IgG avidity assay: A tool for excluding acute toxoplasmosis in prolonged IgM titer sera from pregnant women

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Abstract. An accurate diagnosis for toxoplasmosis is crucial for pregnant women as this infection may lead to severe sequelae in the fetus. The value of IgG avidity assay as a tool to determine acute and chronic toxoplasmosis during pregnancy was evaluated in Universiti Kebangsaan Malaysia Medical Centre (UKMMC). In this study, 281 serum samples from 281 pregnant women in various trimesters were collected. These samples were assayed using specific anti-*Toxoplasma* IgM and IgG antibodies, followed by IgG avidity test. The overall seroprevalence of toxoplasmosis in pregnant women was 35.2% (33.5% for anti-*Toxoplasma* IgG and 1.8% for both anti-*Toxoplasma* IgG and IgM antibodies). Of 5 (1.8%) serum samples positive for IgM ELISA, 4 had high-avidity antibodies, suggesting past infection and one sample with borderline avidity index. Two samples with low avidity were from IgM negative serum samples. The IgG avidity assay exhibited an excellent specificity of 97.6% and a negative predictive value (NPV) of 95.6%. The study also demonstrated no significant correlation between avidity indexes of the sera with IgG (r=0.12, p=0.24) and IgM (r=-0.00, p=0.98), suggesting the complementary needs of the two tests for a better diagnosis outcome. These findings highlight the usefulness of IgG avidity assay in excluding a recently acquired toxoplasmosis infection in IgM-positive serum sample.

INTRODUCTION

Toxoplasmosis is generally asymptomatic in immunocompetent individuals, whereas intrauterine transmission of the parasite from the mother to the fetus during gestation can result in severe fetal and neonatal complications (Pinon *et al.*, 1996). Undiagnosed infection in a new born baby results in further development of the disease in early childhood causing ocular and hearing impairment, neurological symptoms or mental retardation (Karczewski & Golab, 2011).

Since toxoplasmosis is usually asymptomatic, serological test by the demonstration of specific anti-*Toxoplasma* antibodies remains the routine diagnostic procedure in most hospitals. The greatest concern about diagnosing primary toxoplasmosis in pregnancy is deciding whether or not the pregnant woman has acquired acute infection or whether infection has occurred before conception in order to initiate early therapy or other interventions to prevent unwanted complications to the fetus (Iqbal & Khalid, 2007). For a long time, the main marker of recent infection is the presence of specific IgM antibodies. Unfortunately, the detection of IgM specific antibodies may produce unreliable result in determining the time of *Toxoplasma* infection

as IgM anti-*Toxoplasma* antibodies may persist in the serum for years after acute infection (Horvath *et al.*, 2005). False positive result in the diagnosis may lead to unnecessary treatment which may induce teratogenic effect (Julliac *et al.*, 2010), unnecessary expenses on drug (Bueno *et al.*, 2010) and family's anxiety on baby's prognosis before and after the child's birth (Khoshnood *et al.*, 2007).

To date, serological diagnosis of *Toxoplasma* infection has improved by the introduction of anti-*Toxoplasma* IgG avidity test, which is able to discriminate between recent and past infection (Remington *et al.*, 2004; Iqbal & Khalid, 2007). Avidity test measures the functional affinity of specific IgG antibodies, which is initially low after primary antigenic challenge and increases during subsequent weeks and months by antigen-driven B cell selection. Protein-denaturing reagents, including urea are used to dissociate the antibody-antigen complex. The avidity result is determined by using the ratios of antibody titration curves of urea-treated and untreated samples (Liesenfeld *et al.*, 2001).

For diagnosis of toxoplasmosis, most of the hospitals in Malaysia only rely on a single test, by determination of a specific anti-*Toxoplasma* IgM and IgG ELISA. In this study, we investigated the usefulness of testing IgG avidity test in serum samples primarily from pregnant women. Thus far, this is the only study in Malaysia evaluating the usefulness of incorporating IgG avidity together with specific IgM and IgG assay in diagnosis of *Toxoplasma* infection in pregnant women. Our goal was to compare the interpretation of results obtained in an IgG avidity test with those obtained with the IgM and IgG ELISA test in a single serum sample.

MATERIALS AND METHODS

Study population
This study was conducted in the Obstetrics and Gynaecology Department, University Kebangsaan Malaysia Medical Centre (UKMMC), between October 2012 and May 2013. Sera (281 samples) from pregnant women in various trimesters were screened for anti-*Toxoplasma* IgG and IgM antibodies by Platelia Toxo IgG ELISA (Biorad, USA), Platelia Toxo IgM ELISA (Biorad, USA) and for anti-*Toxoplasma* IgG avidity by Platelia Toxo IgG Avidity (Biorad, USA). A second blood specimen was taken at least 3 weeks after the initial sample from all women with suspected active *Toxoplasm*a infection. An exclusion criterion for sample selection is a pregnant women who is immuno-compromised (i.e: patient with HIV infection, autoimmune disease or malignancy). Informed consent was taken from patients prior to the blood taking.

Ethical aspects
This study protocol was approved by the research Ethical Committee of UKMMC (Ethical number: FF-432-2012).

Collection and analysis of blood samples
Three mls of blood was taken from each pregnant woman by venipuncture under sterile conditions. The blood samples were centrifuged at 3000 g for 10 minutes to obtain serum samples for serological test and stored at -20ºC until use.

Platelia Toxo IgG and IgM ELISA
The presence of specific anti-*Toxoplasma* IgG and IgM antibodies in the 281 serum samples were screened using commercial ELISA kits, Platelia Toxo IgG and IgM ELISA (BioRad, USA). The IgG and IgM antibody titers were read at optical density (OD) of 450 nm and 620 nm respectively using automatic ELISA reader (SKANIT Software 2.5.1). Based on the kit interpretation, sera IgG titer < 6 IU/mL is considered negative for anti-*T. gondii* IgG antibodies; between 6 IU/mL and 9 IU/mL is considered equivocal and >9 IU/mL is considered positive. Interpretation for sera IgM titer is determined by calculation of sample ratio (sample OD/cut off OD), whereby any value of sample ratio <0.8 is considered negative; between 0.8 and 1.0 is equivocal and ratio $\geq$1.0 is considered positive.

Platelia Toxo IgG Avidity

The IgG avidity test was performed on ninety nine sera which were positive for both IgM and IgG anti-*Toxoplasma* antibodies with Platelia ToxoIgG avidity kit (Bio-Rad, USA) according to the manufacturer's instructions. Briefly, each serum sample was analyzed in two four-fold titration rows, with one row (row A) starting at a dilution of 1:50 and the other row (row B) starting at a dilution of 1:200. After 1 h of incubation at 37°C, row A was washed three times with 250 ml of 6 M urea in phosphate-buffered saline containing 0.05% Tween 20 in order to remove low-avidity antibodies from their binding sites. Row B, the control row, was washed three times with the washing solution of the kit, but without urea. During each washing step the microtiter plate was vigorously shaken for 5 min. The following EIA steps, including incubation with conjugate, washing, incubation with substrate, and addition of stop solution, were performed according to the recommendations given by the manufacturer. The optical density (OD) of each well was read using an automated ELISA reader (Thermoscientific Multiscan, Finland) at 450 nm, with 620 nm used as the reference wavelength. The avidity is considered high when the antigen-antibody link is not easily dissociated. According to the kit interpretation, and avidity index <0.45 is considered in low avidity zone; between 0.40 and 0.5 is considered as equivocal and ≥0.5 is considered high avidity zone.

Statistical Analysis

Data were entered, cleaned and analysed using SPSS 20.0 for Windows. Sensitivity, specificity and positive and negative predictive values were calculated according to the following formulae:

$$\text{Sensitivity} = \frac{\text{true positives} + \text{false negatives}}{\text{true positives}}$$

$$\text{Specificity} = \frac{\text{false positives} + \text{true negatives}}{\text{true negatives}}$$

$$\text{Positive predictive value} = \frac{\text{true positives} + \text{false positives}}{\text{true positives}}$$

$$\text{Negative predictive value} = \frac{\text{true negatives} + \text{false negatives}}{\text{true negatives}}$$

Sensitivity is the probability that the assay will be positive when the disease is present. Specificity is the probability that the assay will be negative when the disease is not present. Positive predictive value (PPV) is the probability that the disease is present when the assay is positive. Negative predictive value (NPV) is the probability that the disease is not present when the assay is negative. Pearson correlation was used to correlate between avidity IgG and IgM. All hypothesis involved were two-sided tests and P value less than 0.05 was considered as statistically significant.

RESULTS

Serum samples taken from 281 pregnant women in various trimesters were serologically screened to determine their anti-*Toxoplasma* antibody status.

Table 1 shows serological results of serum samples from 281 pregnant women using routine serological method, by measuring anti-*Toxoplasma* IgM and IgG antibody titers. Out of 281 samples, only 5 (1.8%) were found to have probable acute toxoplasmosis. Meanwhile, probable chronic toxoplasmosis were determined in 94 (33.5%) of the subjects. The remaining 182 (64.8%) subjects were free from the infection. Subjects who were seronegative for anti-*Toxoplasma* antibodies were advised to take precautions against toxoplasmosis since their foetus could be at risk. All samples that were determined as probable acute (5/281) and chronic (94/281) toxoplasmosis by anti-*Toxoplasma* IgG and IgM antibodies were further evaluated with anti-Toxo IgG avidity test for confirmation of their antibody status against *Toxoplasma* infection.

Table 2 showed the results of IgM ELISA in comparison with avidity test in 99 serum samples taken from pregnant women in UKMMC. From 99 samples, only 5 (5.0%) were positive for IgM titer. Out of these 5 samples, 4 were of high avidity and 1 sample was in borderline avidity index. The low IgG avidity level however was obtained from samples with negative IgM titer. Follow-up serological testing of low and boderline

Table 1. Anti-*Toxoplasma* antibody status in 281 pregnant women in UKMMC

Probable acute (IgM+ve, IgG+ve) n (%)	Probable Chronic (IgM-ve, IgG+ve) n (%)	No infection (IgM-ve, IgG-ve) n (%)
5 (1.8)	94 (33.5)	182 (64.8)

Table 2. Comparison of results of IgM ELISA with IgG avidity test in 99 serum samples taken from pregnant women in UKMMC

IgG Avidity Result	IgM ELISA Result	
	Positive (n = 5)	Negative (n = 94)
Low	0	2 (2.1)
Boderline	1 (20.0)	9 (9.6)
High	4 (80.0)	83 (88.3)

avidity samples were performed, however no significant increases in IgG antibody titer was revealed.

The validity of results obtained from IgG avidity test was measured and compared with results obtained by anti-Toxo IgG and IgM ELISA as the gold standard. The diagnostic accuracy of the low avidity in the presence of IgG positive antibodies demonstrated a sensitivity of 0%, specificity of 97.75%, positive predictive value (PPV) of 0% and negative predictive value (NPV) of 95.6%. The study also demonstrated that there was no significant correlation between avidity indexes of the sera with IgG (r=0.12, p=0.24) or with IgM (r=-0.00, p=0.98) antibody level.

DISCUSSION

Toxoplasma gondii is one of the most prevalent opportunistic infectious agents in human and is known as a silent threat in Southeast Asia (Nissapatorn *et al.*, 2003). Primary infection during pregnancy constitutes a great diagnostic challenge as interpretation of specific *Toxoplasma*-IgG and IgM antibodies level are unreliable indicators to determine acuteness of the infection. Moreover, early diagnosis is essential since prenatal toxoplasmosis potentially cause tragic outcome to the fetus and newborn. In Malaysia, the latest report on overall seroprevalence of toxoplasmosis among pregnant women was estimated to be 49%, in which 39%, 4% and 6% for anti-*Toxoplasma* IgG, IgM and both anti-*Toxoplasma* IgG and IgM antibodies, respectively (Khairul Anuar *et al.*, 1991; Ravichandran *et al.*, 1998; Nissapatorn *et al.*, 2003). From this study, it was found that the seroprevalence of 35.2% (33.5% for anti-*Toxoplasma* IgG and 1.8% for both anti-*Toxoplasma* IgG and IgM antibodies) was still within the reported range. Since toxoplasmosis is an etiological factor that causes bad obstetric history in asymptomatic pregnant women, a good diagnostic method aiming at precise estimation of the time of infection is therefore crucial for a better clinical management (Singh, 2003; Singh & Pandit, 2004). Health industry and academic researchers have long been trying to develop a serological method to differentiate recent from chronic *T. gondii* infection in pregnant

women using a single serum sample. Such a method would be of particular importance in countries where systematic serological screening during pregnancy is not routinely performed.

At the moment, diagnosis of toxoplasmosis among pregnant women in most hospitals in Malaysia, including UKMMC only relies on determination of the *Toxoplasma*-specific IgM and IgG antibodies. A positive IgM test result in a single serum specimen may reflect an acute infection, however low levels of *Toxoplasma*-specific IgM antibodies may remain up to several years and thus lead to erroneous interpretation. Studies have indicated that the combination of measurement of the IgG avidity antibodies with the sensitive test for *Toxoplasma*-specific IgM antibodies for *T. gondii* had the highest predictive value with regard to the time of infections (Petersen *et al.*, 2005; Press *et al.*, 2005). It improved the diagnosis with the ability to discriminate between acute and chronic infection (Iqbal & Khalid, 2007), as well as to differentiate reactivation from acute infection by using only a single serum specimen, which is important for those pregnant women as well as in immunocompromised patients.

Of the 99 samples used for IgG avidity testing, 5 were positive for IgM. However, none of these had low avidity index but 4 had high avidity indices and one sample with borderline IgG avidity index. All of these 5 cases were in their first trimester of pregnancy. These findings indicated that the results were actually a prolonged titer of IgM. These women actually acquired the infection from a distant past and thus excluded the acquisition of primary infection during early pregnancy. Investigators from European countries reported that high IgG avidity excludes acquisition of the infection in the earlier three months (Liesenfeld *et al.*, 2001) and the risk for acquiring congenital toxoplasmosis is low. The presence of specific *T. gondii* IgM antibodies in the chronic stage of an infection may lead to misinterpretation of the result and cause needless concern which possibly leads to the decision to abort (Liesenfeld *et al.*, 1997). Similar results have been reported in previous studies (Montoya *et al.*, 2002; Iqbal & Khalid, 2007), where they found high percentage of IgM positive samples with high IgG avidity antibodies in the early trimester of pregnancy.

The apparent discrepancy in detecting infection status by ELISA 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. It is also known that low or borderline avidity antibodies may persist for months to more than 1 year due to the difference in the maturation of the IgG response between individuals (Singh, 2003; Singh & Pandit, 2004;). In our study, 2 samples of low avidity were obtained from negative IgM samples and one IgM-positive sample exhibited a borderline avidity index. In these cases, interpretation of the results was tricky and follow-up sera were still needed for confirmation. Although avidity test was claimed to be highly specific and sensitive in detection of recent infection, however in sera with low or borderline-avidity antibodies with negative or positive IgM antibody titer respectively, avidity test is potentially misleading if used alone. This finding is supported by a study conducted by Iqbal & Khalid (2007) who found negative PCR results for IgM negative samples with low avidity antibodies. Furthermore, their study also reported 2 samples with borderline avidity and IgM positive was negative for *T. gondii* DNA.

In our study, we were unable to demonstrate the role of specific IgG with low avidity as an excellent single-serum indicator of recent primary *Toxoplasma* infection since 4 out of 5 of the IgM positive samples were of high avidity indices. Therefore, zero sensitivity and PPV for IgG avidity test was obtained. It is believed by increasing the number of samples in future, we might be able to detect the presence of low avidity cases. However, many studies had verified the significant finding of IgG avidity test as a discriminator of acute infection (Holliman *et al.*, 1994; Jenum *et al.*, 1997; Liesenfeld *et al.*, 2001). On the other hand, excellent specificity of avidity test was exhibited in this study, which has proven its great value in disputing prolonged IgM

result and thus excluded recent infection. The current work clearly showed that all the women who had *Toxoplasma* IgM at the time of pregnancy did not acquire the infection recently. The excellent specificity (97.6%) and NPV (95.6%) indicated that sample with IgM negative titer but high in avidity index is suggestive of chronic infection and thus highlighted the role of IgG avidity test as the best method to determine and exclude chronic infection. The study also demonstrated no correlation between IgG avidity index with IgM and IgG antibodies level, which suggests the need of both tests for a better diagnosis outcome.

In many countries including Malaysia (except for France and Austria), systemic screening in all pregnant women is not routinely performed due to many factors such as cost, demographic characteristics, availability of appropriate tests and the relatively low incidence of acute infection (Montoya & Remington, 2008). Therefore, an accurate diagnosis is vital to initiate early treatment. This study highlights the usefulness of high-avidity IgG test result when only a single sample of serum has been obtained in which *T. gondii* IgM antibodies are present and for which routine specific IgM test reveals an acute or equivocal pattern. High-avidity IgG antibodies develop at least 12–16 weeks after infection. The presence of high-avidity antibodies indicates that infection was acquired 16 weeks earlier (Hedman *et al.*, 1989; Lappalainen *et al.*, 1995; Pelloux *et al.*, 1998). Thus, for the management of pregnant woman, if they have a high-avidity IgG test result in the first months of gestation regardless of the IgM antibody test result, the fetus is essentially not at risk for congenital toxoplasmosis. For pregnancies beyond 16 weeks of gestation, a high-avidity test result may be helpful in establishing that the infection was acquired at least 12–16 weeks earlier in gestation. In this scenario, the transmission rate would be lower (Dunn *et al.*, 1999), the potential for fetal damage would be greater (Dunn *et al.*, 1999) and the negative predictive value of the amniotic fluid PCR would be greater (Romand *et al.*, 2001) than if the infection was acquired later in gestation. Of special note, as IgM can pesist

for more than 1 year following acute infection, low avidity or equivocal test results also can persist for many months or a year or more after the primary infection. Therefore, avidity test must not be used alone to determine whether the infection was recently acquired since it can be misleading (Hedman *et al.*, 1989; Montoya *et al.*, 2004). A panel of tests is required for confirmation of the infection status and thus ease the management of pregnant women.

In conclusion, the measurement of IgG avidity in this study served best in determining chronic infection and thus excluding acute toxoplasmosis in prolonged IgM titer from pregnant women. Therefore, it is highly recommended for all hospitals to implement and apply the IgG avidity test in combination with routine specific serological test in the diagnostic laboratory for diagnosis of toxoplasmosis in pregnant women, newborns or in immunocompromised patients. These tests are complements to each other in achieving a simplified interpretation and better accuracy in diagnosing toxoplasmosis.

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