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APPRAISAL OF PRENATAL ANTI-*TOXOPLASMA GONDII* (IGG+IGM)-IHA/IGM-ELISA SCREENING IN SINGLE SAMPLES VIA IGG AVIDITY TEST

BY

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Abstract

Congenital toxoplasmosis is associated with important morbidity and mortality. Since vertical transmission of *Toxoplasma gondii* can occur in acute cases, antenatal screening for recent infections is vital. Accurate determination of acute toxoplasmosis requires a combination of immunoassays, usually not routinely applied for screening purposes. This study evaluated the anti-*T. gondii* (IgG+IgM)/IgM prenatal screening procedure by IgG avidity assay.

The routine prenatal screening for (IgG+IgM) anti-*T. gondii* by indirect hemagglutination (IHA) in serum samples was done of 2247 pregnant women who attended two hospitals between 2011 and 2013 revealed 487 (21.7%) positive samples. Examination of IHA-positive sera by IgM and IgG/IgG-avidity concurrent ELISA tests revealed 7 positive and 3 border-line IgM-ELISA titers during the initial check-up of 10 women, who were then followed up at 3-4 week-intervals. Among these, 4 (40%) showed simultaneous high avidity IgG antibodies, indicating distant infection by the parasite, and no anti-*T. gondii* specific IgG could be detected in follow-up sera of two cases (20%), indicating false IgM initial positive results. Only 4 (40%) women showed simultaneous IgM and low avidity IgG antibodies indicating active infections. Avoidance of an overdiagnosis of acute toxoplasmosis Anti-*T. gondii* (IgG+IgM)/IgM prenatal screening must be supplemented by a discriminative test like IgG avidity ELISA.

Keywords: Antenatal, Toxoplasmosis, Screening, Hemagglutination, IgM-ELISA, IgG avidity

Introduction

Toxoplasmosis is one of the most frequent zoonoses in the world. It is caused by *Toxoplasma gondii*, an obligate intracellular protozoan parasite infecting man and almost all warm-blooded animals (Montoya *et al.*, 2010). Infection is mainly acquired via oral, by consumption of raw and undercooked meat containing tissue cysts and ingestion of water and food contaminated with oocysts, or by transplacental passage of tachyzoites (Montoya *et al.*, 2010; Dubey and Jones, 2008).

Nearly one third of humanity carries the parasite, but most of infections remain asymptomatic (Sensini, 2006). However, severe symptoms may occur via acute infection or reactivation of latent infections among people with acquired immunodeficiency syndrome or under other immunosuppressive condition (Jones *et al*, 2001; Hill *et al*, 2005; Montoya *et al.*, 2010).

Congenital transmission is associated with abortions or major fetal lesions including malformation, blindness, deafness, mental retardation, hydrocephalus and other neurological sequels (Kravetz and Federman, 2005;Thiebaut *et al*, 2007). The rate of maternal-fetal transmission varies considerably according to the time during

gestation that the mother became infected; it increases considerably with pregnancy duration, varying from 6% at 13 weeks of gestation to 60-80% in late pregnancy (Dunn *et al.*, 1999; Remington *et al*, 2006). Primary maternal toxoplasmosis, if acquired during the first trimester of pregnancy, vertical transmission causes severe feto-placental infections that generally lead to miscarriage or major fetal lesions. Pregnant women who seroconvert in their third trimester of gestation are more at risk of having infected offspring presenting clinical signs that mainly consist of toxoplasmic chorioretinitis. Complications may also appear later during childhood or early adulthood and occasionally jeopardize visual function (Dunn *et al*, 1999; Kravetz and Federman, 2005). The prenatal screening for toxoplasmosis in pregnant women is particularly important (Montoya and Remington, 2008).

Several laboratory tests are available for detection of *T. gondii* infections. Serological tests for detection of anti-*T. gondii* specific antibodies are the most used, particularly for screening purposes (Montoya, 2002;Almogren, 2011). To discriminate between recent (acute) and distant (chronic) infections in pregnant women, a combination of serological tests is normally required. Numerous procedures applied for detection of IgG, IgM, IgA, IgE and/or IgG avidity antibodies have been employed in combinations for this purpose (Sensini, 2006). Simultaneous sero-examination of anti-*Toxoplasma* specific IgG and IgM antibodies is usually the initial approach for antenatal screening. But, as anti-*T. gondii* IgM antibodies may persist in peripheral blood for months or even for more than a year after development, their presence is not confirmative of acute infection (Liesenfeld *et al*, 1997). Also, natural antibodies, predominantly of IgM class, that may occasionally be present in absence of *T. gondii* infection, are believed to react with the parasite's antigens (Konishi, 1987; Sensini, 2006). However, the absence of IgM antibodies is considered exclusive of recent infection, unless an individual is examined only few days post-infection when antibody response is not yet detectable (Pelloux *et al*, 1997). The anti-*T. gondii* specific IgM-positive sera by further tests like IgG avidity ELISA may elucidate the findings. The presence of high avidity anti-*T. gondii* specific IgG antibodies exclude *Toxoplasma* infection during the preceding 3–5 months, depending on the employed test. But, detection of low and borderline IgG-avidity antibodies does not confirm a recent infection because these may persist for more than a year post-infection. Furthermore, it is suggested that maturation of IgG avidity is affected by antibiotic treatment (Cozon *et al.*, 1998). Although toxoplasmosis immune-analysis results may be a labyrinth, approved guidelines for the interpretation of serological tests are found in the literature (NCCLS, 2004).

The present study aimed to evaluate the routine indiscriminative procedures still widely employed for the toxoplasmosis screening among pregnant women, usually based on a systematic serological examination of anti-*T. gondii* IgG and IgM using separate immune-assays or the indirect (IgG+IgM)-IHA test anti-*T. gondii* specific IgM-ELISA in single samples (Almogren, 2011). Positive-sera by (IgG+IgM)-IHA and IgM-ELISA, were further examined by an IgG/IgG-avidity concurrent ELISA test. Also, IgM-positive and doubtful cases were followed up at 3-4 week-intervals in order to differentiate recent from distant infections.

Subjects, Material and Methods

The serum samples from 2247 pregnant women who attended antenatal units in two health care centers at Makkah City between 2011 and 2013 for routine check-up at gestational weeks varying from 6 to 13 were analyzed for anti-*T. gondii* specific (IgG+-IgM) antibodies by indirect hemag-

glutination technique using Cellognost Toxopla-smosis H commercial kit (Siemens Health-care Diagnostics, Germany); the test was considered positive if a layer of agglutinated erythrocytes was formed in wells at serum dilutions of 1:64 or higher. Positive and negative control sera, provided in the kit, were included in each assay.

IHA (IgG+IgM) positive cases were tested for anti-*T. gondii* specific IgM antibodies using a commercial ELISA Kit (Enzywell Toxoplasma IgM, Disease Diagnostica Senese Italy) according to the manufacturer's protocol. Positive, negative and cut-off controls, provided in the kit, were included in each assay. All specimens were tested in duplicate. A sample was considered positive, if the ratio between optical density (OD) value and the cut-off was >1.2; negative, if OD ratio (ODr) was <0.8; and doubtful, if ratio was between the two values.

Pregnant women, whose initial prenatal check-up serum samples were found IHA-positive, were further examined for IgG avidity degrees using a commercial ELISA kit (Enzywell, Diesse Diagnostica Senese Italy). Avidity dissociating buffer-treated samples and non-treated samples were tested at 1/100 dilutions in parallel with avidity test controls supplied in the kit. IgG avidity was determined in base of the ratio between the OD readings of avidity buffer-treated sample and non-treated sample, subtracting the value of the test blank, and expressed in percentage. An avidity ratio (Ar) over 35% reflects the presence of high avidity IgG antibodies in the sample, less than 30% ratio indicates the presence of low avidity IgG antibodies, and a ratio between both values was considered as borderline. Following the recommendations of the manufacturer, non-treated 1/100 diluted serum samples showing an OD $\leq$ 0.4 were repeated at 1/25 dilution. In the present study, OD obtained for non-treated wells were considered as an anti-*T. gondii* IgG ELISA test results in which a cut-off value was calculated as the mean OD of four negative samples included in the test plus 2SD (Standard deviation).

Follow-up specimens were obtained at 3-4 weeks intervals from women with positive or equivocal IgM-ELISA results at initial prenatal check-up, and examined simultaneously by IHA, IgM-ELISA & IgG-avidity.

Results

Of 2247 pregnant women at gestational weeks from 6 to 13, 487 (21.7%) tested positive for anti-*T. gondii* specific (IgG+-IgM) antibodies by IHA assay, indicated a prior infection with *T. gondii*. anti-*T. gondii* IgM-ELISA analysis of IHA-positive sera showed 477 negative, 3 doubtful, & 7 positive cases indicated possible acute *Toxoplasma* infections (Tab.1).

The IHA-positive sera by anti-*T. gondii* IgG and IgG-avidity concurrent ELISA test revealed 472 IgG-positive samples (472/2247: 21.0% anti-*T. gondii* IgG seroprevalence) and 15 IgG-negative cases, of which one was positive by anti-*T. gondii* IgM-ELISA and 14 IgM-negative. 455/487 (93.4%) of IHA-positive cases showed high IgG-avidity ratios (Ar>35%), of which two were found positive and two with borderline levels of anti-*T. gondii* specific IgM antibodies. 7/487 (1.4%) IHA-positive cases showed borderline IgG-avidity ratios and IgM-ELISA negative results. 10/487 (2.1%) samples showed low IgG-avidity ratios, of which 4 cases were positive and one doubtful for anti-*T. gondii* IgM. 15/487 (3.1%) as mentioned above, IgG-ELISA negative cases (Tab. 2).

Among first group, a woman (TxM+3) showed negative IgG-ELISA at initial antenatal check-up (11 weeks post-conception) and in two more visits later at 15 & 19 weeks post-conception, meanwhile IgM-ELISA results were maintained positive along the follow-up. Other 6 women showed positive IgG levels in all follow-up specimens, and their IgM titers decreased with time and reached negative ELISA-

values for two of them, TxM+4 at 15 weeks & TxM+7 at 19 weeks post-gestational. Of them, two showed high avidity ratios since the first visit and 4 cases showed low avidity IgG antibodies at first check-up, but 3 of them converted to high avidity IgG antibodies during follow-up. Follow-up assessment of women with equivocal initial IgM-ELISA revealed one *Toxoplasma* negative case (TxMd2) at second visit by three immunodiagnostic assays, and two (TxMd1 & TxMd3) cases converted to negative IgM titers with maintained positive levels of IgG and high IgG avidity ratios along the follow-up.

Table 1: anti-*T. gondii* (IgG+IgM)-IHA, IgG-ELISA & IgM-ELISA results of single serum sample from pregnant women at gestational times earlier than 13 weeks.

Item	IHA-positive (n = 487)			IHA-negative (n=1760)	Examined samples (n=2247)
anti-*T. gondii*	Positive	Doubtful	Negative		
IgG-ELISA	472	-	15	untested	487
IgM-ELISA	7	3	477	untested	487

Table 2: anti-*T. gondii* IgM & IgG-avidity ELISA assessment of IHA-positive single serum samples of 487 pregnant women at their first antenatal checkup.

IgG-Avidity results	IgM ELISA results			Total
	Positive (§ODr>1.2)	Doubtful (0.8≤ODr≤ 1.2)	Negative (ODr<0.8)	
Low (£Ar<30%)	4 (0.8)	1 (0.2)	5 (1.0)	10 (2.1)
Borderline (30%≤Ar≤35%)	0 (0.0)	0 (0.0)	7 (1.4)	7 (1.4)
High (Ar>35%)	2 (0.4)	2 (0.4)	451 (92.6)	455 (93.4)
IgG-ELISA Negative	1 (0.2)	0 (0.0)	14 (2.9)	15 (3.1)
Total	7 (1.4)	3 (0.6)	477 (97.9)	487 (100)

Data: n (%),§ODr: OD/Cut-off ratio, and £Ar: Avidity ratio.

Table 3: (IgG+IgM)-IHA, IgM-ELISA, IgG & IgG-avidity concurrent ELISA follow up of pregnant women with anti-*T. gondii* IgM positive or equivocal primary test.

	P.W.	G.W.	IHA (titer)	IgM ELISA (ODr)	IgG ELISA (OD)	IgG Avidity (Ar)
Positive IgM cases	**TxM+1**	12	1:512	5.8	1.9	26%
		15	1:2048	4.4	1.8	24%
		19	1:512	3.2	1.4	28%
	TxM+2	8	1:2048	4.6	1.8	18%
		11	1:1024	2.8	1.5	41%
	TxM+3	11	1:128	3.1	0.2	-
		15	1:64	2.2	0.2	-
		19	1:128	2.7	0.3	-
	TxM+4	8	1: 1024	2.4	1.3	43%
		12	1:512	1.5	1.4	48%
		15	1:256	0.6	1.1	56%
	TxM+5	9	1:1024	2.1	1.6	22%
		13	1:512	1.7	1.2	40%
	TxM+6	9	1:128	1.8	0.9	46%
		13	1:128	1.5	1.0	63%
	TxM+7	13	1:256	1.7	1.3	24%
		16	1:256	1.4	0.9	39%
		19	1:256	0.7	0.9	47%
Doubtful IgM cases	**TxMd1**	8	1:128	1.1	0.8	49%
		11	1:64	0.6	0.7	64%
	TxMd2	10	1:64	0.9	0.5	21%
		13	Neg	0.5	0.3	-
	TxMd3	11	1:128	1.2	0.9	51%
		14	1:128	0.7	1.0	57%

P.W.: pregnant women, G.W.: gestational weekIgM-ELISA ODr: Neg.<0.8≤doubtful≤1.2<Pos., IgG-ELISA OD: Neg.<0.5<Pos., and IgG-Avidity ratio: Low<30%≤Borderline≤35%<High.

Discussion

The mortality and morbidity associated with congenital toxoplasmosis have prompted several studies involving serological screening for *Toxoplasma gondii* infection during pregnancy. Timing the onset of *T. gondii* infection is also crucial in pregnant women since congenital transmission is mainly associated with post-conceptional acquisition of the infection (Sensini, 2006). In this study we evaluated classical antenatal screening procedures still of use in many health care services, based on systematic serological examination of anti-*T. gondii* IgG and IgM antibodies either by separate immune-assays or by (IgG+IgM)-IHA combined test, followed by IgM-ELISA investigation in single samples. During antenatal toxoplasmosis follow-up, specific IgM antibodies can be seen approximately 14 days post-infection and decline to undetectable levels several months to more than a year later (Dunn *et al.*, 1999; Gras *et al*, 2004). In contrast, anti-*T. gondii* specific IgG antibodies start appearing approximately 14 days after the first positive IgM result (Montoya *et al.,* 2002), and persist indefinitely. Consequently, examination of anti-*T. gondii* specific IgG antibodies offers an efficient method for estimating the seroprevalence of *T. gondii* infection. In turn, examination of anti-*T. gondii* specific IgM antibodies is widely used to estimate the timing of infection, particularly in antenatal care units.

In the present study, of 2247 pregnant women who attended the hospital, 1760 pregnant ones were negative for anti-*T. gondii* specific (IgG+IgM) antibodies by the IHA test, showing no evidence of previous exposure to the parasite, and 487/2247 (21.7%) tested positive by IHA assay, indicating a prior infection with *T. gondii*. The IHA-positive sera by anti-*T. gondii* IgM-ELISA showed 477 IgM-negative sera, which were determined as solely IgG positive and consequently considered as chronic infections. 3/487 cases showed IgM borderline levels and 7 IgM positive results; these women would be considered respectively as suspicious and confirmed active *Toxoplasma* infections when screenings are limited to (IgG+IgM)-IHA/IgM-ELISA tests in single samples. Although, detection of anti-*T. gondii* specific IgM antibodies is generally considered as a sensitive indicator of an ongoing or recent infection, it must be interpreted either as a true-positive result indicating an active infection; a true-positive result indicating a chronic infection; or a false-positive result due to the presence of naturally acquired antibodies, predominantly of the IgM class (Montoya, 2002; NCCLS, 2004) . The variability between individuals in the duration of IgM positivity is notable; residual anti-*T. gondii* specific IgM antibodies can persist for months or even years after acute infection (Bobic *et al*, 1991; Liesenfeld *et al*, 1997). IgM positive results have been detected beyond 2 years in 9.1% to 27.1% women depending on the type of immunoanalysis used (Gras *et al*, 2004). Besides, false-positive results of IgM antibodies immunoassays were reported (Emna *et al*, 2006). Thus, diagnosis of primary infection should be improved by inclusion of other parameters such as IgG avidity, IgA, and IgE immunoassays or polymerase chain reaction for a conclusive diagnosis and timing of infection (Montoya, 2002).

In the present study, the IgG avidity ELISA test as an approach for determination of factual acute *T. gondii* infection in cases showed positive or equivocal IgM-ELISA results. IgG avidity analysis is broadly used for its ability to discriminate between recent and distant *Toxoplasma* infections (Lappalainen *et al*, 1993). The technique has been proven highly useful in antenatal screening for active toxoplasmosis, particularly in pregnant women presenting both anti-*T. gondii* IgG and IgM antibodies in their sera (Liesenfeld *et al*, 2001). In such cases the

analysis of a second serum sample after 3 weeks is also recommended (Sensini, 2006). Accordingly, we collected further samples from the IgM positive (TxM+) and IgM borderline (TxMd) cases at 3-4 weeks follow-up intervals. Anti-*T. gondii* specific IgG antibodies functional affinity in individuals with primary infection is low during the first weeks post-infection, but increases during subsequent weeks and months by antigen-driven B-cell selection (Cozon *et al*, 1998; Pelloux *et al*, 1998). In the present study, avidity ratios with a value Ar>35% was associated with distant infection and could absolutely exclude an acute toxoplasmosis condition. Two IgM-positive specimens (TxM+4 & 6) and two cases with initial IgM borderline levels (TxMd1 & 3) showed high IgG avidity values at their first check-up during their first gestational trimester, which indicates a distant infection, previous to conception. Although the time required for conversion from low to high avidity antibodies depends on the method used, in pregnant women, the presence of high avidity IgG antibodies is highly predictive of past infection and excludes the possibility of infection during the last 3-5 months (Sensini, 2006; Montoya and Remington, 2008). The presence of IgM antibodies is not always an indication of a recent infection (Montoya *et al,* 2002; Gras *et al*, 2004). The presence of high avidity IgG antibodies was confirmed in follow-up samples of these cases and IgM levels decreased considerably with time. Thus, these cases could be considered as pre-conception infections without risk of trans-placental transmission of parasites.

The first visit serum sample of one IgM positive women (TxM+3) tested negative for anti-*T. gondii* IgG antibodies rising two possibilities, either a very recent infection or a false positive result; false-positive IgM antibody test results have been reported previously (Emna *et al.*, 2006). Examination of follow-up samples obtained 3 and 7 weeks later from this woman also tested IgG negative, indicating an anti-*T. gondii* IgM false positive case. Similarly, follow-up serum sample of one woman (TxMd2), with an initial check-up IgM-ELISA equivocal result, was found IgM and IgG negative by IHA and ELISA, indicating a false positive IgM initial result at her first check-up. Since most studies did not consider the overall precision of *anti-T. gondii* specific IgM antibodies detection immunoassays, their factual specificity might be overestimated (Liesenfeld *et al*, 1997). These two, IgM positive and borderline, cases are to be considered as negative for toxoplasmosis.

In the present study, four women (TxM+1, 2, 5 & 7) showed initially low avidity IgG antibodies in presence of IgM; low or borderline IgG avidity antibodies are known to persist for more than a year and, thus their unreliability for systematic discrimination between old and acute infections. But, presence of simultaneous low avidity IgG antibodies and anti-*T. gondii* IgM antibodies were highly predictive of recent infection with risk of congenital transmission in pregnant women (Montoya and Remington, 2008). Serological follow-up of these cases was imperative to confirm their infection status. Specimens obtained at intervals of 3-4 weeks later showed high avidity IgG antibodies in 3 of these women. Low avidity IgG antibodies were still detected 7 weeks later for one case (TxM+1), but a decreasing tendency of IgM & IgG levels was noted. These results confirmed the situation of recent *Toxoplasma* infection in the 4 cases at their first antenatal check-up. Even though, it was reported that maturation of IgG avidity may be influenced by antibiotic treatment, discordant opinions were found in the literature (Cozon *et al*, 1998).

Generally speaking, toxoplasmosis is a worldwide distributed disease, characterized by a complex epidemiology. The risk of infection for humans depends on their contact with infective oocysts in a contaminated environment and on the amount of tissue cysts located within consumed meat. The toxoplasmosis in pregnancy and females bearing

children are a worldwide health problem (CDC, 2016). In Arab countries congenital toxoplasmosis was reported as examples in Egypt (Saleh *et al*, 2014), Iraq (Razzak *et al*, 2005), Jordan (Obaidat *et al*, 2015), Kuwait (Iqbal *et al*, 2016), Lebanon (Fox and Bzik, 2002), Libya (Gashout *et al*, 2016), Somalia (Ahmed *et al*, 1988), the United Arab Emirates (Dubey *et al*, 2010), Tunisia (Khammari *et al*, 2010), and Yemen (Abdul-Ghani, 2011).

Conclusion

The detection of anti-*T. gondii* specific IgM proved to be a strong marker for recently acquired *Toxoplasma* infections, but as observed in this study, interpretation of positive or equivocal IgM-ELISA results during (IgG+IgM)/IgM antenatal single sample screening has shown to be a serious challenge. Complementing such procedure by an IgG/IgG-avidity concurrent ELISA proved to be an excellent method for the discrimination between distant and recent infections and detection of the false IgM-positive cases.

The evaluation of 3-4 weeks follow-up specimens would be the ideal procedure to ascertain the stage of the *Toxoplasma gondii* infection and in consequence to limit over-diagnosis of acute cases and avoid unnecessary interventions.

The health education about toxoplasmosis should be tailored to women of childbearing age may help to prevent its complications.

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