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Original Article

The Necessity of Confirmatory Testing in Serodiagnosis of Toxoplasmosis in Iran

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Abstract

Background: *Toxoplasma* specific IgM antibodies; the common serologic marker in diagnosis of acute toxoplasmosis has its own limitations. Confirmatory testing with other markers, introduced as a complementary tool in distinguish acute and chronic infections is unusual in Iran. In the present study, we investigated the correlation between the results of IgM ELISA, IgA ELISA, and IgG avidity tests in the diagnosis of toxoplasmosis to demonstrate the necessity of confirmatory testing in serodiagnosis of infection in the country.

Methods: A total of 107 positive *Toxoplasma* IgG and IgM sera were obtained from patients referred to private laboratories and stored at -20 ℃ for futures use. Serologic tests were set up in duplicate to analyze the serum levels of IgG, IgM, IgA, and IgG avidity antibodies using commercial ELISA kits. The results were presented as semi quantitative for IgG, IgM and IgA ELISA, and Relative Avidity Index in percentage for IgG avidity test. Pearson's correlation coefficient (r_p) was applied to analyze the data.

Results: Of 107 serum samples, *T. gondii* specific IgM and IgA antibodies were positive in 67.3% and 53.3%, respectively. Besides, 29.9% of the sera displayed low avidity for IgG antibodies. The r_p was - 0.572 (*P*<0.01) between the IgG avidity and IgM ELISA, - 0.364 between the IgG avidity and IgA ELISA (*P*<0.01), and 0.564 between the IgM and IgA ELISA (*P*<0.01).

Conclusion: The study strongly highlights the necessity of confirmatory testing in differential diagnosis of acute and chronic toxoplasmosis in Iran.

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Introduction

Toxoplasma gondii (*T. gondii*) is a ubiquitous protozoan parasite that infects at least 30% of humans in most parts of Iran (1). Infections with *T. gondii* in immunocompetent subjects are most often presented as asymptomatic however approximately 10 to 20% of the acquired infections are associated with clinical syndromes without pathognomonic signs for toxoplasmosis. Moreover, the parasite is a main opportunistic pathogen of humans and life-threatening in immunocompromised patients. On the other hand, in primary *Toxoplasma* infections during pregnancy, the tachyzoites of *T. gondii* may transplacentally transmit to fetus and cause severe damages in brain and eyes (2). The parasite can persist lifelong in various organs of hosts, especially brain and muscle, as tissue cyst (chronic infection), which is responsible for the durability of antibody response in chronic phase of infection, and reactivation in immunocompromised cases (3).

Seroconversion from a negative to positive titer and a four-fold rise in antibodies titer over a 3-week interval are considered as the most reliable indicators for acute toxoplasmosis (4-6). However, the former is mainly restricted to monitoring program of women during pregnancy which is unusual in developing countries. Limitation of the latter is related to antibody level in serum samples of patients at the time of sampling which is usually at maximum. In spite of remarkable development in the serology of toxoplasmosis, distinguishing between acute and chronic infections is also a main challenge in serodiagnosis of the disease. *T. gondii* specific IgM, IgA, IgE, and low IgG avidity antibodies, which are considered as the serologic markers for recently acquired infections, have a common limitation that tend to persist in chronic phase of infection (7-12).

An IgG avidity test has been widely used to differentiate between the recent and latent infections of *T. gondii*, especially during pregnancy (13 -23). In this test, a high avidity index can certainly exclude the occurrence of infection within recent 3 to 5 months and therefore it is considered as a reliable indicator to rule out acquired infection during pregnancy during the first trimester (15). However, a low avidity index which regards the infection as acquired recently may persist for more than one year and thus cannot be used as a definite marker for recent infections with *Toxoplasma* (17, 21). Protracted low avidity of IgG antibodies that is attributed to delayed maturation of IgG avidity antibodies (17) may affect the anti-*Toxoplasma* therapy in pregnant women (24). A high IgG avidity is considered as a single reliable marker to exclude the acquired infections during pregnancy in women who are tested within the first trimester (14-16, 19-21).

In view of the limitations propounded for each of the serological markers in differentiating the recent and past infections of *T. gondii*, the complementary testing is considered as a tool for increasing the accuracy in the detection of clinical toxoplasmosis or to determine primary infections during pregnancy (10-21). In USA, a panel of serological tests may be used to confirm *Toxoplasma* IgM positive samples in a serology reference laboratory (25), whereas in Iran, the simultaneous measurement of IgG and IgM antibodies in a single serum is the only criterion for judging and making a decision over toxoplasmosis. In a report from Iran, only 18 serum samples with positive results for IgM antibodies to *T. gondii* were evaluated with IgG avidity test (26), a sample size insufficient to show an importance for complementary tests in diagnosis of toxoplasmosis in the country. The present study was performed to clarify the necessity for confirmatory testing in serodiagnosis of toxoplasmosis in Iran. To achieve the objective of the study, the authors evaluated IgA ELISA and IgG avidity as confirmatory tests

in diagnosis of recently acquired infections of *T. gondii* in specimens with positive results for IgM antibodies to *T. gondii*.

Material and Methods

Serum samples

A total of 131 serum samples were collected from patients suspected of having toxoplasmosis and referred to 3 private laboratories in Tehran and Qazvin Province, located 150 km northwest of Tehran, during 2011 to 2012. The sera were unknown in the existing clinical settings and stored at -20 °C until use. Initially, all serum samples were examined for anti-*T. gondii* IgG antibodies by enzyme-linked immunosorbent assay (ELISA) in reference lab and those negative for antibody, excluded from the study. Of 131 serum samples, 107 were found positive in the initial screening of specimen and thus used for the measurement of *T. gondii* IgM, IgA, and IgG avidity antibodies.

IgG, IgM, and IgA ELISA

Anti-*T. gondii* IgG, IgM, and IgA antibodies were detected by commercial kits (Euroimmun, Germany). The tests were performed according to the manufacturer's instructions. All samples were tested in duplicate. The optical density (OD) of wells was measured in a microplate reader (Awareness, Stat Fax 3200, USA) at 450 nm while a wavelength of 620 nm was used as reference. The results were expressed semi-quantitatively by calculating the ratio of the extinction value of the control or patient sample over the extinction value of calibrator and considered positive for ratio$\geq$1.1, negative<0.8, and borderline$\geq$0.8 - <1.1 for all three classes of antibodies.

IgG Avidity ELISA Test

Avidity determination of IgG antibodies against *T. gondii* was conducted by ELISA method using commercial kits (Euroimmun, Germany). Serum samples were examined according to the manufacturer's instructions.

The results obtained were presented by calculating the percentage of relative avidity index (RAI %) of the extinction of sample treated with urea over the extinction of sample without urea treatment. A RAI of <40% was indicative of low antibodies; a RAI of >60% indicated the presence of high avidity antibodies; and a RAI of 40%-60% considered as borderline.

Statistical Analysis

Data were analyzed using SPSS version 13 statistical package. The descriptive results were presented as mean$\pm$ SD (standard deviation). Also, Kappa coefficient, Pearson's correlation coefficient (r_p) and regression were used. *P*<0.05 were considered as significance level.

Results

Of 107 serum samples from patients suspected of having toxoplasmosis, specific IgM and IgA antibodies to *T. gondii* were positive in 67.3% (n=72) and 53.3% (n=57), respectively. Similarly, 29.9% (n=32) of the sera displayed low avidity for IgG antibodies. The minimum, maximum, and mean$\pm$SD of ratio indexes for positive sera were 1.20, 8.56, and 4.47$\pm$1.64 for IgG antibodies; 1.10, 9.12, and 2.65$\pm$1.70 for IgM antibodies, and 1.10, 6.82; and 2.83$\pm$1.52 for IgA antibodies, respectively. In addition, the minimum, maximum, and mean$\pm$SD of RAI% for sera with low avidity were 6.9, 38.2, and 20.4$\pm$7.89, respectively.

In 24.3% (n=26) of serum samples, both IgM and IgA antibodies were positive while the avidity index showed low reaction for IgG antibodies. On the other hand, 14% (n=15) of the sera were negative for both IgM and IgA antibodies, demonstrating a high avidity reaction for the IgG avidity (Table 1).

Pearson's correlation coefficient (rp) between the RAI% of IgG avidity and the ratio index of IgM was -0.572 (*P*<0.01) and the equation of regression line was drawn as {IgG

avidity= 70.16-8.2 × (IgM ratio)}, i.e. it is expected that one unit increase in IgM ratio to be accompanied with reduction of RAI% by 8.2 units (Fig. 1).

Table 1: Comparison of IgM ELISA, IgA ELISA, and IgG avidity tests for diagnosis of toxoplasmosis in 107 patients suspected of having toxoplasmosis

IgM-ELISA	IgA-ELISA	IgG avidity		
		Low	High	Borderline
Positive	Positive	26	16	9
	Negative	3	9	4
	Borderline	1	3	1
Negative	Positive	ND*	2	0
	Negative	ND	15	3
	Borderline	ND	1	0
Borderline	Positive	1	3	0
	Negative	0	6	1
	Borderline	1	2	0
Total		32	57	18

*ND: not done

The rp was calculated at -0.364 between the indexes of IgG avidity and IgA ($P<0.01$) with the equation of regression line drawn as {IgG avidity= 63.18 – 5.43× (IgA ratio)}. Also, the rp between the IgM ratio and IgA ratio was 0.564 ($P<0.01$), and the equation of regression line drawn as {IgM ratio= 0.98 + 0.59 × (IgA ratio)} (Fig. 2).

The results of IgM ELISA and IgG avidity were completely in agreement with the findings obtained for 45.8% (n=49) of samples. Of 72 specimens with positive IgM ELISA results, 41.6% (n=30) demonstrated low IgG avidity whereas among those with negative IgM ELISA results (n=21), 85.7% (n=18) showed high IgG avidity.

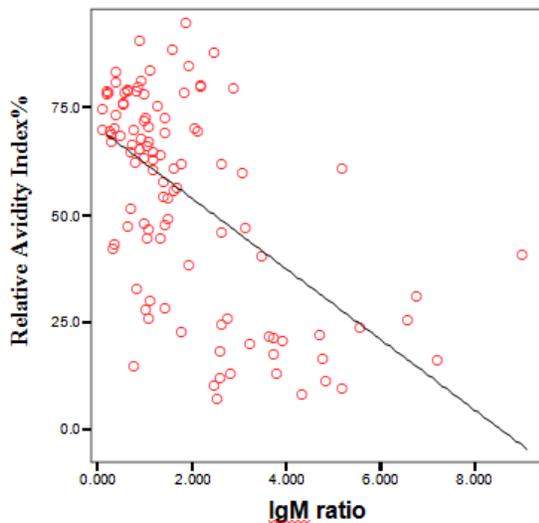**Fig. 1:** Scatter diagram and regression line for IgM and IgG avidity antibodies to *T. gondii* in 107 patients suspected of having toxoplasmosis
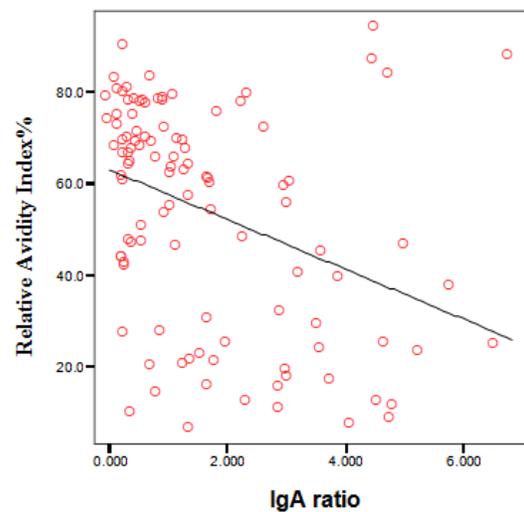

Fig 2: Scatter diagram and regression line for IgA and IgG avidity antibodies to T. gondii in 107 patients suspected of having toxoplasmosis

On the other hand, none of the 21 samples with negative results for IgM did not show low avidity reaction (Table 2). Kappa coefficient between the results of two tests was 0.192.

Table 2: Comparison of IgM ELISA and IgG avidity tests for diagnosis of toxoplasmosis in 107 patients suspected of having toxoplasmosis

IgG avidity	IgM-ELISA		
	Positive	Negative	Borderline
Low	30	0	2
High	28	18	11
Borderline	14	3	1
Total	72	21	14

The results of IgA ELISA and IgG avidity showed a full agreement in 54.2% (n=58) of cases. In other words, 47.4% (n=27) out of 57 specimens with positive results for IgA ELISA, showed low IgG avidity whereas a high level IgG avidity was found in 73.2% (n=30) out of 41 serum samples whose results for IgA ELISA were negative. However, 7.3% (n=3) of samples with negative results for IgA antibodies displayed low avidity (Table 3). Kappa coefficient between the results of two tests was calculated at 0.264.

Table 3: Comparison of IgA ELISA and IgG avidity tests for diagnosis of toxoplasmosis in 107 patients suspected of having toxoplasmosis

IgG avidity	IgA-ELISA		
	Positive	Negative	Borderline
Low	27	3	2
High	21	30	6
Borderline	9	8	1
Total	57	41	9

The highest agreement was observed between the results of IgM and IgA ELISA, i.e., 66.4% (n=71) of 107 cases demonstrated similar patterns. Of 72 serum samples positive for IgM ELISA, 69.4% (n=50) also showed positive results for IgA ELISA. Of 57 samples

with positive results for IgA ELISA, 87.7% (n=50) also found to be positive for IgM ELISA. Moreover, 85.7% (n=18) of the 21 specimens whose IgM ELISA were negative, had similar results for IgA ELISA. Of 41 samples with negative results for IgA antibodies, 43.9% (n=18) showed negative results for IgM ELISA. Kappa coefficient between the results of IgM and IgA ELISA was 0.406.

Of 72 specimens positive for IgM to *T. gondii*, the ratio indexes were 1.1-2 in 32.7% (n=35), 2-4 in 23.4% (n=25), and > 4 in 11.2% (n=12) cases. Of 35 samples with ratio indexes of 1.1-2, 17.1% (n=6) demonstrated low avidity reaction whereas the reaction rate of 83.3% (n=10) from 12 samples with IgM ratio indexes > 4 showed low avidity for IgG antibodies. On the other hand, of 57 serum samples with positive results for IgA ELISA, the ratio indexes were 1.1-2 in 42.1 (n=24), 2-4 in 35.1% (n=20), and > 4 in 22.8% (n=13) cases. Of 24 samples with IgA ratio indexes of 1.1-2, 33.3% (n=8) had low avidity reaction whereas the avidity reaction of 61.5% (n=8) from 13 samples with IgA ratio indexes > 4, was low.

Table 4: Comparison of IgA ELISA and IgM ELISA for diagnosis of toxoplasmosis in 107 patients suspected of having toxoplasmosis

IgM-ELISA	IgA-ELISA		
	Positive	Negative	Borderline
Positive	50	17	5
Negative	2	18	1
Borderline	5	6	3
Total	57	41	9

Discussion

In the present study, there was a remarkable difference between IgA ELISA and IgG avidity tests in confirming the specimens with positive IgM antibodies against *T. gondii*. In other words, almost 70% of IgM positive samples were confirmed by IgA whereas nearly 40% of

the specimens showed low avidity for IgG antibodies. Higher frequency of positive samples for IgM and IgA antibodies in comparison with low avidity for IgG antibodies has been also shown in other studies (7, 10), a finding probably due to difference in durability of the antibodies in chronic phase (10) and diversity in sensitivity of the assays (18). In a study by Kodym et al., *Toxoplasma* IgM and IgA antibodies remained positive for 12-18, and 6-9 months, respectively (10), and Auer et al. reported a higher sensitivity for IgM ELISA compared with IgA ELISA (18). Hence, it seems that the measurement of IgM antibodies has superiority to other serological markers for the initial diagnosis of *T. gondii* infections.

Our findings support other studies that emphasize on confirmatory testing in serodiagnosis of toxoplasmosis (4-6,15, 16, 18, 19, 21), however, the interpretation of the test results is difficult in some cases. At present, there is no unique serological pattern for confirming toxoplasmosis. The limitations of serological tests and their interpretive problems in distinguishing between acute and latent infections require the primary results to be confirmed in a reference laboratory. At *Toxoplasma* Serology Laboratory in Palo Alto Medical Foundation, known as the reference laboratory for the U.S. Centers for Disease Control and Prevention (CDC) and Food and Drug Administration (FDA), a battery of tests as *Toxoplasma* serology profile (TSP) is used which is slightly different in clinical settings. The TSP has been demonstrated to be a useful tool to distinguish between acute and chronic infections (2).

We showed a significant association between increasing the IgM and IgA antibodies indexes and decreasing the avidity index for IgG antibodies, a finding in agreement with the results of previous studies (16, 21, 27, 28). In our study, a low avidity was demonstrated in 83.3% (10/12) and 61.5% (8/13) of samples with index > 4 for IgM and IgA antibodies, respectively. It seems that a high IgM antibody index is a more reliable marker than a high IgA antibody index in confirming acute toxo-

plasmosis. However, our results cannot argue with certainty which of the IgA ELISA and IgG avidity tests is more appropriate to conform the positive samples for IgM to *T. gondii*. To answer this question, serum samples of known history and clinical setting is required which was a main limitation of the present study.

Considering high avidity as an indicator to rule out recent infections in samples with positive results for IgM antibodies to *T. gondii*, 38.9% of 72 IgM positive samples in our study may have been implicated in latent infections. However, a high avidity for IgG antibodies cannot be regarded as a definite criterion for excluding acute toxoplasmosis in individuals who are positive for IgM antibody to the parasite. For example, lymphadenitis due to *T. gondii* may sustain for more than 6 months, whereas, the avidity index of IgG antibodies may convert from low to high prior to the time when the swollen lymph nodes in the disease subside (11, 19, 30). Also, the results of high IgG avidity test can be misleading in patients with ocular toxoplasmosis caused by reactivation of *T. gondii* (31).

The frequency of a high IgG avidity in serum samples containing specific *T. gondii* IgM antibodies has been diverse in different studies, for example, 66.3% (18), 51.9% (21), 48.7% (15), 56% (32), and 61.3% (20). These differences have been attributed to several possible factors, including; heterogeneity in clinical setting of toxoplasmosis, heterogeneity in population, function of immune system at time of blood collection, interval between sampling time and beginning of infection, lack of standardization of serological tests, diversity in antigen preparation, anti-*Toxoplasma* therapy, and variations in calculating the avidity index (33).

In Iran, a developing country located in western Asia, where at least 30% of the populations, widely scattered in many parts of the country, are seropositive for *T. gondii* (34, 35), and the diagnosis of toxoplasmosis is facing two main challenges. Firstly, clinicians may be unaware of serodiagnostic problems of the

parasitic infection. Secondly, there is not a reference center for confirmatory testing of the disease in the country. Therefore, management of patients suspected of having toxoplasmosis and making a decision about pregnant women who are screened for congenital toxoplasmosis may lead to unnecessary interventions with anti-toxoplasma drugs which are mostly with adverse impact on hematopoiesis (2).

Conclusion

The results of our study highlight a necessity for confirmatory testing in diagnosis of toxoplasmosis in Iran. We recommend the *Toxoplasma* serology laboratory to be established within the university reference centers in all provinces of the country while being supervised by a specialist in parasitology. Also, training courses for clinicians will help enhance their knowledge in the field.

Acknowledgment

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