Serological and molecular diagnosis of *T. gondii* in complicated pregnant Egyptian women

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**ABSTRACT**

In pregnant women, primary *Toxoplasma* infection or reactivated chronic infection can lead to transplacental transmission with potential risk of congenital toxoplasmosis depending on the stage of pregnancy. Blood and matched placental and fetal tissue samples were collected from one hundred and six Egyptian women from the labor ward of Beni-Suef General Hospital and Kasr El-Eini Hospital-Egypt, which are suffering from complicated pregnancy. The study includes G1, G2, G3 and G4 are symbolized the abortion corresponding to the 1st, 2nd and 3rd trimesters with congenital anomalies. All women were assayed serologically by IgM and IgG ELISA and molecular identification by PCR for confirming *T.gondii* infection. The sero-positive total percentage was 42.4%, 35% and 33% using ELISA, Latex agglutination and PCR respectively. It was concluded that all *T.gondii* sero-positive women were harboring DNA or compatible placental cysts; denote that serological assay was not accurate alone for confirming toxoplastic abortion.

**Keywords**: *Toxoplasma gondii*, complicated pregnancy, Egyptian isolates, Latex, ELISA, PCR.

**INTRODUCTION**

In pregnant women, primary *Toxoplasma* infection or reactivated chronic infection secondary to immune-suppression can lead to transplacental transmission with potential risk of Congenital toxoplasmosis results in manifestations varying in severity from asymptomatic infection to spontaneous abortion or fetal death to severe congenital defects depending on the stage of pregnancy (Innes, 2010; Robert-Gangneux and Dardé, 2012). Pregnancy is an exceptional immune-suppression condition attributable to hormonal shift where sharp progesterone level deviation thinks to stimulate silent bradyzoites to motivate the recurrent acute symptomatic stage toward tachyzoites re-conversion (Carneiro et al., 2009 and Elfadaly et al., 2012). Parasite isolation is so difficult. Therefore, laboratory diagnosis mainly based on DNA detection by Polymerase Chain Reaction (PCR) or by detection of IgM and IgG immunoglobulin titers, which can differentiate between recent and chronic infection.
especially in pregnant women, but few studies have focused on the direct detection of parasites in the placenta (Weiss and Kim, 2007 and Robert-Gangneux et al., 2010). However, no laboratory test performed alone is self-sustained, but the combination of serological, parasitological and molecular techniques is important for firm diagnosis of toxoplasmosis (Pignanelli, 2011). IgM antibodies are characteristic marker of acute infection and begin within the first week of infection, rapidly increase, reaching peak after 2 weeks then a plateau within 1 month. Levels of specific IgM antibodies usually decrease after 1-6 months where they become negative in 25% of patients within less than 7 months but commonly remain detectable for a year or longer (Gras et al., 2004).

Detection of IgG without IgM defines the classical serological pattern of past infection at least from six months (Montoya, 2002). During the 1st or 2nd trimesters of pregnancy, this most often reflects an infection acquired before the present pregnancy with no risk for congenital infection (Tenter et al., 2000). In the 3rd trimester, this result is more difficult to interpret. Although it is more consistent with an infection acquired before pregnancy, in some patients, this result may reflect an infection acquired early in gestation that was accompanied by an increase in the IgM titer and a decrease to non-detectable levels within a relatively brief period of time (Montoya and Remington, 2008).

The present study deals with diagnosis of toxoplasmosis among complicated pregnant women by serological and molecular techniques.

PATIENTS, MATERIALS AND METHODS

Study population

A cross sectional observational hospital-based study was conducted on one hundred and six females presenting with complicated pregnancy, with age range between 18 and 40 years. These patients were selected from the labour ward of Beni-Suef General Hospital and Kasr El-Eini Hospital in the period from July 2011 to October 2012. All studied cases were subjected to complete obstetric history using a questionnaire sheet.

The study population was classified according to the obstetric history of pregnancy into four groups:

- Group 1; females presenting with abortion in the 1st trimester [< 12 weeks of gestation] (No.=56)
- Group 2; females presenting with abortion in the 2nd trimester [13- 26 weeks of gestation] (No.=30)
- Group 3; females presenting with intrauterine fetal death [27 weeks of gestation to full term] (No.=15)
- Group 4; females gave birth to babies with congenital anomalies (No.=5)

Exclusion criteria included recent trauma, positive consanguinity, systemic diseases, Rh incompatibility and other risk factors suggestive of complicated pregnancy. The study was approved by Medical Research Ethics Committee of the National Research Center, and written consent was taken regarding this issue prior to the sample collection.

Collection of blood and tissue samples

For each studied case, Blood and tissue samples were collected. Venous blood samples (3 ml) were drawn aseptically from all enrolled cases into dry, sterile screw capped tube in the immediate post abortion or postpartum period. Collected blood samples were centrifuged at 3000 rpm for 5 min for sera preparations, and stored at [-20°C] until serologically assayed. Also, matched tissue samples were collected from the same cases.

For group 1: about 50 gm of the conception products were obtained by dilatation and curettage (D and C) (Dubey, 1998). For group 2, 3 and 4: about 50 gm portions from placentae were collected, washed three times in phosphate buffered saline (PBS), and divided into two compartments; the first was kept at the refrigerator (4C) till being digested and microscopically examined while the second compartment was kept frozen at (-80 C) for further Toxoplasma DNA detection (Fallahi et al., 2009).

Serological assay for T. gondii antibodies detection

A total of 106 serum samples were serologically assayed by Latex agglutination test (LAT) as a screening test for detection of total Toxoplasma immunoglobulins, according to the manufacturer's instructions (Toxocheck-MT; Eiken Chemical, Tokyo, Japan). It was considered positive when agglutination observed at dilutions of 1:64 and greater. While specific Toxoplasma IgM and IgG were detected using enzyme linked immunosorbent assay (ELISA), the original according to (Lind et al., 1997 and Hillyer et al., 1992).

Polymerase chain reaction (PCR)

The genomic DNA from Placental tissues collected from women was extracted DNA Extraction Kit (Vivantis Co., Malaysia).PCR Amplification of B1 Gene using primers 1 (5'-TCG GAG AGA GAA GTT CGTGC AT-3' and 2 (5'-AGC CTC TCT CTT CAA GCA GCG TA-3') (Burg et al., 1989).The following reaction mixture was added in a 0.2 ml PCR tubes: The reactions were set up to a final volume of 25 µL containing 2µL of Toxoplasma RH strain DNA as standard control or 10µL of tissue specimen DNA, 1 µL of each primer (100 pmol) and 12.5 µL Pyo-
Table 1. Average results of positive ELISA IgM and IgG immunoglobulin, LAT total immunoglobulin tests and positive PCR for detecting both anti-Toxoplasma antibodies and DNA in various four groups of complicated pregnant females.

<table>
<thead>
<tr>
<th>Test</th>
<th>Groups</th>
<th>Total/</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1/%</td>
<td>G2/%</td>
<td>G3/%</td>
</tr>
<tr>
<td>Total samples</td>
<td>56</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>+ve IgM ELISA</td>
<td>12 (21.4)</td>
<td>5 (16.7)</td>
<td>2 (13.3)</td>
</tr>
<tr>
<td>+ve IgG ELISA</td>
<td>17 (30.4)</td>
<td>8 (26.7)</td>
<td>7 (46.7)</td>
</tr>
<tr>
<td>+ve LAT</td>
<td>22 (39.3)</td>
<td>9 (30)</td>
<td>5 (33.3)</td>
</tr>
<tr>
<td>+ve PCR</td>
<td>18 (32.1)</td>
<td>10 (33.3)</td>
<td>6 (40)</td>
</tr>
</tbody>
</table>

Table 2. Interpretation of average IgM and IgG varied immunoglobulin in the 4 groups.

<table>
<thead>
<tr>
<th>IgM +ve / IgG -ve</th>
<th>G1/%</th>
<th>G2/%</th>
<th>G3/%</th>
<th>G4/%</th>
<th>Total/</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recent infection or acute opportunistic relapse. Require IgM follow up.</td>
<td>7 (12.5)</td>
<td>3 (10)</td>
<td>1 (6.7)</td>
<td>----</td>
<td>11 (10.4)</td>
<td></td>
</tr>
<tr>
<td>IgM -ve / IgG +ve</td>
<td>13 (23.2)</td>
<td>6 (20)</td>
<td>5 (33.3)</td>
<td>1 (20)</td>
<td>25 (23.6)</td>
<td>Old immune cases (≥3 YPI).</td>
</tr>
<tr>
<td>Active or immune cases (≤1 YPI). Need IgM/ IgG follow up.</td>
<td>5 (8.9)</td>
<td>2 (6.7)</td>
<td>1 (6.7)</td>
<td>1 (20)</td>
<td>9 (8.5)</td>
<td></td>
</tr>
<tr>
<td>IgM -ve / IgG -ve</td>
<td>31 (55.3)</td>
<td>19 (63.3)</td>
<td>8 (53.3)</td>
<td>3 (60)</td>
<td>61 (57.5)</td>
<td>Uninfected</td>
</tr>
</tbody>
</table>

Table 3. Correlation and compatibility between the average total positive and negative cases by PCR and ELISA.

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Positive%</th>
<th>Negative%</th>
<th>Total/</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>33 (94.3)</td>
<td>2 (5.7)</td>
<td>35 (100)</td>
</tr>
<tr>
<td>Positive</td>
<td>12 (16.9)</td>
<td>59 (83.1)</td>
<td>71 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>45 (42.5)</td>
<td>61 (57.5)</td>
<td>106 (100)</td>
</tr>
</tbody>
</table>

Statistical analysis

The results for the quantitative variables were expressed as percentages. The comparison of quantitative variables between different groups was done by Chi-Square. The data was considered statistically significant if P value ≤ 0.05.

RESULTS

Table (1) shows the highest toxoplasma positivity was for LAT (35.8%) followed by PCR (33%), ELISA targeting IgG antibodies (32.1%) and finally ELISA targeting IgM antibodies (18.9%).

Regarding the used groups, the highest sero-positivity was for G1, G3, G4 and G3 using IgM ELISA, IgG ELISA, LAT and PCR respectively. While the lowest sero-positivity was for G3, G2, G2 and G4 for the same tests respectively.

From table (2), Concerning the ELISA results, most of animals were uninfected (57.5%), followed by IgM –ve / IgG +ve ELISA (23.6%) as old immune cases, IgM +ve / IgG –ve ELISA cases (10.4%) as acute infections, while positive in both IgM and IgG ELISA were the lowest (8.5%). Moreover, G4 group were the highest in both positive and both negative IgM and IgG ELISA results. G1 were the highest in IgM +ve / IgG –ve ELISA results and G3 were the highest in IgM –ve / IgG +ve ELISA results.

From table (3), concerning the correlation between the results of both ELISA and PCR, the positive correlation between both tests was 94.3%. PCR negatively missed 12 positive ELISA samples, while ELISA negatively missed 2 positive PCR samples.

By comparing the positive results of ELISA and PCR, it was revealed that 33 PCR positive cases were also sero-positive; including 20 cases that were positive only for IgG antibodies, 5 cases that were positive only for IgM antibodies.
Table 4. Comparison between average results of PCR and ELISA IgM/IgG immunoglobulin.

<table>
<thead>
<tr>
<th>ELISA-variation</th>
<th>total</th>
<th>PCR compatibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive/%</td>
</tr>
<tr>
<td>IgM-positive</td>
<td>11</td>
<td>5(45.5)</td>
</tr>
<tr>
<td>IgG positive</td>
<td>25</td>
<td>20(80)</td>
</tr>
<tr>
<td>IgM and IgG positive</td>
<td>9</td>
<td>8(88.9)</td>
</tr>
<tr>
<td>IgM and IgG negative</td>
<td>61</td>
<td>2(3.3)</td>
</tr>
<tr>
<td>Total</td>
<td>106</td>
<td>35(33)</td>
</tr>
</tbody>
</table>

Figure 1. An agarose gel electrophoresis showing PCR amplification product of *T. gondii* using B1 gene. Lane N: Negative control, Lane P: Positive control, Lane M: 100 bp Marker, Lane 1--12: *Toxoplasma* positive PCR product (94bp).

antibodies and the 8 samples that were positive for both IgM and IgG antibodies while the remaining 2 cases were ELISA-seronegative (Table 4).

DISCUSSION

The present study concerns ELISA IgM and IgG sero-monitoring with PCR as a tools for comparing both *T. gondii* antibodies titers with the parallel DNA recognition among one hundred and six Egyptian women suffering complicated pregnancy at different trimesters.

The high *T. gondii* sero-prevalence (40-60%) of Egyptian pregnant women during last year's (Elfadaly, 2007; Hassanain et al., 2013), could be linked to higher prevalence of primary infection through undercooked meat, where feeding habits among the Egyptian population have been extra changed during the last years, due to the establishment of restaurants serving quick meat meals that may be insufficiently cooked Abd El-Razik et al., (2014). Confirming our concept, in the study of 131 mothers in the USA who had given birth to children infected with *T. gondii*, 50% recalled having eaten uncooked meat (Tenter et al., 2000).

Wang et al. (2003); Wyatt et al. (2005) confirmed spontaneous abortions occur in the first trimester and frequently decreases with increasing gestational age. The current study insure this concept, where the highest percent (52.8%) of aborted women was in their 1st trimester with consequence decline in the 2nd and 3rd trimesters (28.3% and 14.2%) respectively. While only (4.7%) were deliveries babies with congenital anomalies. This possibly related to hormonal factors, where, the acute opportunistic relapse during pregnancy possibly set sequence to sharp expolits of progesterone and estrogen levels. Wherever, the silent bradyzoites were liberating from tissue cysts and motivate the recurrent acute symptomatic stage through tachyzoites re-conversion (Roberts et al., 2001; Elfadaly et al. (2012) confirms the materno-fetal diffusion in pregnant rats depends mainly on pregnancy stages, and on estrogen/progesterone deviation.

The results signify that not all sero-positive women were harboring placental bradyzoites; Possibly due to
incompatibility between placental cysts distribution and the taken tissue samples or may be sequence to maternal antibodies against avirulant none cyst forming strains.

Although isolation of *T. gondii* tissue cysts from woman placenta is so difficult (Ferreira et al., 2011), but, success isolation was strongly correlates with fetal infection (Fricker-Hidalgo et al., 2007).

In the present study 57.5% women were sero-negative for both IgM and IgG immunoglobulins, representing the group at risk for developing acute toxoplasmosis at any time, require follow up of both IgM and IgG for accurate interpretation. Other 23.6% cases were positive IgG / negative IgM, indicate old unturmed chronically infected cases, reflect an infection acquired before pregnancy, which must be excluded as a cause of abortion, with high suspicion of other non-Toxoplasmic abortifacient agents.

On the other hand, another 10.4% cases were IgM positive / IgG negative, which confirmed either acute primary infection during pregnancy or acute opportunistic relapse, and are considered the most suspected group for developing toxoplasmic abortion in the current study. However, the most challenging situation occurs when IgG and IgM were positive and the serological status before pregnancy was unknown. In the present study, both immunoglobulins were detected in 9 (8.5%) cases. In four cases, IgM titre was higher than IgG titre which may indicate either primary or reactivated infection. But in two cases, IgG titre was more than IgM titre which means either immune status with decreasing IgM titre or novel reactivation of chronic latent infection. This group must be exposed to further follow up of IgM titre where additional elevation of IgM titre confirms latent opportunistic toxoplasmosis.

In the present study, LAT results were not accurate enough for interpretation or diagnostic purposes. Wherever, LAT test principally depends on the detection of total immunoglobulin via naked eye. Thus differ from one person to another; LAT was detecting lower seropositive results compared to ELISA (35.8% versus 42.4%). The results were accordance with numerous studies that were comparing LAT and ELISA (Elfadaly, 2002).

Although PCR is the most common molecular technique used for Toxoplasma diagnosis, it is typically limited to acute or reactivated infections but is of little value in chronic infections because parasite DNA will not be present in readily obtainable samples (e.g. peripheral blood) (Boothroyd, 2009). In the present study, PCR was performed on all 106 DNA samples extracted from aborted placental tissues using B1 gene which revealed that 35 (33%) of the studied women had Toxoplasma nucleic acid in their tissues. Similar results were detected by Abdel-Hameed and Hassanein (2004); El Askari (2007); Hassanain et al. (2013). In the present study, PCR positivity in two seronegative cases might be explained by delayed production of immunoglobulins which may not be produced until several weeks of parasitaemia as reported by Hussein et al. (2002). Previous studies confirmed that the presence of Toxoplasma in the placental tissues was the only argument at birth (IgM was negative) in 15% and 11% of toxoplasmosis positive cases (Fricker-Hidalgo et al., 2007; Robert-Gangneux et al., 2010) respectively. Negative Toxoplasma PCR in twelve seropositive cases (Table 4 and 5) could be attributed to the presence of PCR inhibitors (Terry et al., 2001), or possibly false sero-positive results due to cross-reactivity with closely related parasites (Chahan et al., 2003). 57.1% of the PCR positive women were only IgG positive. This may be explained by persistence of encysted forms of Toxoplasma in chronically infected uteri added to unfeasibility to separate the conception products from the placental tissues in aborted fetuses. Thus positive tissue PCR results might be due to maternal infection. Confirming this concept, Kaul et al. (2004) reported that PCR amplification isn’t very helpful in predicting the time of infection because the clearance of DNA from tissue samples isn’t well established. Also, Filisetti et al. (2010) reported that the placenta being a feto-maternal organ, PCR can’t identify the maternal or fetal source of infected cells.

We are concluded that; all *T.gondii* sero-positive women were harboring DNA or compatible placental cysts; denoting that serological assay was not accurate alone for confirming toxoplasma as a cause of abortion. PCR detected *T.gondii* DNA in placental tissues, but not absolutely a confirmation of fetal infection.

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