

Comparison of two assays in the diagnosis of toxoplasmosis: immunological and molecular

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مقارنة بين المقياس المناعي والمقياس الجزيئي في تشخيص داء المقوسات

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الخلاصة: لقد كانت الاختبارات المصلية للمقوسات الغوندية غير كافية نظراً لأن إنتاج المضادات كان إما يفشل أو يتأخر بشكل كبير. وقد تناولت هذه الدراسة التي أجريت في شرق العراق اختبار مقياس المناعة المرتبط بالأنزيم لشدة الرغبة للغلوبولين المناعي IgG بغية اكتشاف حالات العدوى الحديثة بالمقوسات الغوندية لدى السيدات الحوامل، مع مقارنتها بالطرق المناعية والتفاعل المتسلسل للبوليميراز في تشخيص المقوسات الغوندية. وتم أخذ العينات المصلية من 130 سيدة حامل ممن يشتبه بإصابتهم بداء المقوسات إلى جانب 25 امرأة حامل حملاً طبيعياً كمجموعة شاهدة. فأظهر اختبار شدة الرغبة للضد IgM و IgG لنحو 50 عينة إيجابية، أن 15 عينة فقط فيها أضداد IgG منخفضة الرغبة. وتم تطبيق تقنية التفاعل المتسلسل للبوليميراز على 25 عينة مختارة. واكتشف دنا المقوسات في 15 من أصل 15 عينة إيجابية للضد IgM ذات الضد المنخفض الرغبة IgG وواحدة من أصل ثلاث عينات موجبة للضد IgM ذات الضد IgG عالي الرغبة. في حين لم يكن دنا المقوسات موجوداً في عينات سالبة للضد IgM ذات الضد IgG عالي الرغبة. ومما تقدم يمكن استنتاج أن استخدام اختبار شدة الرغبة للضد IgG عندما يستخدم بالتزامن مع اختبار الضد IgG و IgM بتقنية الامتزاز المناعي للرغبة المرتبط بالأنزيم، يوفر مقياس قيمة في استبعاد الإصابة السابقة أو تلك التي اكتسبت مؤخراً بالمقوسات لدى الحوامل.

ABSTRACT Serological tests for *Toxoplasma gondii* are inadequate because antibody production either fails or is significantly delayed. This study in eastern Iraq investigated the IgG-avidity ELISA test for detecting recent *T. gondii* infections among pregnant women and compared immunological methods and PCR as molecular assays in the diagnosis of *T. gondii*. Serums samples were taken from 130 pregnant women at risk of toxoplasmosis and a control group of 25 women with normal pregnancy. Of 50 IgM- and/or IgG-positive samples, only 15 showed low IgG-avidity antibodies. PCR was performed on 25 selected samples. *Toxoplasma* DNA was detected in 15/15 IgM-positive with low IgG-avidity and 1/3 IgM-positive with high IgG-avidity. None of the IgM-negative with high IgG-avidity showed any *Toxoplasma* DNA. ELISA IgG-avidity when used in combination with ELISA IgG/IgM is a valuable assay for the exclusion of ongoing or recently acquired *T. gondii* infection in pregnant women.

Comparaison de deux dosages dans le diagnostic de la toxoplasmose : immunologique et moléculaire

RÉSUMÉ Les tests sérologiques pour *Toxoplasma gondii* sont inadaptés car la production d'anticorps soit n'a pas lieu, soit se produit très tardivement. La présente étude menée dans la partie orientale de l'Iraq a examiné le test ELISA d'avidité des anticorps IgG pour dépister les infections à *Toxoplasma gondii* récentes chez les femmes enceintes, puis a comparé les méthodes immunologiques et la méthode PCR en tant que dosages moléculaires pour la pose du diagnostic de l'infection à *T. gondii*. Des échantillons de sérum ont été prélevés chez 130 femmes enceintes à risque de toxoplasmose et 25 femmes appartenant à un groupe témoin dont la grossesse était normale. Sur 50 échantillons positifs pour les IgM et/ou les IgG, seuls 15 ont présenté un faible degré d'avidité des anticorps IgG. La méthode PCR a été utilisée sur 25 échantillons sélectionnés. L'ADN de *Toxoplasma* a été détecté dans 15 échantillons positifs aux IgM sur 15 ayant une forte avidité des IgG et dans un échantillon positif aux IgM sur trois ayant une haute avidité des IgG. Aucun des échantillons négatifs pour les IgM et ayant une forte avidité des IgG ne contenait d'ADN de *Toxoplasma*. Le test ELISA d'avidité des IgG, en utilisation combinée à un test ELISA d'avidité des IgG/IgM, constitue un dosage valable pour l'exclusion des infections à *T. gondii* contractées récemment ou actives chez les femmes enceintes.

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Introduction

Infection with the protozoan *Toxoplasma gondii* is one of the most common parasitic infections of humans worldwide [1]. In general, *T. gondii* infections are asymptomatic and self-limiting, especially among healthy immunocompetent hosts; however the infection may cause severe complications in pregnant women and immunocompromised patients [2,3]. Fetal toxoplasmosis, particularly in early pregnancy can cause miscarriage, stillbirth and birth defects [4]. Early first trimester maternal infection is less likely to result in congenital infection, but the sequelae are more severe [5,6]. The detection of recently acquired infection in pregnant women is therefore critical for clinical management of the mother and her fetus [7].

The diagnosis of toxoplasmosis is routinely based on serological tests for the presence of immunoglobulin (Ig)M and IgG-specific antibodies to *Toxoplasma* by enzyme-linked immunosorbent assay (ELISA) [8]. These tests, however, are not ideal because antibody production either fails or is significantly delayed. The IgG avidity test was developed to help discriminate between past and recently acquired infection [9]. The results are based on the measurement of the avidity (functional affinity) of *Toxoplasma*-specific IgG antibodies. Following an antigenic challenge, the antibodies produced usually have a low average affinity. During the course of the immune response, there is maturation of antibody affinity that increases progressively over weeks or months. IgG avidity, or the strength with which IgG binds to *T. gondii*, usually shifts from low avidity to high avidity at about 5 months after infection [10].

Most cases of active toxoplasmosis are due to reactivation of latent infections, which is why direct demonstration of the parasite in tissues or other fluids by polymerase chain reaction

(PCR) assay is a major breakthrough for the diagnosis of toxoplasmosis in these patients [11,12]. PCR was first developed for diagnosis of congenital toxoplasmosis in amniotic fluid [13]. The detection of *T. gondii* DNA in blood has highlighted the possibility of anticipating the diagnosis compared with radiological findings and histology [14]. PCR assay is an important technique to evaluate the prevalence of *Toxoplasma* reactivation when the detection of circulating DNA is the only clue to its reactivation [15].

A comparison between IgM and IgG-avidity measurements can help in the detection of past or recent toxoplasmosis as verified by PCR. The aim of this study was to evaluate the utility of ELISA IgG-avidity test for detecting recent *T. gondii* infections among pregnant women and to compare immunological methods and PCR as molecular assays in the diagnosis of *T. gondii*.

Methods

Study subjects

This study was carried out on patients attending private clinics in Al-Suwaira province, Wassit governorate, in eastern Iraq from 1 December 2010 to 31 August 2011. A total of 130 pregnant women, with ages ranging from 18–36 years and median age 27 years, who were considered to be at high-risk of *T. gondii* infection (abnormal pregnancy outcomes), were enrolled into this study. Informed consent to participate in the study was taken from participants.

Data collection

A 5 mL venous blood sample was collected from all participants. Serum was separated from half of each sample and kept at -20°C , while the other half of the sample was placed in a sterilized EDTA tube and stored at -80°C for amplification by PCR. The entire study

groups were screened for *Toxoplasma* infection with a rapid latex agglutination test (Latex-Toxo kit, Biokit Company).

Determination of IgM and IgG by ELISA

Presence of IgM and IgG antibodies were determined using ELISA *Toxoplasma* kits (ELISA Toxo-IgG and IgM, IBL International).

Determination of avidity index of IgG anti-*T. gondii* antibodies

Measurement of *Toxoplasma* IgG-avidity was performed and interpreted according to the directions of the manufacturer (Toxo-IgG-avidity; EUROIMMUN) using the ELISA system. The avidity index allows specimen classification as low (avidity index < 0.4 indicating an acute infection), borderline (avidity index $0.4-0.6$) or high (avidity index > 0.6) avidity. A high-avidity index excludes primary infection within the previous 16 weeks.

Confirmatory testing for *T. gondii* by PCR

PCR assay was performed on 25 selected samples as a confirmatory test of *Toxoplasma* infection by targeting a recently discovered repetitive 529 bp DNA fragment in *T. gondii*. This sequence is more repetitive than the B1 gene, approximately 200 to 300 times, and is highly conserved. This region of the *T. gondii* genome has been reported to be a very specific and sensitive target for the diagnosis [16].

DNA was extracted from whole blood using a commercial purification system (AccuPrep Genomic DNA Extraction Kit, Bioneer). Conventional PCR was performed on all DNA samples to amplify a fragment of restriction endonuclease sequence. The specific primers for used for amplification of the sequence of *T. gondii* DNA were: (forward primer) 5'-AAG-GCG-AGG-GTG-AGG-AT-<g>-3', MW 5693, melting temperature 65.3°C ; (reverse primer)

5'-GCG-TCG-TCT-CGT-CTG-GAT-<c>-3', MW 5786, melting temperature 66.2 °C.

Sample cross-contamination problems were avoided following a number of precautions including performing DNA extraction in laminar flow hood with subsequent irradiation by ultra-violet light and use of 3 separated areas for the DNA extraction, preparation of PCR mixture, PCR amplification and running gels.

Statistical analysis

The experimental data are presented in terms of observed numbers and percentages. The Student *t*-test was used for statistical analysis. A *P*-value ≤ 0.05 was considered statistically significant.

Results

With the rapid latex agglutination test 72/130 women (55.4%) showed a seropositive result.

Of the women tested for specific anti-*Toxoplasma* IgM and IgG antibodies by ELISA, 50/130 pregnant women (38.5%) were positive for IgM and/or IgG using ELISA *Toxoplasma* Ab kit; 18/130 (13.8%) had *Toxoplasma*-specific IgM antibodies. When the IgG-avidity ELISA test was applied to sera from the 50 positive IgM and/or IgG individuals, only 15 of them showed low IgG-avidity.

The 25 samples selected for PCR included 15 IgM-positive with low IgG-avidity antibodies, 3 IgM-positive with high-avidity antibodies and 7 IgM-negative with high IgG-avidity antibodies (Table 1). PCR analysis detected *Toxoplasma* DNA in 16 of the selected samples, 15 (93.8%) of these were IgM-positive with low IgG-avidity antibodies and 1 (6.3%) was IgM-positive with high IgG-avidity antibodies. PCR was negative for 9 of the samples. None of the 7 IgM-negative with high IgG-avidity antibodies

Table 1 Comparison between enzyme-linked immunosorbent assay (ELISA) IgM and IgG-avidity results in the detection of past or recent toxoplasmosis according to polymerase chain reaction (PCR) results in samples from pregnant women at risk of toxoplasmosis (*n* = 25 samples)

ELISA results		Total	PCR results	
IgG avidity	IgM status		DNA +ve (<i>n</i> = 16)	DNA -ve (<i>n</i> = 9)
		No.	No.	No.
Low	-ve	0	0	0
	+ve	15	15	0
High	-ve	7	0	7
	+ve	3	1	2
Total		25	16	9

+ve = positive; -ve = negative.

showed any *Toxoplasma* DNA (Table 1 and Figures 1, 2 and 3).

Calculating the accuracy of the test according to the PCR results showed that the sensitivity of IgM ELISA was 100.0% (16/16) and specificity was 77.8% (7/9), while for IgG-avidity sensitivity was 93.8% (15/16) and specificity was 100.0% (9/9).

Discussion

Routine serological diagnosis of toxoplasmosis provides high sensitivity, but the specificity varies depending on the test used. In this study, 18 (13.8%)

pregnant women had *Toxoplasma*-specific IgM antibodies, suggesting an acute infection warranting appropriate therapeutic intervention. Generally, detection of anti-*Toxoplasma*-specific IgM antibodies is a sensitive indicator of an ongoing or recent infection. However, false-positive IgM antibody test results have been reported previously [14]. In such cases, the diagnosis of primary infection with *T. gondii* in early pregnancy can be improved by determination of anti-*Toxoplasma* IgG-avidity, which has the ability to discriminate between recent and prior infections. On avidity testing, 15 out of the 18 (83.3%) IgM-positive

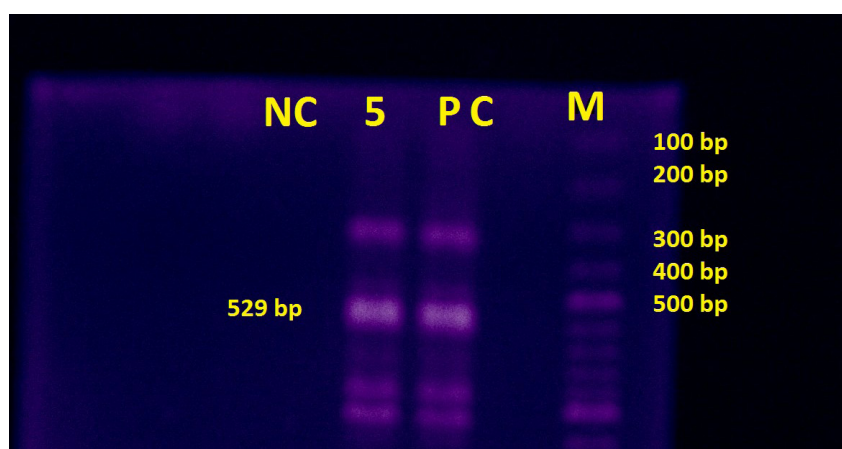


Figure 1 Amplification of 529 bp from *Toxoplasma gondii* DNA in the blood of abnormal pregnancy. Lane M, molecular weight marker (100 bp ladder), Lane NC, negative control, Lane PC, positive control, Lane 5, positive sample. Running conditions: agarose gel (2%), 5 v/cm for 2 h, stained with ethidium bromide

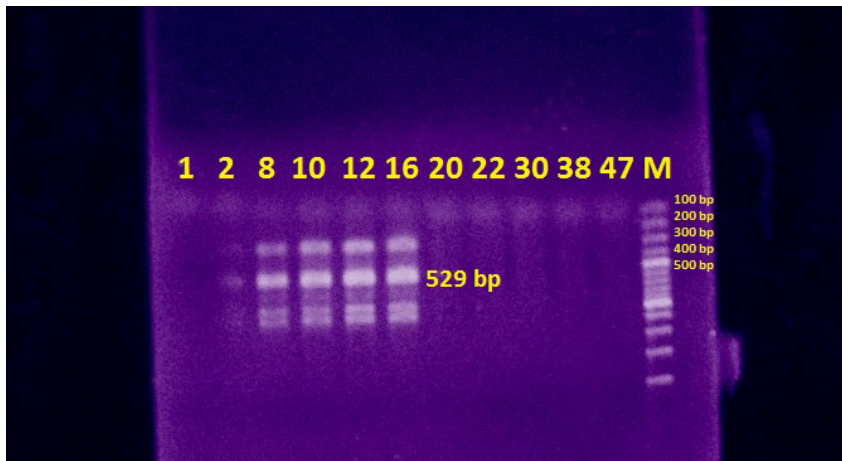


Figure 2 Amplification of 529 bp from *Toxoplasma gondii* DNA in the blood of abnormal pregnancy. Lane M, molecular weight marker (100 bp ladder), Lanes 8, 10, 12 and 16, positive samples. Lanes 1, 2, 20, 22, 30, 38 and 47, negative samples. Running conditions: agarose gel (2%), 5 v/cm for 2 h, stained with ethidium bromide

women had low-avidity IgG antibodies suggesting a recent *T. gondii* infection. More importantly, 3 (16.7%) of the 18 IgM-positive women had high-avidity antibodies suggesting that the infection was acquired before gestation. The apparent discrepancy in detecting infection status by IgM serology and avidity tests may be due to the fact that IgM antibodies may

persist for months or even years following the acute phase of an infection in some individuals; thus the presence of IgM antibodies is not always an indication of a recent infection [17]. The presence of specific *T. gondii* IgM antibodies in the chronic stage of an infection, as observed in 16.7% of IgM-positive cases in this study, may have resulted in unwarranted concern

and a misdiagnosis particularly in early pregnancy.

Previously it has been reported that the ELISA IgG-avidity test is highly sensitive and specific for detecting a recent *T. gondii* infection in IgM-positive cases [18]. Such results have confirmed in the present study by a sensitivity of 100%, while specificity was found to be 77.8%. It is also known that the maturation of the IgG response varies considerably between individuals and thus low-avidity antibodies may persist for months to more than 1 year [19]. In such patients, an avidity test result, if used alone, would have been misinterpreted as suggestive of an acute infection. Previous studies have documented that PCR can actually detect *T. gondii* in the blood of women before or during pregnancy [20,21]. Based on this, the presence of *Toxoplasma* DNA in the maternal blood probably indicates a recent infection or apparent parasitaemia, which is likely to be clinically significant. The clearance time for *Toxoplasma* DNA from the blood of patients with acute toxoplasmic lymphadenopathy was estimated to be 5.5–13 weeks [22].

Conclusions

The results described in this study showed that the ELISA IgG-avidity test, when used as a confirmatory test along with the ELISA IgG/IgM tests in pregnant women, was useful in distinguishing a recently acquired infection from chronic infection. Confirmatory testing for ongoing or recent *Toxoplasma* infection with the ELISA IgG/IgM antibody test and the ELISA avidity method in pregnant women has the potential to decrease the need for follow-up sera and for unnecessary therapeutic interventions in pregnant women. Despite the ease of use of ELISA tests, false positives were found among the high-avidity, IgM-positive samples, and therefore in well-equipped laboratories the application of PCR is recommended.

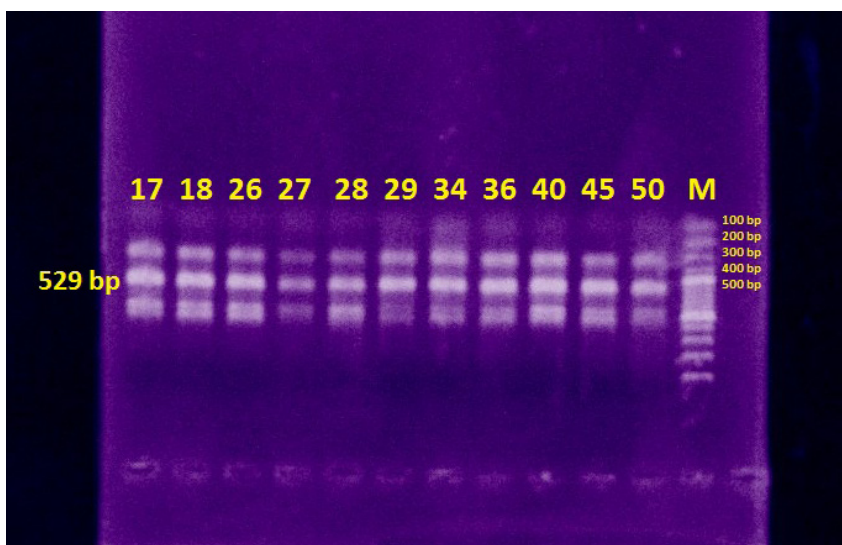


Figure 3 Amplification of 529 bp from *Toxoplasma gondii* DNA in the blood of abnormal pregnancy. Lane M, molecular weight marker (100 bp ladder), Lanes 17, 18, 26, 27, 28, 29, 34, 36, 40, 45, and 50, positive samples. Running conditions: agarose gel (2%), 5 v/cm for 2 h, stained with ethidium bromide

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