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ORIGINAL PAPER

A loop-mediated isothermal amplification (LAMP) assay for detection of *Toxoplasma gondii* infection in women with spontaneous abortion

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Abstract

The present study aimed to use the loop-mediated isothermal amplification (LAMP) technique in comparison with serological tests to determine the rate of *T. gondii* infection in women suffering from spontaneous abortion (SA). A total of 140 women suffering from their first SA were included in this study. The collected aborted fetal remains and blood samples from each patient were examined in sterilized conditions using the LAMP technique and ELISA. Of the 140 women, 80 (57.1%) tested seropositive for anti-*Toxoplasma* antibodies by ELISA, 72 (51.4%) women tested seropositive for the IgG antibody, 8 (5.7%) tested seropositive for the IgM antibody. Among the eight women who'd had their first SA who tested seropositive for IgM antibody by ELISA, only five cases (62.5%) reported positively to the LAMP test. The difference in the frequency distribution of the LAMP results for measuring the *Toxoplasma* infection in pregnant women under study was statistically significant ($P < 0.001$) from the results of the serological test (ELISA). Although there was a significant difference between age and positivity in the LAMP test ($P = 0.017$), no significant difference was observed between positivity in the LAMP test and other variables. The findings of the present investigation suggest that LAMP is a preferred method for determining *Toxoplasma* infection in pregnant women suffering from SA compared with other routine serological tests. Even in a field with limited facilities and equipment, this technique can be effective and efficient in accurately and specifically diagnosing *Toxoplasma* infections in women at high risk of SA.

Keywords ELISA · LAMP · Pregnant women · Spontaneous abortion · Toxoplasmosis

Introduction

Toxoplasmosis caused by *Toxoplasma gondii* is well known as one of the most widespread parasitic diseases, which has now infected nearly a third of the world's population (Gilbert 2000; Fallahi et al. 2014a; Kheirandish et al. 2016). The main ways to contract this disease are consumption of undercooked meat with *T. gondii* tissue cysts, drinking of water and food contaminated with excreted oocysts of cat feces, as well as congenitally (Dunn et al. 1999; Arab-Mazar et al. 2016a, b; Hanifehpour et al. 2019). Although toxoplasmosis is almost asymptomatic in healthy and immunocompetent people, it results in severe complications in immunocompromised individuals (Dunn et al. 1999; Kheirandish et al. 2016). Among the serious complications of toxoplasmosis, congenital infection is considered the most important complication in pregnant women, with a worldwide prevalence rate of nearly 200,000 cases every year (Remington et al. 2006). During pregnancy, especially in the first trimester,

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if a mother gets infected with the *T. gondii*, the parasite is capable of passing through the placental barriers and causing stillbirth or spontaneous abortion (SA) in the fetus (Robbins et al. 2012). SA, also known as miscarriage and pregnancy loss, is the natural death of an embryo or fetus before 20 weeks of gestation, after which fetal death is known as a stillbirth was reported in about 1 to 2% of pregnant women (Ford and Schust 2009). A number of factors, such as genetic factors, anatomical disorders, endocrine disorders, autoimmune syndromes, as well as infections can cause abortion (Nigro et al. 2011).

Among the infectious agents, *Chlamydia trachomatis, Ureaplasma urealyticum, Mycoplasma hominis,* cytomegalovirus, human papillomaviruses, and *T. gondii* are the main causes of SA (Singh et al. 2010; Torgerson and Mastroiacovo 2013). Previous studies have shown the association between toxoplasmosis and SA (Singh et al. 2010; Nigro et al. 2011; Torgerson and Mastroiacovo 2013; Elamin Elhag and Elturabi 2015; Fallahi et al. 2018; Nasirpour et al. 2020). However, there are few studies regarding the connection between toxoplasmosis and SA in Iran. The majority of studies regarding the prevalence of toxoplasmosis in SA have been carried out based on serological and some molecular tests, such as conventional polymerase chain reaction (PCR) (Zargar et al. 1998; Sharif and Ajami 1999; Torgerson and Mastroiacovo 2013; Saki et al. 2015; Matin et al. 2017; Fallahi et al. 2018; Soltani Tehrani et al. 2020; Valian et al. 2020). The loop-mediated isothermal amplification (LAMP) technique has some unique properties, such as high sensitivity and specificity, field usability, and isothermal reaction conditions, without the need for expensive equipment, such as thermal cycling. It is well known as one of the important techniques for determining microbial pathogenesis (Lau et al. 2010; Fallahi et al. 2014b, 2015; Rostami et al. 2018). The present study aimed to use the LAMP technique together with serological tests to determine the rate of *T. gondii infection* in women suffering from SA in Lorestan Province, Western Iran.

Materials and methods

Participants

A total of 140 women suffering from their first SA, and who had been referred to the Obstetrics and Gynecology Department, Asalian Hospital, Khorramabad, Iran, were investigated. The fact of SA was diagnosed by a gynecologist, based on a case history and clinical tests, and also from the results of a sonography, to rule out other possible causes of SA for example Rh-incompatibility, threatened abortion, incompetent cervix, as well as some uterine disorders. All the enrolled participants were informed about study and written informed consent was obtained. For women under 15 years of age, written consent was given to their spouse.

Samples collection

Aborted fetal remains and blood samples (5 ml) were collected from each patient in sterilized conditions and stored at a temperature of − 20 °C until testing. Moreover, a questionnaire including some demographic and risk factors such as the mother's age, gestational age, contact with cats, etc., was completed by each patient.

Serological test

The serum samples of all patients were examined to determine the specific IgM and IgG anti-*Toxoplasma* antibodies using ELISA commercial kits (de EIA de *Toxoplasma* IgG Foresight® ACON) according to the manufacturer's instructions.

Molecular evaluation by LAMP technique

In this study, the genomic DNA was extracted from the remains of aborted fetuses using a DNA Extraction Kit (Yekta Tajhiz Azma, Iran). The nucleotide sequences of four *Toxoplasma*-specific primers targeting the six conserved regions within the sequence of the B1 gene of 35-fold repeats used in the LAMP reaction are as follow:

F3: 5′–CAGATGTGCTAAAGGCGTCA–3'

B3: 5′–ACGTGACAGTGAAGAGAGGA–3'

FIP: 5′–AGGCGGAACCAACGGAAATCCTTGCTG TTCTGTCCTATCGC–3'

BIP: 5′–TGTTCGCTGTCTGTCTAGGGCAGGTGG TCGACTTCATGGGA–3′ (Lau et al. 2010).

The LAMP reaction mixture was prepared in 25 µl containing: 2 µl of template DNA, 40 pmol each of FIP and BIP inner primers, 5 pmol each of F3 and B3 outer primers, 8 U (1 µl) of *Bst DNA polymerase* (New England Biolabs (NEB), Ipswich, MA, USA) in 2.5 µl of buffer [20 mM Tris–HCl (PH 8.8), 8 mM $MgSO_4$, 10 mM $(NH4)_2SO_4$, 0.1% Tween 20], 10 mM KCl, 0.8 M betaine (Sigma-Aldrich), in addition to 1.4 mM deoxynucleoside triphosphates (dNTP). *T. gondii* RH-strain genomic DNA and distilled water (D.W) were used as positive and negative controls, respectively. Using a water bath, the reaction mixture of the isothermal LAMP samples were incubated at 65 °C for 60 min and then was inactivated at 80 °C for 2 min. The resulting amplicons were visually detected by adding 3 µl of 1:10 diluted 10,000 × concentration fluorescent dye SYBR Green I (Invitrogen Carlsbad, CA, USA) to the reaction tubes. In a positive LAMP sample, green fluorescence was observed, while in the negative one, it remained the original pinkish-orange (Fig. 1).

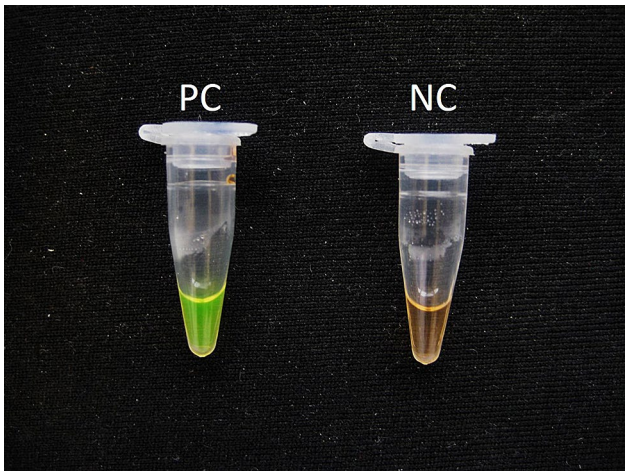

Fig. 1 Evaluation of LAMP products after adding SYBR Green I DNA stain under natural light. *PC* Positive control, *NC* Negative control

Statistical analysis

The SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the collected data. Descriptive statistics were shown in terms of percent (for categorical) and mean (SD) (for continuous) variables. The Chi-square test was applied to determine the univariate association between independent variables and the outcome. Multifactorial logistic regression models were also used to assess the association between *T. gondii* positivity and the present risk factors. $P < 0.05$ was considered to be statistically significant.

Results

Participants

In this study, a total of 140 pregnant women suffering from their first SA were evaluated for toxoplasmosis infection by molecular (LAMP) tests. The mean age of the participants was 26.7 ± 6.6 years, ranging from 15 to 44 years old. In term of education, the majority of participants had secondary education and diplomas (39.3%). The majority of participants were also housewives (70.7%), resident in urban areas (75.7%). Table 1 shows the demographic characteristics of the participants in the study.

The epidemiologic and clinical characteristics of pregnant women suffering from their first SA are presented in Table 1. Out of 140 women, 24 (17.1%) had a history of keeping cats at home. In terms of pregnancy rate, 67 (47.9%) were pregnant for the first time, 41 (29.3%) were pregnant for the second time, 19 (13.6%) were pregnant for the third time, 8 (5.7%) were pregnant for the fourth time and 5 (6.3%) had experienced their fifth pregnancy. The history of abortion in previous pregnancies was not positive in any of the women studied, and none of the women reported a history of the specific disease in their previous offspring at birth. The history of the underlying disease was not reported in any of the women.

Table 1 Demographic characteristics and prevalence of *T. gondii* infection among the 140 pregnant women with the first spontaneous abortion

Variables	Frequency No. (%)	Positive in LAMP No. (%)	*P* value
Age (years)			
≤25	63 (45)	5 (7.9)	0.017*
<25	77 (55)	0 (0.0)	–
Residence			
Rural	106 (75.7)	3 (2.8)	–
Urban	34 (24.3)	2 (5.9)	0.595
Education			
Illiterate	0 (0.0)	0 (0.0)	–
≤guidance	47 (33.6)	4 (8.5)	0.073
Diploma	55 (39.3)	1 (1.8)	–
Academic	38 (27.1)	0 (0.0)	–
Job			
Housewife	99 (70.7)	4 (4)	0.813
Employee	32 (22.9)	1(3.1)	–
Student	9 (6.4)	0 (0.0)	–
Contacting with cat			
Yes	24 (17.1)	0 (0.0)	–
No	116 (82.9)	5 (4.3)	0.588
Pregnancy grade			
First	67 (47.9)	4 (6.0)	0.193
Second	41 (29.3)	1 (1.4)	–
Third	19 (13.6)	0(0.0)	–
Forth	8 (5.7)	0(0.0)	–
Fifth	5 (3.6)	0(0.0)	–

*Difference was statistically significant

Prevalence of *T. gondii* infection

Of the 140 women, 80 (57.1%) tested seropositive for anti-*T. gondii* antibodies by ELISA, 72 (51.4%) women tested seropositive for the IgG antibody, 8 (5.7%) tested seropositive for the IgM antibody (Table 2). Among the 8 women suffering from their first SA who tested seropositive for the IgM antibody by ELISA, only five cases (62.5%) reported positively by the LAMP test (Fig. 2). Based on Fisher's exact test, the difference in the frequency distribution of the LAMP results for measuring the toxoplasma infection in the pregnant women under study was statistically significant ($P < 0.001$) with the serological test (ELISA).

Table 2 Frequency of *T. gondii* infection among the 140 pregnant women with the first spontaneous abortion by ELISA and LAMP methods

Test	Positive No. (%)	Negative No. (%)	Total No. (%)
Toxoplasma IgM Ab	8 (5.7)	132 (94.3)	140 (100)
Toxoplasma IgG Ab	72 (51.4)	68 (48.6)	140(100)
LAMP	5 (3.6)	135 (96.4)	140 (100)

In term of age, there was a significant difference between age and positivity in the LAMP test ($P=0.017$) so that all positive cases for *Toxoplasma* parasite infection based on LAMP test were reported from the age group of lower than 25 years. The majority of positive cases (8.5%) were observed in women with lower education (≤ diploma). However, there was no significant difference between positivity to toxoplasmosis by LAMP and education ($P=0.073$).

Based on the LAMP test, the highest number of positive cases was observed in housewives (4%). However, no statistically significant difference was observed between positivity to toxoplasmosis by LAMP and profession ($P=0.813$). The highest number of positive cases of *T. gondii* infection was observed in pregnant women who lived in rural areas (5.9%), but by Fisher's exact test, the difference between positivity to toxoplasmosis by LAMP and residence was not statistically significant ($P=0.595$). Moreover, the results demonstrate that no statistically significant difference was observed between positivity to toxoplasmosis by LAMP and contact with cats ($P=0.588$) and pregnancy grade ($P=0.193$) (Table 1).

Discussion

Congenital toxoplasmosis, an infectious disease that is found in fetuses infected with *T. gondii*, may result in severe consequences, such as ocular, hearing, cognitive, and mental complications, and may even cause SA, miscarriage or stillbirth (Gilbert 2000). Since the accurate mechanism of transmission of the *Toxoplasma* across the human placenta is not completely understood, the rate of congenital toxoplasmosis in pregnant women varies, depending on the trimester during which the congenital infection occurred (Dunn et al. 1999; Gilbert 2000). So far, it has been proven that in the first trimester, the rate of transmission of toxoplasmosis is nearly 25%, while the transmission rates in the second and third trimester are 50 and 65%, respectively (Remington et al. 2006; Robbins et al. 2012). SA occurring before 20 weeks of pregnancy has been reported in about 1–2% of pregnant women (Ford and Schust 2009). A number of factors such as genetic factors, anatomical disorders, endocrine disorders, autoimmune syndromes, as well as infections can contribute to abortion (Ford and Schust 2009; Fallahi et al. 2018). Among the infectious agents, *C. trachomatis, U. urealyticum, M. hominis,* cytomegalovirus, human papillomaviruses, and *T. gondii* are the main causes of SA (Singh et al. 2010; Nigro et al. 2011; Torgerson and Mastroiacovo 2013; Elamin Elhag and Elturabi 2015; Menati Rashno et al. 2016; Rashno et al. 2017; Fallahi et al. 2018). Despite the fact that previous studies have shown the association between toxoplasmosis and SA (Torgerson and Mastroiacovo 2013; Elamin Elhag and Elturabi 2015), there are few studies regarding the association between toxoplasmosis and SA in Iran. The majority

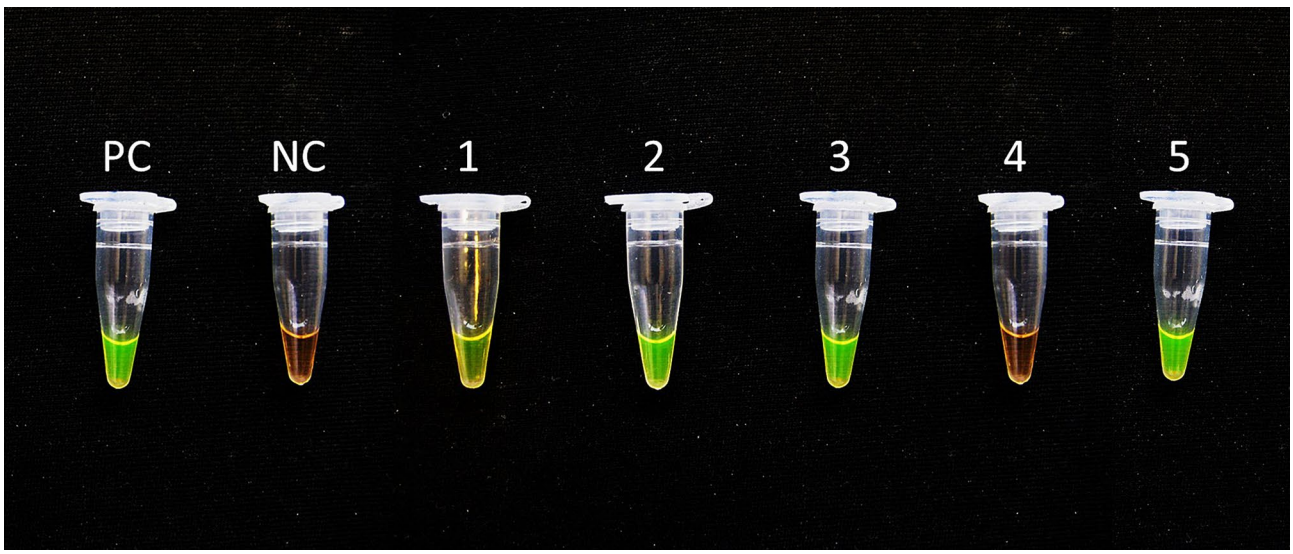

Fig. 2 Analytical characteristics of LAMP test results using SYBR Green I DNA stain under natural light: *PC* Positive control, *NC* Negative control, *Tubes 1–5* represent randomly selected genomic DNA extracted from aborted fetal remains samples from patients

of the studies regarding the prevalence of toxoplasmosis in SA have been carried out based on serological and some molecular tests, such as conventional PCR.

In this study, 140 pregnant women suffering from their first SA were examined for *Toxoplasma* infection by serological (ELISA) and molecular (LAMP) tests. The results from ELISA demonstrated that anti-*Toxoplasma* IgG and IgM were found in 72 (51.4%) and 8 (5.8%) of a group of pregnant women. Usually, identification of acute primary infection, especially in pregnant women, is very challenging, but the IgM antibody can be used to identify early acute toxoplasmosis (Mousavi et al. 2018). In line with our results, Elamin Elhag and Elturabi (2015) showed that among 99 women who had suffered SA in Khartoum State, *T. gondii* IgM was found in 5 women (5.3%), whereas 27 women (28.4%) were found to be positive for *T. gondii* IgG. Zargar et al. (1998) have demonstrated the prevalence of the IgM anti-*Toxoplasma* antibody in 49.47% of women with recurrent SA (49.47%) in Kashmir, Pakistan.

In Iran, in a study conducted by Matin et al (2017) in Ardebil Province, Northwest of Iran, the prevalence of anti-*Toxoplasma* IgG and IgM in women with a history of SA or stillbirth was 4.0% and 43.0%, respectively. Saki et al (2015) reported that IgG and IgM anti-*Toxoplasma* antibodies were found in 32 (24.6%) and 1 (0.77%) of women suffering SA in Ahvaz, Southwest of Iran. Sharif and Ajami demonstrated that the prevalence of anti-*Toxoplasma* IgG and IgM in women with a history of SA or stillbirth in Sari, Iran was 34.2% and 7.9%, respectively. Jahromi (2007) have reported that among 124 women with a history of SA in Bandar Abbas, southern Iran, anti-*Toxoplasma* IgG, and IgM antibodies were found in 98 (79.03%) and 19 (15.32%) cases, respectively. In another study conducted by Saeedi et al (2009) in Gorgan province, Iran, the prevalence of anti-*Toxoplasma* antibodies among women with abnormal pregnancy was 44.1% and 21% for IgG and IgM antibodies, respectively.

The results of current study showed that among 140 pregnant women suffering their first SA, the *T. gondii* DNA was found in 5 (3.6%) women by LAMP (Fig. 2). Similarly, Abdoli et al (2017) demonstrated that *T. gondii* DNA was detected in 3.8% of in formalin-fixed, paraffin-embedded fetoplacental tissues (FFPTs) of women with recurrent SA in Tehran, Iran. On the other hand, Asgari et al (2013) have reported that among 542 FFPTs, the B1 gene of *T. gondii* was amplified from 78 (14.4%) of spontaneous aborted fetuses in Shiraz, Southern Iran by semi-nested PCR. In recent years, the LAMP technique has become well known as one of the important techniques to determine microbial pathogens because of its unique properties, such as high sensitivity and specificity, field usability, and isothermal reaction condition that do not need expensive equipment, such as thermal cycling (Nagamine et al. 2002; Fallahi et al. 2014b; Arab-Mazar et al. 2016a, b). However, the difference between the serological tests and LAMP for the detection of acute infection may be affected by some factors, including the short duration of parasitemia and the low numbers of trophozoites circulating in peripheral blood, which can cause a sampling error that will create false-negative results in such cases (Badparva et al. 2009; Mahmoudvand et al. 2016; Fallahi et al. 2016, 2017; Arab-Mazar et al. 2016a, b).

Among the demographic and risk factors studied in the present investigation, such as age, residence, education, contact with cats, etc., a significant correlation was only observed between age (< 25 years) and positivity to *T. gondii* ($P = 0.017$). However, there is no significant association between other risk factors and positivity to *T. gondii*. Consistent with our results, Elamin Elhag and Elturabi (2015) showed that among 99 women suffering SA in Khartoum State, the highest percentage of a positive result was observed between 20 and 29 years. Moreover, in the study conducted by Saki et al (2015) in line with our results, there was no significant association between some risk factors such as contact with cats and positivity to *T. gondii* in women who had spontaneously aborted. However, to reach a more accurate conclusion the sample size needs to be increased.

Conclusion

In conclusion, the results of the present study in line with previous studies indicate the importance of this parasite as a cause of SA. The findings of the present investigation suggested that LAMP is a preferred method to determine *Toxoplasma* infection in SA by pregnant women, compared with other routine serological tests.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The present study was approved by The Ethics Committee of Lorestan University of Medical Sciences (IR.LUMS.REC.1395.193) and before sampling the written informed consent was obtained from all the participants.

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