Detection of acute *Toxoplasma gondii* infection in early pregnancy by IgG avidity and PCR analysis

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Acute *Toxoplasma gondii* infection in early pregnancy carries the risk of transmitting the infection to the fetus with serious sequelae. However, serological testing for IgG/IgM anti-*Toxoplasma* antibodies may fail to differentiate between a recent and past infection. Two hundred and twenty-four Kuwaiti women in their first trimester were screened for IgG/IgM antibodies by the Vitek Immuno Diagnostic Assay System (VIDAS) and VIDAS IgG-avidity tests. On serological screening, 119 (53.1 %) women were positive for IgG antibodies and 31 (13.8 %) for IgM antibodies. Nine of the IgM-positive and 7 IgM-negative women had low-avidity antibodies. However, the IgG-avidity test detected low-avidity antibodies only in 9 (29 %) of the 31 IgM-positive women, suggesting a recent infection; 19 (61.3 %) women had high-avidity antibodies, indicating that the infection was acquired in the distant past. Based on IgM serology alone, at least 31 IgM-positive women may have been wrongly labelled as having acute *Toxoplasma* infection thus warranting appropriate therapeutic intervention. All the 19 IgM-positive women with high-avidity antibodies were confirmed negative for *Toxoplasma* DNA on PCR analysis. Compared with PCR analysis, the VIDAS avidity test was a helpful tool for the diagnosis of recent *Toxoplasma* infection in IgM-negative women with low-avidity antibodies and IgM-positive women with high-avidity antibodies; the specificity was >85–100 %. It is concluded that the VIDAS avidity test when used in combination with VIDAS IgG/IgM tests is a valuable assay for the exclusion of ongoing or recently acquired *T. gondii* infection in pregnant women in their first trimester and that it decreases significantly the necessity for follow-up testing and unnecessary therapeutic intervention.

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INTRODUCTION

Acute primary maternal toxoplasmosis if acquired during the first trimester of pregnancy can cause significant morbidity and mortality in developing fetuses (Singh, 2003; Thiebaut *et al.*, 2007). The principal modes of transmission are from mother-to-fetus, through food or water contaminated with cat faeces or by eating undercooked meat of infected animals (Bahia-Oliveira *et al.*, 2003; Singh, 2003). Serological studies show a considerable variation in the prevalence of *Toxoplasma* infection from 7.5 to 95 % in different parts of the world, and indeed between different population groups within the same country (Asthana *et al.*, 2006). The diagnosis of *Toxoplasma gondii* infection is most commonly made by detecting IgG and IgM antibodies in the blood; however, these tests can not estimate the time of infection precisely enough to properly manage the risk to the fetus of a maternal infection. Congenital infection of the fetus in women infected just before conception is extremely rare, and even during the first few weeks of pregnancy, the maternal–fetal transmission rate is low (Emna *et al.*, 2006; Press *et al.*, 2005). It is therefore essential to estimate the

time of infection as precisely as possible to properly manage the risk to the fetus of a maternal infection. A positive IgM test result in a single serum specimen may reflect an acute infection; however, low levels of *Toxoplasma*-specific IgM antibodies may be found for up to several years, and thus may lead to erroneous interpretation. Recently, it has been suggested that the combination of a sensitive test for *Toxoplasma*-specific IgM antibodies and measurement of the avidity of IgG antibodies for *T. gondii* had the highest predictive value with regard to the time of infection (Petersen *et al.*, 2005; Press *et al.*, 2005).

The State of Kuwait is demographically a rapidly changing society with a mix of different racial and national groups due to a large population of migrant workers. The majority (>80 %) of the foreign workforce comes from Asia and other Arab countries. The prevalence of *Toxoplasma* infection in pregnant women in Kuwait has not been documented. However, an earlier serological study found an unusually high prevalence rate (>75 %) in the Kuwaiti population (Behbehani & Al-Karmi, 1980). The present study was undertaken to detect an ongoing or recent *T.*

gondii infection in pregnant Kuwaiti women during the first 16 weeks of gestation by using the VIDAS *Toxoplasma*-specific IgG-avidity test and nested PCR.

METHODS

Study group. This study was performed at the Faculty of Medicine, Kuwait University, between October 2002 and November 2005. Sera (224 samples) from 224 Kuwaiti pregnant women in their first 16 weeks of gestation were screened for anti-*Toxoplasma* IgG and IgM antibodies by the Vitek Immuno Diagnostic Assay System (VIDAS) and for antibody avidity by the VIDAS IgG-avidity test. A second blood specimen was taken at least 3 weeks after the initial sample from all women with suspected active *Toxoplasma* infection. When available, the follow-up serum samples were also used to resolve discrepant results between individual tests. All such sera were tested concurrently with the initial sample. The screening tests were essentially performed following manufacturers' instructions.

All women participating in this study gave their informed consent. Information on age, gestational age and number of children, and type of area of residence and other relevant socio-demographic information were recorded from all women enrolled for the study. The Ethical Committee of the Faculty of Medicine, Kuwait University, approved the study.

VIDAS *Toxoplasma* IgG/IgM (bioMérieux Vitek). This assay combines the enzyme immunoassay method with fluorescence detection (ELFA) using alkaline phosphatase-labelled monoclonal anti-human IgG/IgM antibodies. These enzyme-conjugated antibodies react with the substrate (4-methylumbelliferyl phosphate) and the relative fluorescence value of the solution is measured and interpreted automatically. The test was performed on a fully automated Vitek Immuno Diagnostic Assay System as described by the manufacturer. The test results were interpreted as follows: for IgG, ≥ 10 IU ml⁻¹ was positive, 8–10 IU ml⁻¹ was equivocal and < 8 IU ml⁻¹ was negative; and for IgM, > 0.65 IU ml⁻¹ was positive and < 0.55 IU ml⁻¹ was equivocal.

The current *Toxoplasma* screening policy practised in our centre is that all women with a VIDAS IgG reading of > 15 IU ml⁻¹ and IgM-negative are requested for a second follow-up specimen collected at least 3 weeks after the initial sample to detect any significant increase in IgG antibody titre. The women with positive IgM antibodies and/or two- to threefold increase in IgG antibodies in the follow-up specimen are prescribed spiramycin throughout their pregnancy.

VIDAS Toxo-IgG avidity. The IgG-avidity test was developed to help discriminate between past and recently acquired infection. Measurement of *Toxoplasma* IgG avidity was performed and interpreted according to the directions of the manufacturer (VIDAS Toxo-IgG avidity; bioMérieux) using the fully automated VIDAS machine. The test strip contains 6 M urea to remove the low-avidity IgG antibodies from their binding sites. Only antibodies with high avidity remain bound to the solid phase. The ratio between the quantity of high-avidity antibodies (test strip) and the quantity of total antibodies (reference strip) provides an index which indicates the avidity of the antibody in the tested sample. The avidity index allows specimen classification as low (avidity index < 0.2 indicating an acute infection), borderline (avidity index 0.20–0.25) or high (avidity index > 0.25) avidity. A high-avidity index excludes primary infection within the previous 16 weeks.

Nested-PCR. The PCR assay was done on a selected number of specimens. Buffy coat was prepared from the peripheral blood. Buffy coat samples were lysed in 2 vols TNN lysis buffer (0.5% Tween 20,

0.5% Nonidet P-40, 10 mM NaOH) with 10 mM Tris and 250 µg proteinase K ml⁻¹ for 5–15 h. The DNA was extracted by the phenol/chloroform method (Lachaud *et al.*, 2000). The final pellet was resuspended in 25 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 7.2) and stored at -70 °C until used.

The nested-PCR amplifications were performed on all DNA samples to amplify a fragment from the B1 gene as described earlier with slight modifications (Burg *et al.*, 1989). Briefly, the primers used in the first round of the PCR (inner primers) were 5'-GGAAGTGCATCC GTTCATGAG-3' and 5'-TCTTTAAAGCGTTCGTGGTC-3', which correspond to nucleotides 694–714 and 887–868, respectively. The primers used in the second round (outer primers) were 5'-TGCATAGGTTGCAGTCACTG-3' and 5'-GGCGACCAATGTGCG AATAGACC-3', which correspond to nucleotides 757–776 and 853–831, respectively. Five microlitres of template DNA was added to a final volume of 50 µl PCR mixture consisting of 5 µl 10 × PCR buffer (50 mM Tris/HCl, pH 9.1, 3.5 mM MgCl₂), 8 µl 1.25 mM deoxy-nucleoside triphosphates, 0.5 µl *Taq* DNA polymerase (5 units µl⁻¹) and 1.5 µl (20 pmol) of each of the outer primers. The amplification was performed in the GeneAmp 9700 PCR System (Applied Biosystems). The cycling conditions for both PCRs were 95 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 90 s and 72 °C for 1 min, and a final extension at 72 °C for 10 min.

Five microlitres of the first-round PCR product was used as template for the second-round PCR in a total volume of 50 µl under the same conditions as in the first round, using the inner primers. DNA extracted from the RH strain of *T. gondii* obtained from the *Toxoplasma* Reference Centre (Singleton Hospital, Swansea, UK) was used as a positive control. One negative and positive control was included in each PCR experiment. The PCR product was analysed on a 1% agarose gel stained with ethidium bromide.

Statistical analysis. Data were analysed with the chi-square and Fisher's exact tests to evaluate the possible differences between the various diagnostic assays. A *P* value < 0.02 was considered significant.

RESULTS

Two hundred and twenty-four women, median age 27 years (range 19–41 years), in the first 16 weeks of gestation were enrolled for this study. The mean length of pregnancy was 9.3 weeks (median 10 weeks, range 1–16 weeks) at the time of specimen collection. There were 47 (21%) primigravida women and 117 (79%) multigravida women. For 46 women, a second follow-up serum specimen obtained at least 3 weeks after the initial sample was available which was tested concurrently with the initial sample. All 224 women were screened for *Toxoplasma* infection with VIDAS IgG, IgM and IgG-avidity tests.

One hundred and nineteen of the 224 women (53.1%) were positive for *Toxoplasma*-specific IgG antibodies and 31 (13.8%) for IgM antibodies (Table 1). Twenty-three of the 119 (19.3%) IgG-positive cases had an IgG titre of > 15 IU ml⁻¹ and thus were retested for IgG antibody titre on a second follow-up specimen. Seven of the 23 (30.4%) cases showed a threefold rise in IgG titre with the follow-up specimen, suggesting an active *Toxoplasma* infection (data not shown). Four of the seven (57%) cases were also positive for IgM antibodies. These women were prescribed spiramycin throughout their pregnancy to

Table 1. Comparison of the VIDAS IgM and VIDAS IgG-avidity test results for a single serum specimen from 224 Kuwaiti pregnant women during the first 16 weeks of gestation

Avidity results	No. (%) of specimens with VIDAS IgM result		
	Positive (n=31)	Equivocal (n=5)	Negative (n=188)
Low	9 (29)	1 (20)	7 (3.7)
Borderline	3 (9.7)	0	10 (5.3)
High	19 (61.3)	4 (80)	171 (91)

prevent congenital transmission of infection to the fetus. Of the 16 low-avidity IgG-positive specimens, 9 were also IgM-positive, and 5 showed a two- to threefold rise in IgG titres in the second follow-up specimen collected at a 3-week interval; however, 2 specimens did not show any significant change in IgG titres in the second specimen. Follow-up specimens from the two women with IgG-equivocal levels showing low avidity did not show any significant rise in IgG titres (data not shown). Based on avidity test results alone, at least 4 of these 18 (22%) women with low-avidity test results (2 IgG-positive and 2 IgG-equivocal levels) may have been wrongly labelled as having acute *Toxoplasma* infection.

Thirty-one (13.8%) women were IgM-positive; however, only 9 of these 31 (29%) women had low-avidity IgG antibodies suggesting a recent infection (Table 1). Nineteen of the 31 (61.3%) IgM-positive women showed high-avidity IgG antibodies indicating that the infection was acquired in the distant past. Furthermore, follow-up sera did not show any significant change in IgG antibody titre. All these women had uneventful pregnancies and delivered normal healthy babies. Comparative results of the VIDAS IgM and IgG avidity tests are presented in Table 1.

PCR analysis was performed on the 41 selected women that required further analyses for confirmation of their *Toxoplasma* infection status. These included seven IgM-negative women with low-avidity antibodies, two women with equivocal IgG levels with low-avidity antibodies, ten IgM-negative and three IgM-positive women with borderline-avidity antibodies, and 19 IgM-positive women with high-avidity antibodies (Table 2). These women were selected because the *Toxoplasma* infection status could not be defined by the IgG/IgM antibody and avidity assays. PCR analysis detected *Toxoplasma* DNA in one IgM-positive case with borderline-avidity antibodies and in five of the seven IgM-negative women with low-avidity antibodies. None of the 19 IgM-positive women with high-avidity antibodies showed any *Toxoplasma* DNA (Table 2). Comparing with PCR analysis, five of the seven IgM-negative women (71%) with low-avidity antibodies showed *Toxoplasma* DNA ($P < 0.05$); all of the 19 IgM-positive women with high-avidity antibodies were negative for *Toxoplasma* DNA (specificity 100%).

Socio-demographic data collected did not specify any specific risk factor in women with active *Toxoplasma* infection. All women with suspected active *Toxoplasma* infection were prescribed spiramycin throughout their pregnancy. The newborn were screened for active *Toxoplasma* infection and monitored accordingly for an extended period of 18–24 months.

DISCUSSION

The diagnosis of primary toxoplasmosis in pregnant women early in the first trimester is of utmost importance in order to offer them early therapy or other interventions to prevent congenital infection of fetuses (Akoijam *et al.*, 2002; Emna *et al.*, 2006; Reis *et al.*, 2006; Singh, 2003). Hence it is desirable to know the *Toxoplasma*-specific antibody status of a woman before or during the first trimester of pregnancy. The results described in this study show that the VIDAS IgG-avidity test, when used as a confirmatory test along with the VIDAS IgG/IgM tests in women during the first trimester, was highly useful in distinguishing a recently acquired infection from a chronic infection.

Routine serological diagnosis of toxoplasmosis provides high sensitivity, but specificity varies depending on the test used. In this study, 31 (13.8%) women in their early pregnancy 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 (Emna *et al.*, 2006). 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, only 9 of 31 (29%) IgM-positive women had low-avidity IgG

Table 2. Comparison of VIDAS avidity test and nested-PCR assay results to confirm ongoing or recent *Toxoplasma* infection in the selected group of pregnant women

Ig avidity	Serology status (no.)	Nested PCR analysis	
		DNA positive	DNA negative
VIDAS low-avidity	IgM-negative (7)	5	2
	IgG-equivocal (2)	0	2
VIDAS borderline	IgM-positive (3)	1	2
	IgM-negative (10)	0	10
VIDAS high-avidity	IgM-positive (19)	0	19
Total	41	6	35

antibodies suggesting a recent *T. gondii* infection in these women. More importantly, 19 (61.3 %) of the 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 (Gras *et al.*, 2004; Montoya *et al.*, 2002). The presence of specific *T. gondii* IgM antibodies in the chronic stage of an infection as observed in 61.3 % of the IgM-positive cases in this study may have resulted in unwarranted concern and a misdiagnosis particularly in women in early pregnancy.

Previously it has been reported that the VIDAS avidity test is highly sensitive and specific for detecting a recent *T. gondii* infection in IgM-positive cases (Petersen *et al.*, 2005). However, in sera with low- or borderline-avidity antibodies and negative IgM antibodies, the VIDAS IgG-avidity test was potentially misleading, if used alone. In this study, seven IgM-negative women with low-avidity antibodies were confirmed negative for *Toxoplasma* DNA on PCR analysis. These seven women continued their pregnancies and no congenitally infected newborn were delivered. Similar results have been reported in previous studies (Montoya *et al.*, 2002). Montoya *et al.* (2002) reported high-avidity antibodies in 74.8 % of the IgM-positive serum samples from pregnant women during the first 16 weeks of gestation. It is also known that the maturation of the IgG response varies considerably between individuals and thus low- or borderline-avidity antibodies may persist for months to more than 1 year (Singh & Pandit, 2004; Singh, 2003). A study from France found a mean IgG-avidity index of 0.2 in pregnant women infected within 5 months (Lecolier & Pucheu, 1993). 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 blood specimens of women before or during pregnancy (Chabbert *et al.*, 2004; Slawska *et al.*, 2005). 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 (Guy & Joynson, 1995). In this study, all the 19 IgM-positive women with high-avidity antibodies and 2 of the 3 IgM-positive women with borderline-avidity antibodies were negative for *Toxoplasma* DNA on PCR analysis, confirming the high sensitivity and specificity of the avidity test for detecting recent *Toxoplasma* infection in early pregnancy.

Generally most clinicians determine an active *Toxoplasma* infection by detecting *Toxoplasma*-specific IgM antibodies

and/or by detecting a threefold increase in IgG antibodies (IgM-negative) in pregnant women during the first trimester. However, relying solely on an IgG and/or IgM test to detect an acute infection may result in unnecessary interventions in pregnant women. Using nested-PCR analysis to detect *Toxoplasma* DNA to confirm the ongoing or recent infection, our data further validate earlier studies showing that the avidity test represents an additional confirmatory method, most useful if high-avidity antibodies are detected in IgM-positive women and also in IgM-negative women with low-avidity antibodies. Our study further validates that the avidity test should not be used as the only confirmatory test for pregnant women with IgG and/or IgM antibodies because of the potential to misinterpret low- or borderline-avidity antibody results. Confirmatory testing for ongoing or recent *Toxoplasma* infection with the VIDAS IgG/IgM antibody test and the VIDAS avidity method in pregnant women during the first 16 weeks of gestation has the potential to decrease the need for follow-up sera and for unnecessary therapeutic intervention in pregnant women.

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