Sero-genetic Variation for *Toxoplasma gondii* diagnostic among women with early spontaneous abortions.

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**Abstract:**

**Background:** *Toxoplasma gondii* is common foodborne parasite that generally infects wild, domestic and companion animals. Toxoplasmosis is traditionally identified by serum anti-*Toxoplasma* antibodies using serological methods, such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA). However, IFA and ELISA toxoplasmosis diagnosis are time exhausting, costly and these assays have variable sensitivity and specificity. Moreover, *T. Gondii* specific antigens and antibodies may not appear early, particularly immunodeficient individuals, and during parasite reactivation, specific antibodies, especially IgM, may not appear. Thus, serological methods have certain limitations. Contrary to technology of polymerase chain reaction (PCR), in which the reaction is executed with cycles or alternating chain of temperature processing. Amplification of isothermal does not demand a cycle or is performed at a stable temperature. Thus, the current work aimed to evaluate the challenge in *Toxoplasma* diagnosis in human sample.

**Material and methods:** One hundred thirteen enrolled women acquired by aborted women and 23 controls in Babel Hospital for Women and Children. All samples were checked by LAMP assay (SAG1, B1, and SAG2) and nPCR. Same sample was also tested by ELISA and enzyme-linked immunofiltration assay. **Results:** The presented LAMP method successfully diagnosed *T. gondii* infection; the LAMP results corresponded to those of indirect fluorescent antibody test (IFAT) and ELISA. In the nPCR assays, all samples were positive for *T. gondii*. This finding was completely opposite those of IFAT and ELISA. Furthermore, two bands were observed in 1.5% agarose gel electrophoresis of nPCR. **Conclusion:** The LAMP assay supplies easy, sensitive, specific and rapid technique for *T. gondii* genomic DNA diagnosis.

**Keywords:** *T. gondii*, LAMP, Nested PCR, DNA, Aborted women


1. **Introduction**

*Toxoplasma gondii* is common foodborne parasite that generally infects wild, domestic and companion animals. (1). It is the agent of toxoplasmosis, which is life-threatening to immunocompromised individuals (2). Toxoplasmosis is the cause of up to approximately 20% of the total foodborne disease burden, especially in...
Europe (3, 4). During human gestation, the disease can be serious, as the parasite can cross the barrier of the placenta and infect the embryo with severe and even fatal effects (5). Humans become infected by consuming food contaminated with faeces from the definitive host are cats, or by ingesting raw and undercooked flesh from infected animals. Microwaves use for quick cooking has become a widespread household practice, but certain parasites and bacteria reside in active even after microwave using (6). Due to clinical features of toxoplasmosis are often not specific, the diagnosis of disease is challenging. Thus, to detect the *T. gondii* early is significant the use of specific molecular and serological techniques (5, 7, 8, 9). Diagnosis of *T. Gondii* can be confirmed by isolation of parasite, examination of histological and serologically by IFAT (10), as well as by ELISA (11). Genetically, nPCR, rPCR and LAMP have been utilized to diagnosis DNA of *T. gondii* in material of animal (12), water (13), soil (14) and clinical specimens (15). The LAMP technique consider specificity, sensitivity and newly developed method (16). LAMP depend on large fragment Bst DNA polymerase with high strand separation and replication efficiency, which allows small traces of nucleic acid to amplification under isothermal temperature (60–65 °C). Set of primers at least four (2 outer and 2 inner) annealing with six parts target position to increases the specificity (16). This two loop primers allow reaction time minimized and sensitivity maximized (17). The most sensitive toxoplasmosis detection method has been reported followed by hybridisation was nPCR (18, 19). In the current research, three LAMP tests were developed targeting the SAG1, B1 and SAG2 genes *T. gondii* genetic DNA identifications. Blood specimens were utilized to estimate the sensitivities of the LAMP tests as compare with other techniques. The data presented here consider depends on our knowledge, the first study in Iraq.

2. Material and methods

2.1. Clinical samples

A total of 136 women recorded in Babel Hospital for Women and Children were studied. The first group included 32 aborted women with chronic toxoplasmosis (rise *Toxoplasma* IgG antibody titres) serologically determined by IFAT and ELISA. Commercial diagnostic kits of *T. gondii* IgM and IgG (Biotech, Trinity, Ireland). The second group consisted of 23 controls (10 normal unmarried women and 13 healthy normal delivery women). The last group included 81 aborted women with unknown or known causes of abortion other than toxoplasmosis.

2.2. ELISA (IgG and IgM)

Anti-*Toxoplasma* and anti-*Cytomegalovirus* IgG and IgM were measured by using an ELISA kit (Calbiotech, USA).

2.3. Immunofluorescence assay (IgG and IgM)

In serum, anti-Toxoplasma and anti-Cytomegalovirus IgG and IgM were revealed by utilizing enzyme-linked fluorescent assay (ELFA). In this report, IgG quantitative and qualitative was determined by automated VIDAS family instruments. The principle of this test joins technique of an enzyme immunoassay by immunocapture with final fluorescent exposure (ELFA). This test was established using a commercial kit (Biomerieux, France).

2.4. DNA extraction:

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DNeasy blood kit (Qiagen, Germany) was used to DNA Genome extraction from 200 µl solution in correspondence with the manufacturer’s instructions. 200 µl elution buffer was employed to elute Genomic DNA. The reactions were performed in triplicate.

2.5. LAMPassay:

The cycles to amplify three genes SAG1, B1, and SAG2, three LAMP primer sequences specific for *Toxoplasma* were prepared employing the software Primer Explorer (Table 1), were 25 µl of a mixture was done by each of primers BIP and FIP (40 pmol), DNA (2 µl), each of primers F3 and B3 (5 pmol) and Bst DNA polymerase (NEB) (1 µl) in master mix buffer (2.5 µl) for LAMP optimisation. The reaction of LAMP was established at 65°C for (30–60) min and at 80°C for 2 min (inactivated). By the SYTO-9 fluorescence dye, SYBR green I addition and visual detection, in real time the resulting amplicons were discovered. LAMP products was resolved by electrophoresis in 1.5% agarose gels as well as by photography.

2.6. nPCR

To accomplish nPCR, two pair’s primers were designed against the B1 gene, one of specific primer for diagnosis *T. gondii* (20). The first round mixture for PCR amplification contained from 10 mM Tris-HCl at pH 8.3; 1.5 mM MgCl2; 50 mM KCl and 0.1 µM of each primer (Table 1). In addition, 0.2 mM of each dNTP, 2 µl of DNA, and Taq DNA (2.5 U) polymerase. Denaturation (35 cycle at 94°C for 1 min), annealing (at 54°C for 30 s) and a final extension (1 min at 72°C) were cycled for all samples (controls and patients). Mixtures of nested PCR contained 2 µl of first-round products, 0.5 µM of each primer and 1 U Taq DNA polymerase. The nPCR was done by 35 cycle of 94°C for 10 s (a denaturation step), followed 60°C for 10 s (annealing step) and 72°C for 15 s (extension). Negative-control samples for two round, first and second-round amplification of sterile water were included in the PCR and nPCRs. The products of PCR were electrophoresed in agarose gel (1% TAE, Tris-acetate-EDTA) stained with 1 µg/ml ethidium bromide solution. Product of positive reaction for nested PCR was expected at 213 bp.

Table 1: LAMP primers nucleotide sequences in the current study.

<table>
<thead>
<tr>
<th>Technique primer</th>
<th>sequence (5’–3’)</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SAG1-LAMP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>CGAGGGTCAATGTAGTGGGT</td>
<td><em>T. gondii</em> SAG1 gene (GenBank)</td>
</tr>
<tr>
<td>F2</td>
<td>GACAACGCAGACGTTCGTTTGTG</td>
<td></td>
</tr>
<tr>
<td>BIP</td>
<td>AATGTGCAAGGGTGCTCTCTACGTCTCAGCACAGACACTTGACA</td>
<td></td>
</tr>
<tr>
<td>FIP</td>
<td>ACCGATGAGGGCTCTGCTTAGTGGTTGAGTCCGTTGCAATCAAGGGAG</td>
<td></td>
</tr>
<tr>
<td><strong>SAG2-LAMP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>GTAGCAGGACCTTTCGCG</td>
<td><em>T. gondii</em> SAG2 gene (GenBank)</td>
</tr>
<tr>
<td>F3</td>
<td>GCGCAACGAAAGACTTGTGGA</td>
<td></td>
</tr>
<tr>
<td>BIP</td>
<td>TGGCAAGGAAATGCACAGACTCGGACCTTACGTCTGTAAGACCCG</td>
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</table>
3. Results

All enrolled women aborted an healthy women, that was confirmed by three LAMP tests based on three genes, SAG1, B1 and SAG2, were showed negative results by using this techniques. All samples (from aborted women and controls)did not turn green upon the addition of SYBR green I to the PCR tube and instead remained orange (Figure 1). On the agarose gel all nPCR reactions samples created a multiple bands typical ladder (Figure 2).

The ages range of the aborted women involved in the research was 17–40 years. Nineteen subjects were under 20 years old, 65 were between 21 and 30 years old, and 29 were over 31 years old. According to the collected information, most of the aborted women (65.4%) were in the first trimester of gestation. In addition, 34.5% and 00.0% were in the second and third trimesters of gestation, respectively (Table 2). Furthermore, 28.3% of them did not have prior miscarriage experience whereas 71.6% had no history of diseases except abortion.
Figure 1: T. gondii detection in clinical and control samples by LAMP

The turbidity of the LAMP reactions was investigated visually. After the addition of SYBR green, negative reactions under UV light unturned green. Tubes 34–76 represent the clinical samples, and tube 77 represents the negative control.

Figure 2: nPCR products electrophoresis amplified from T. gondii tachyzoite genomic DNA. Lanes 1–36 represent the clinical samples, and lane M represents a 100-bp DNA ladder.

Table 2: Demographic features of mothers who had miscarriage and results of diverse diagnostic techniques.

<table>
<thead>
<tr>
<th>N</th>
<th>O</th>
<th>ELISA</th>
<th>VIDUS</th>
<th>Nested PCR</th>
<th>LAMP PCR</th>
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<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>17-20</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>19</td>
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<td>21-30</td>
<td>65</td>
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<td>0</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>31-40</td>
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<td>0</td>
<td>0</td>
<td>22</td>
<td>65</td>
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<td>Gestational age</td>
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<td></td>
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<td>74</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>29</td>
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<tr>
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<td>39</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>History of abortion</td>
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<td>No</td>
<td>81</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>32</td>
</tr>
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</table>

4. Discussion

The specific genes, B1, SAG1 and SAG2 were examined for detecting T. gondii, by used three LAMP tests and their results were compared with findings by nPCR and immunoflorescence test (IFT). The each route importance in toxoplasmosis human transmission is so far underdetermined due to infections induced by oocysts cannot be distinguished yet from those induced by tissue cysts using serologic investigations (21). The proposed
LAMP method successfully diagnosed \emph{T. gondii} infection. Current result is in accordance with those of Sotiriadou and Karanis\textsuperscript{22} (2008) and Lau et al.\textsuperscript{23} (2010), who also reported that the LAMP method demonstrated its effectiveness for the same purpose. In our result, all samples (136 control and aborted women) showed a negative reaction in LAMP method. This finding corresponds with the results of IFAT and ELISA. Furthermore, all aborted women with \emph{T. gondii} demonstrated IgG in their blood (not IgM), as tested via IFAT and ELISA. According to the questionnaire, all individuals in our study were immunocompetent and not suffering from any immunodeficiency or AIDS. Therefore, the serological examination results are accurate. By using electrophoresis, no results were obtained in LAMP products amplified from DNA of \emph{T. gondii} tachyzoite, which established our idea above Figure 1. Due to the four to six primers, the LAMP technique considers high sensitivity, additionally, the auto-cycling amplification can produce huge amounts of different sizes of DNA (24). DNA amplification could be achieved using simple incubators (water bath or block heater) due to the isothermal conditions in the LAMP assay. Thus, the LAMP test could be applied for field assays. LAMP assay its tolerance to extracted DNA, in additions for biological substances such as plasma, serum, and vitreous substances (25,26). Another characteristic of the proposed LAMP assay needless gel electrophoresis. They are differentiated easily by recognizing the turbidity of product in the tube. All samples in our results revealed no turbidity. Current result is agree with those reported by Mori et al.\textsuperscript{27} (2001), who recorded the turbidity because the LAMP assay produces magnesium pyrophosphate, which is visible as white precipitation, as a by-product. These features allow LAMP to be utilised as a precise and fast technique. On the basis of blood samples, in current project, we evaluated the clinical sensitivities and specificities (100%) of the LAMP tests and compared the results with those of nPCR. LAMP test results were showed that they have high susceptibilities in blood samples toxoplasmosis diagnosis. By contrast, nPCR demonstrated low sensitivities for the same purpose. In the nPCR assays, all samples were positive for \emph{T. gondii}. This result is completely opposite those of IFAT and ELISA. The reported nPCR is not specific for detecting \emph{T. gondii} in blood samples. Our data are not consistent with those obtained previously study (Angel et al.1997)\textsuperscript{28} utilizing the same technique with blood specimens from lymphadenopathy patients. Lau et al. (2010)\textsuperscript{29} also confirmed that all LAMP assays and nPCR are 100% specific (Figure 2). The series of techniques applied in this work (Table 2) showed that direct LAMP methods, especially in immunodeficient individuals, are dependable for \emph{T. gondii} diagnosis due to the lack of immunoglobulin production, which causes serological tests to become unreliable. These finding are coordinate with those reported by Matin et al. (2017)\textsuperscript{30}.

5. Conclusion

Our findings show that the reported LAMP methods can be utilised as a dependable and easily diagnostic technique of \emph{T. gondii}. LAMP is a relatively new DNA amplification method that could provide major advantages because its simplicity, specificity, sensitivity, ruggedness and low cost. It's a promising method that can provide quantitative findings comparable with those of nPCR.

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Competing interests:

The authors declare that they have no competing interests.
References:


