrifugation at 440 × g for 20 min, and adding sterile distilled water to the pellet containing the tachyzoites (18).

### *Preparation of TLA and protein quantification*

The tachyzoites obtained in the previous step were sonicated (20× 10 s at 30 s intervals) in the vicinity of ice. The solution underwent centrifugation at 5000 × g for duration of 30 min, the supernatant was removed and the concentration of the protein was measured by the Bradford assay with a commercial kit (Nadford, Urmia, Iran; cat# NS-15074).

### *Preparation of suitable dilution of antigen for coating*

A volume of 100 µL of 2.5, 5, 10, 20, and 40 µg/ml of TLA in coating buffer [carbonate buffer, 0.05 M, pH 9.6] were added into the 96-well microplate [sterilized; SPL, South Korea] and it was kept overnight at 4 °C. Then it was washed three times with Tris buffered saline with 0.05% Tween 20 (TBST) and 200 µL of blocking solution (1% bovine serum albumin (BSA) with 0.5% Triton X-100 in PBS; Triton X-100 was included to enhance antigen coating permeability and reduce non-specific binding, improving the signal-to-noise ratio, as reported in previous ELISA protocols for lysate antigens (19, 20) was added and incubated for 2 h at room temperature. A volume of 100 µL of Standard D (200 IU/mL) of a commercial kit (NovaLisa®: Avidity *T. gondii* IgG ELISA) was added to the wells and incubated at 37 °C for 45 min. A volume of 100 µL of conjugated enzyme (anti-human IgG peroxidase, Pishtaz Teb, Tehran, Iran) was coated into all the wells and placed at 37 °C for 30 min. The wells were washed 3 times and after adding 100 µL of chromogen/substrate, O-phenylenediamine dihydrochloride (OPD) (Pishtaz Teb, Tehran, Iran), the plate was kept for 30 min in the dark at room temperature. After coating 100 µL of 0.1 M sulfuric acid [stop solution] into all wells, the optical density (OD) of the microplates (450 nm with a 630 nm reference filter) was read by Elisa Reader (BioTek, LX800, USA). The concentration yielding the highest OD (Fig. 1) was determined as the optimal antigen coating concentration (20 µg/mL).

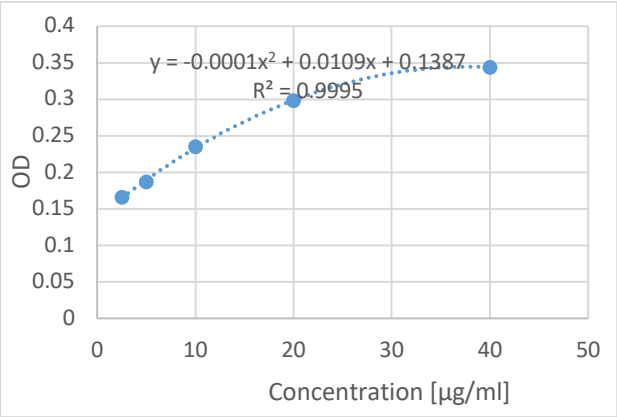


**Fig. 1:** Determining the best antigen concentration for ELISA microplate coating

### *Evaluation of the in-house IgG ELISA avidity*

Two parallel tests (row A and row B) were conducted on the plate after coating the TLA (20 µg/mL). Following a 45 min incubation and three washes, 200 µL of blocking solution was added to the wells, followed by the addition of human serum diluted 1:100 in the blocking solution. The plate was then incubated for 30 min at 37 °C. One of the wells was treated with 100 µL of 6 M urea solution, while the other wells received the washing solution. Both wells were incubated at 37 °C for 10 min. After another round of washing, 100 µL of conjugate was coated into to the wells, followed by 30 min incubation at room temperature. The plate underwent three washes, and 100 µL of chromogen/substrate was added to each well. The plate was then kept in the dark for 15 min before being read by an ELISA reader using a 450 nm filter and a reference filter of 630 nm. The avidity index (AI) was calculated as a percentage: AI % = (Absorbance (Abs) washed with urea /Abs washed with PBST) × 100.

### *Cut-off calculation*

After calculating the optimal cut-point value compared to the commercial kit (NovaLisa®) the borderline avidity obtained between cut-off values of ±10% (21). As it is seen in Table 1, the optimal cut-point for three indices was obtained 57, and the range of values for them was 51.30 – 62.70 be called borderline. The results were categorized into three levels: low, high, and intermediate. These categories have been applied in previous studies (16, 22, 23).

**Table 1:** Optimal cut-off points for IgG avidity ELISA based on Youden index, accuracy, sensitivity, and specificity (n = 75)

| Method | Optimal cut-point | Category | Avidity index (AI %) | | Index value | Accuracy | AUC (95% CI) | Spe, % (95% CI) | Sen, % (95% CI) |
|---|---|---|---|---|---|---|---|---|---|
| | | | High | Low | | | | | |
| **Youden index** | 57 | < 51.30: L<br>51.30 - 62.70: B<br>> 62.70: H | H: 40<br>B: 10<br>L: 0 | H: 2<br>B: 0<br>L: 23 | 0.92 | 0.973 | 0.977 (0.951–1.000) | 100 (86–100) | 92 (74–99) |
| **Maximum accuracy** | 57 | < 51.30: L<br>51.30-62.70: B<br>> 62.70: H | H: 40<br>B: 10<br>L: 0 | H: 2<br>B: 0<br>L: 23 | 0.973 | 0.973 | 0.977 (0.951–1.000) | 100 (86–100) | 92 (74–99) |
| **Sensitivity + Specificity** | 57 | < 51.30: L<br>51.30-62.70: B<br>> 62.70: H | H: 40<br>B: 10<br>L: 0 | H: 2<br>B: 0<br>L: 23 | 1.92 | 0.973 | 0.977 (0.951–1.000) | 100 (86–100) | 92 (74–99) |

## Results

### *Acute and chronic toxoplasmosis group*

Initial testing using the TLA followed with suitable antibodies detection performance in a panel of low and high IgG avidity in sera. In the final step, among 25 serum samples suspected to be acute toxoplasmosis (IgM+, low avidity), 23 samples had low avidity (92%) and 2 (8%) had an avidity index higher than 62.7. All serum samples were positive for anti-*Toxoplasma* IgG and IgM antibodies. Moreover, 40 (80%) of 50 serum samples were suspected to be chronic toxoplasmosis (IgG+, high avidity) had an avidity index higher than 62.7%, and 10 (20%) of them were between 51.3 - 62.7% (Table 1). No cross-reactivity was detected among the collected samples, and all measurements were performed in duplicate using a commercial assay kit.

### *Reproducibility of the in-house IgG avidity ELISA*

The reproducibility of the in-house IgG avidity ELISA was evaluated by determining intra-assay and inter-assay precision using three representative low-avidity and three representative high-avidity serum samples. For intra-assay precision, each sample was tested in eight replicates within a single run. The coefficients of variation (CVs) ranged from 4.1% to 5.8% for the evaluated samples. For inter-assay precision, the same samples were tested in eight independent runs, yielding CV values ranging from 7.5% to 9.5% (Tables 2, 3).

**Table 2:** Intra-assay precision of the in-house *Toxoplasma* IgG avidity ELISA

| Sample Type | Number of Samples | Replicates per Sample | CV Range (%) |
|---|---|---|---|
| Low-avidity | 3 | 8 | 5.8 |
| High-avidity | 3 | 8 | 4.1 |

**Table 3:** Inter-assay precision of the in-house *Toxoplasma* IgG avidity ELISA

| Sample Type | Number of Samples | Replicates per Sample | CV Range (%) |
|---|---|---|---|
| Low-avidity | 3 | 8 | 7.5 |
| High-avidity | 3 | 8 | 9.5 |

### *Statistical analysis*

The analysis of the distribution of the samples shows that the values of the three indices were 0.92, 0.973, and 1.92. In addition, based on Receiver Operating Characteristic (ROC) curve analysis (Fig. 2), the area under the curve (AUC), accuracy, sensitivity (Sen %), and specificity (Spe %) of these indices were reported in Table 1.

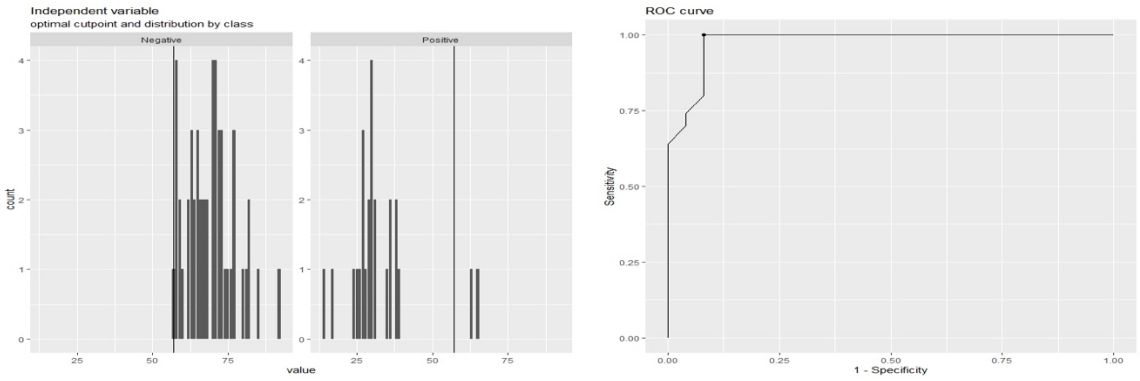


**Fig. 2:** The ROC curve and distribution plot of samples by IgG avidity ELISA test according to the calculated cut-point. The ROC curve analysis showed an AUC of 0.977 (95% CI: 0.94–1.00)

Quantitative variables were presented as means ± standard deviations, while qualitative variables were expressed as frequencies (%). For validation purposes, the commercial ELISA kit (NovaLisa®) was used as the reference standard. The in-house IgG avidity ELISA results were compared against this commercial kit.

ROC analysis, cut-off determination, and calculation of diagnostic performance indices were performed using the 75 samples classified as acute (n = 25) and chronic (n = 50) toxoplasmosis. The additional 25 RF/ANA-positive, *Toxoplasma*-seronegative samples were included exclusively for assessment of potential cross-reactivity and were not incorporated into the ROC analysis or diagnostic performance calculations. Then, the Youden index, maximum accuracy, and the sum of sensitivity and specificity were used to determine the optimal cut-off point. Statistical analyses were performed using R software (version 4.3.1), with statistical significance set at $P < 0.05$.

## Discussion

Diagnosing current and past *Toxoplasma* infections, particularly in pregnant women and immunocompromised patients with nonespecific symptoms, primarily relies on serological evaluations of *Toxoplasma*-specific IgG and IgM immunoglobulins. Due to the potential for false-positive IgM outcomes, interpreting results in individuals with low anti-*Toxoplasma* IgG titers can be challenging (24-26). Therefore, measuring IgG avidity is recommended for a more definitive diagnosis. This test works based on urea's ability to disrupt hydrogen bonds between antigens and antibodies (12). In this study, the IgG avidity assay was standardized and evaluated using serum samples and a commercial kit to differentiate between acute and chronic infections.

IgG avidity diagnostic tools primarily utilize TLA, because of their high sensitivity and specificity. The effectiveness of the ELISA method largely depends on antigen coating quality and antigen-antibody binding affinity (27). The use of TLA offers several advantages, including ease of use, rapid obtaining of results, and no need for expensive equipment. Our study demonstrated 92% sensitivity and 100% specificity, closely aligning with the findings of Teimouri et al. (21) and Ferra et al. (19). The success of our results can be attributed to factors such as the use of a standard *Toxoplasma* strain, freshness of the extracted antigen, and adherence to ELISA protocol requirements, including fresh solutions and strict control of temperature and pH.

The methods used to maintain and extract the antigen as well as the parasite strain significantly affects the results. Contamination with non-parasitic cell debris (28) or other protozoan antigens (29, 30) during antigen extraction can cause inter-assay variability (31, 32). Therefore, purification of tachyzoites from other cellular debris is essential to obtain pure antigens and measure the accuracy and diagnostic performance of the designed kit.

The RH strain, which is type 1 and the most immunogenic strain in humans, was used in this study (33). Researchers chose this strain as a reference strain for research studies due to the characteristics of the RH strain, including the absence of oocyte formation in final hosts or bradyzoites in secondary hosts (34) due to the long periods of the passage process in mice (35).

Selecting an appropriate microplate is essential for optimal antigen binding and accurate spectrophotometric readings. In this study, high-binding flat-bottomed SPL microplates were selected to enhance antigen binding. Microplate selection should consider the requirements of the ELISA reader and the characteristics of the antigen–antibody reaction (36).

Determining the optimal antigen coating concentration by titration is essential for reliable assay performance. The use of relatively

impure antigens for coating may reduce assay performance because impurity proteins can occupy binding sites on the microplate surface (37, 38). Previous studies using recombinant proteins reported coating concentrations of 2.5, 5, and 10 μg/mL (23, 19, 20); however, these concentrations are not directly comparable with the TLA used in the present study. In our study, 20 μg/mL was selected based on preliminary optimization experiments, while removal of tachyzoite-derived impurities enhanced antigen binding and assay sensitivity.

Recent studies have explored cut-off rates, yielding various values. Specifically, Ferra et al. (19) reported a cut-off point of 30%, Rahbari et al. (20) found 50%, and Teimouri et al. (21) identified 52%. Our findings indicate that the optimal cut-off is 57%, determined using three indicators: Youden index, maximum accuracy, and the sum of sensitivity and specificity.

A review of previous studies indicates that the interpretation of results is significantly influenced by the determination of the cutoff range. To accurately interpret IgG avidity ELISA results, it is essential to define ranges for low, borderline, and high avidity. While commercial kits now provide guidelines for determining these ranges, the default method or ROC analysis can also be employed. However, according to Costa et al. (39), the accurate interpretation of the test depends on the specificity of the antigen used.

In this study, ROC analysis was used to assess the discrimination ability of the in-house IgG avidity ELISA using TLA (39). The ROC curve (Fig. 2) revealed an AUC of 0.977, demonstrating high diagnostic accuracy. An AUC near 1 signifies that the data predominantly lie above the median, indicating a high true positive rate and excellent diagnostic accuracy.

IgG avidity testing provides a useful approach for distinguishing acute from chronic *T. gondii* infection and improving serological diagnosis. The in-house ELISA showed promising performance; however, its evaluation was limited by the small sample size and the lack of an independent gold standard, as sample classification relied on the commercial NovaLisa® assay. Further validation using molecular methods, clinical follow-up, and larger populations is needed to confirm its diagnostic performance and ensure assay standardization. Nevertheless, the findings support the potential application of this method, particularly in resource-limited settings.

## Conclusion

The IgG avidity ELISA developed in this study using TLA demonstrated promising potential for distinguishing acute from chronic *Toxoplasma* infections. Standardization and further validation in larger, multicenter studies are warranted to improve its diagnostic accuracy, reproducibility, and clinical applicability, particularly in vulnerable populations.

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## Conflict of interest

The authors declare that there is no conflict of interests.

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