Molecular and Immunological Detection of Toxoplasma Gondii among Sudanese Pregnant Women

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Abstract:

Background: Toxoplasma gondii protozoon is widely distributed around the world and can infect all mammals and birds. While acquired toxoplasmosis is usually asymptomatic in healthy subjects, acute infection during pregnancy may lead to abortion, stillbirth, fetal neurological and ocular damages. This study aimed to determine the molecular and serological prevalence of Toxoplasma gondii and associated risk factors in women attending antenatal care in Khartoum during period 2015 to 2016.

Method: This was a cross-sectional descriptive study carried out between April 2015 and October 2016 involving 200 pregnant women attending antenatal care units at selected health centers in

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Khartoum state Sudan to determine prevalence of toxoplasmosis among pregnant women using serological and PCR techniques.

**Results:** The positive cases of T. Gondii recorded 26 (13%) by Molecular Technique PCR with cut off level of IgG 9.35 with significant P. value 0.0000001 using independent T test which shows Negative PCR tests were 174 (6.02471±1.35242). In contradiction with IgG antibody, IgM antibody and PCR test showed insignificant association with p value of (0.549).

**Conclusion:** PCR is considered to be the golden standard test for Toxoplasmosis gondii. Advocating for the implementation of control and preventive measurements and routine screening services for T. gondii infection to be integrated with other antenatal care services to identify potential effects of the infection and its management should be considered as the main strategy to minimize congenital toxoplasmosis and abortion.

**Keywords:** Toxoplasma gondii, Toxoplasmosis, Molecular diagnosis, IgG, IgM, PCR (Polymerase chain reaction)

**INTRODUCTION**

The protozoan parasite Toxoplasma gondii (T. gondii) is one of the most common parasites worldwide due to its ability to infect all warm blooded animals including humans. Serological Detection of T. gondii technique involves the detection of T. gondii antibodies in the serum of infected patients. Key among the serological methods employed in diagnosing toxoplasmosis include the Sabin-Feldman Dye test, the Indirect Hemagglutination assay, the Indirect Fluorescent antibody assay (IFA), the direct agglutination test (DAT), the Latex agglutination test (LAT), the Enzyme-Linked Immunosorbent assay (ELISA) and the Immunosorbent agglutination assay test (IAAT).

According to Hill and Dubey (2002), the most effective and preferred method and hence the gold standard for diagnosing toxoplasmosis is the Sabin-Feldman Dye test. This method is extensively used due to its high sensitivity and specificity for toxoplasmosis (1). However, it is costly and highly hazardous when used on live humans compared to ELISA (1,2). The initial evaluation is
to analyse toxoplasma IgG, IgM antibodies and IgG avidity. If toxoplasma infection is suspected in pregnancy, a new sample, ideally after three weeks, should be analysed for immunological changes and confirmation of the result. IgG seroconversion and at least a two-fold rise in IgG amount indicates primary infection. IgG and IgM positivity and low IgG avidity indicate the possibility of a primary infection (3,4). The strength of the antibody binding to the antigen (-avidity) increases with time. Different tests measure the antigen binding avidity of IgG antibodies. The method is most commonly used in order to decide whether the pregnant woman acquired the infection prior to conception or during pregnancy. Avidity cannot give precise information on the duration of the infection, but high avidity clearly indicates the occurrence of infection more than four months earlier. Low avidity indicates a possible but unconfirmed primary infection since low avidity in some patients may last for several months (5,6,7) The presence of T. gondii in a specimen can be confirmed by detection of the parasite DNA with the PCR method. In 1990, Grover et al. described the efficacy of the PCR method for rapid diagnosis of congenital infection. PCR has been performed on amniotic fluid after amniocentesis with sensitivity 59 - 97% and specificity 94 -100% depending on gestation, method and laboratory. In the study by Jenum et al., the sensitivity was 59% and the specificity 94% (8). Conventionally, the B1 gene of the parasite has been the target of PCR, but recently the 529-bp fragment has been shown to be more informative. The sensitivity has been found to be highest in the second trimester and lowest in the first trimester (9,10,11,12).

The aim of this study to detect prevalence of toxoplasmosis infections among Sudanese pregnant women by Serological and Molecular Techniques PCR.

MATERIALS AND METHODS

This was a cross-sectional descriptive study which was carried out between April 2015 and October 2016 among 200 pregnant women who attended health care centers located in Khartoum state. With those who agreed to participate by signing the consent form were included in the study while subjects with autoimmune disorders or those in immunosuppressive therapy were excluded from this study.
Furthermore, regarding collection of Data, the study participants were interviewed by administration of a standard questionnaire to obtain the socio-demographic and economic status information as well as epidemiological risk factors. As for detection of T. gondii antibodies (IgM and IgG) using ELISA assays, 5mL of venous blood were collected aseptically from each of the included pregnant women and divided into two EDTA anticoagulated containers. Plasma was separated from the whole blood in one container by centrifugation at 3,000 rpm for 5 minutes.

**Detection of T. gondii infection by nested polymerase chain reaction**

Real Time PCR was conducted using a pair of T. gondii-specific primers:

GAR6-F1: 5’-ATTTGTGTGTTTCCGAGCAGGT-3’ and R1: 5’-GCACCTTCGCTTTGTTGTT-3’.

Nested-PCR was performed with primers:

GAR6-F2: 5’-TTCGAGGACGGTGACCT-3’ and R2: 5’-TCGCCGAAGAGTTGACATAG-3’ (15).

Amplifications were conducted in a final volume of a 20 μL reaction mixture that contained 10 μL of 2x Taq DNA polymerase Master Mix with 2 mM MgCl2 (Cat. no. A170301, Ampliqon, Denmark), 10 pmol of each primer, 5 μL of distilled water, and 3 μL of template DNA. For nested-PCR, one μL of the first round PCR product was used as the template. For each reaction, two positive controls (DNA extracted from T. gondii paraffin-embedded tissues and the RH strain of T. gondii) and a negative control (double distilled water) were included. Amplification was performed with initial denaturation for 5 minutes at 95°C, followed by 35 cycles at 95°C for 30 seconds (denaturation), annealing at 59°C in the first round, and 57°C in nested PCR for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 10 minutes. A total of 5 μl of nested-PCR products along with a 100-bp DNA ladder were electrophoresed in 1.5% safe stain (2) agarose gels and visualized under ultra-violet trans-illumination.
Genotyping of positive samples by restriction fragment length polymorphism
Positive samples were genotyped using GRA6 and SAG3 markers (13). First, we digested the nested-PCR products of GRA6 positive samples using TruI (MseI) restriction enzyme (Cat. No. ER0982, Thermo Fisher Scientific, USA) as previously described (2). Digestion was conducted in a final volume of 16 μL reaction mixtures that contained 5 μL of the nested-PCR products, 1μL of TruI enzyme, 1 μL of 10X Buffer R, and 9 μL of nuclease-free water. Then, the reaction mixtures were incubated at 65°C for 1 hour according to the manufacturer’s instructions. A total of 10 μl of restriction fragments were electrophoresed by Tris-acetate-EDTA (TAE) buffer through 3% (w/v) agarose gel stained with safe stain and visualized under UV transillumination. We conducted genotyping of the positive samples by the SAG3 marker (2,13). Nested-PCR was carried out for positive samples using the SAG3 marker as previously described (14). Next, the products were digested using BcnI (NciI) restriction enzyme (Cat. No. ER0061, Thermo Fisher Scientific, USA) at 37°C for 6 hours according to the manufacturer’s protocols. The restriction fragments were electrophoresed and visualized under UV transillumination. The type of T. gondii was determined according to the restriction patterns after digestion with restriction enzymes (2,13,15). In order to determine better illustration patterns of the genotypes, the GRA6 and SAG3 sequences of three types of T. gondii (RH type I, ME49 type II, and NED type III) were obtained from GenBank and digested by their restriction enzymes using NEBcutter V2.0 (39).

Nucleotide sequence analysis of the GRA6 gene
We extracted two positive nested-PCR products of the GRA6 gene from the gel (Vivantis Gel Purification kit, Selangor DarulEhsan, Malaysia) according to the manufacturer’s instructions. The products were sequenced in the forward and reverse directions by the Sequetech Corporation (Mountain View, CA, USA), edited with BioEdit software, (33) and compared with GRA6 partial sequences of T. gondii available in GenBank.

For analysis of data, Statistical Package for Social Sciences software, version 23.0 (IBM SPSSInc., Chicago, IL) and STATA 11 were used. Initially, all information gathered via Data master sheet and Interview based questionnaire then coded into variables. Both
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descriptive and inferential statistics involving Pearson Fishers exact test, Independent T-test, Mann-Whitney U-Test, binary logistic regression and ROC curve for specificity and sensitivity were used to present results. A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In both tables [1] and table [2], PCR testing to detect IgG antibody shows great significance with p value of 0.0000001 (p value less than 0.05) by using different types of statistical tests such as independent T test that illustrated that Negative PCR tests were 174 (6.02471±1.35242) and the positive were 26 (86.28846±35.780769) and the Mann-Whitney statistical U-Test that showed negative samples were 174 with mean rank of (87.5) and positive samples with a mean rank of (187.5)In contradiction with IgG antibody, The measurement of IgM antibody by PCR test showed insignificant association as shown in both tables [3] and table[4] which also use different statistical tests. By using independent T test it shows that Negative PCR tests were 174 (0.02588±0.0557) and the positive were 26 (0.02258±0.017909) p value of (0.549) which is more than p value (0.05) and the Mann-Whitney statistical U-Test that showed negative samples were 174 with mean rank of (99.22) and positive samples with a mean rank of (109.04) and gave insignificant association of p value (0.419) which is more than p value 0.05

In Figure [1] the ROC Curve represents sensitivity and specificity of IgG and IgM antibodies by using PCR molecular diagnosis test. The smallest cutoff value is the minimum observed test value minus 1, and the largest cutoff value is the maximum observed test value plus 1. All the other cutoff values are the averages of two consecutive ordered observed test values.

It clearly shows that:

- IgG was considered as Positive test for toxoplasmosis if IgG ≥ 9.35000 (Sensitivity=100% and Specificity=100%) under area 100 % (CI 95%; 100%-100%) statistically significant with P value 0.0000001<0.5.
- IgM was considered Positive test for toxoplasmosis if IgM ≥ 0.1950 (Sensitivity=53.8% and Specificity=60.9%) under area
54.9% (CI 95%; 42.2%-67.6%) statistically insignificant with P value 0.065>0.5.

The coordinate of the curve states that the test result variable(s): IgM has at least one tie between the positive actual state group and the negative actual state group.

Table [1] Shows molecular detection of IgG antibody by PCR testing using independent T-test

<table>
<thead>
<tr>
<th>Variables</th>
<th>Number</th>
<th>IgG</th>
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<tbody>
<tr>
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<td></td>
<td></td>
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<tr>
<td>Negative</td>
<td>174</td>
<td>6.02471</td>
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<tr>
<td>Positive</td>
<td>26</td>
<td>86.28846</td>
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P value = 0.0000001**

Table [2] Shows molecular detection of IgG antibody by PCR testing using Mann-Whitney U-Test

<table>
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</tr>
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<tbody>
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<tr>
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<td>87.5</td>
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<tr>
<td>Positive</td>
<td>26</td>
<td>187.5</td>
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P value = 0.0000001**

Tables [3] Shows molecular detection of IgM antibody by PCR testing using independent T-test

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<tr>
<td>Positive</td>
<td>26</td>
<td>0.02258</td>
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P value = 0.549*

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Table [4] Shows molecular detection of IgG antibody by PCR testing using Mann-Whitney U-Test

<table>
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<th>Mean Rank</th>
<th>Sum of Ranks</th>
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<tr>
<td>Positive</td>
<td>26</td>
<td>109.04</td>
<td>2835</td>
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P value = 0.419*

Figure [1] ROC Curve that represents sensitivity and specificity of IgG and IgM antibodies by using PCR molecular diagnosis test.

DISCUSSION

In current study out of 200 samples, the overall seroprevalence of anti-T. gondii antibodies in women was 13%. A significant relation between non pregnant and pregnant women for IgG (P<0.05), the majority of IgG positive cases were observed in non-pregnant, as well as there were significant differences (P<0.05) between types of Toxoplasma antibodies for each non-pregnant and pregnant groups whereas the healthy women were 0% for all antibodies by ELISA technique. Although, in present study the highest seroprevalence was in anti-T. gondii IgG, this is due to that in T. gondii infection the maturation of antibody (T. gondii IgG) in response to infection generally are slow.
However, seroprevalence of toxoplasmosis in the present study was similar to many studies around the world and some Arabic countries in women with Bad Obstetric History (BOH), such as 44.8% 17, 35.1% 18, 41.9% 19 and 49.52% 20 (1.12.14,22, 23).

Although, the result of present study is agreement with other studies in Iraq, that deal with a seropositivity by using ELISA were 37.5% for IgG in Erbil. While, in Diyala province found high infection were 37 (46.26%) women out of eighty were infected with toxoplasmosis. A recent study in Iraq showed a different result than present study in which the ratio of infected female with toxoplasmosis was 330 (98.51%).

The present study revealed low anti- IgM in pregnant women, this finding is similar to the results of a study in Salah- Adden among pregnant women, the acute infection was 7(3.1%) of cases24 and in Zanjan, Northwest of Iran 38.6% (IgM and IgG were positive in 1.4% and 37.2% respectively).

The variation in the prevalence rates of T. gondii among studies can be attributed to the differences in the study populations and study areas, as well as the different diagnostic methods used for blood examination. Also, the differences in toxoplasmosis rates can be interpreted on the basis of similarity or differences of climatic conditions of location and that the various ages of patient’s study, nutritional and immune status, hygienic habits, sanitary supplies and socioeconomic conditions in study's region. In general, the prevalence of protozoans was strongly associated with a variety of risky factors including host, sociodemographic environmental and zoonotic transmission 24,25.26.27.28.29).

**CONCLUSION:**

The findings of this study demonstrated a relatively lower prevalence of molecular-seropositivity than studies reported from other countries. Existence of disease in rural areas with contact with of cats and animal at homes and farms, consumption of undercooked meat and unpasteurized milk were identified as risk factors for T. gondii infection (31,32,33). Therefore, a health education program to increase the mother’s knowledge about toxoplasmosis towards the source of infection, modes of transmission and prevention methods by avoiding
eating raw or undercooked meat, contact with cats and consumption of unpasteurized milk during pregnancy is recommended. Encourage the laboratories to implement Real time PCR as golden standard test for Toxoplasma Gondii (34,35,36,37). Advocating for the implementation of control and preventive measures and routine screening services for T. gondii infection to be integrated with other ANC services to identify potential effects of the infection and its management should be considered as the main strategy to minimize congenital toxoplasmosis. Furthermore, our results suggested that the implementation of newborn screening and follow-up testing can lead to reducing of toxoplasmosis associated complications (38).

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