

Sero-prevalence of toxoplasmosis and Role of Cathepsin L-like and Cathepsin B-like Genes as Risk Factors for Abnormal Pregnancy Outcome

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Abstract

Background and objectives: *Toxoplasma gondii* (*T. gondii*) is a ubiquitous apicomplexan parasite. As an obligate intracellular parasite, *T. gondii* must invade host cells to survive and replicate, five cathepsin proteases are encoded in the genome of *T. gondii*, cathepsin L like protein (TgCPL), cathepsin B like protein (TgCPB), and three cathepsin C like proteins (TgCPC 1, 2, and 3). The present study was aimed to investigate the prevalence of toxoplasmosis among women in Erbil, and to study the role of cathepsin B and cathepsin L genes in the pathogenesis of toxoplasmosis as well as their role as risk factors for abnormal pregnancy outcome. A total of 230 women at their reproductive age who attended Maternity Teaching Hospital and Nazdar Bamarni primary Health Center were enrolled in this study. Anti- toxoplasma IgG and IgM antibodies were detected by cobas 6000. Toxoplasma cathepsin B and cathepsin L – like genes were selected to be targets in PCR. **Results:** Anti-toxoplasma IgG and IgM were seropositive in 105 (45.7%) and 18 (7.8%) women, respectively, and only 15(6.5%) of them for both anti-toxoplasma IgG and IgM. No significant association of toxoplasmosis and educational level, socioeconomic level, age, history of abortion, and abnormal baby birth weight were observed. PCR targeting cathepsin L-gene was more sensitive to be used in the diagnosis of toxoplasmosis. **Conclusion:** Sero-prevalence of toxoplasmosis is relatively high in Erbil and cathepsin L gene is an efficient target for PCR and could be used as risk factor for abnormal pregnancy outcome.

Keywords: *Toxoplasma gondii*, Cathepsin L like, Cathepsin B like, Polymerase chain reaction.

Introduction

Toxoplasma gondii is a ubiquitous apicomplexan parasite that infects a wide range of warm-blooded animals including humans. It is estimated that one third of the world's population suffer from toxoplasmosis. Which in general asymptomatic are or may presents as flu-like symptoms in immunocompetent individuals. However, it may result in severe complications in pregnant woman and immunocompromised patients [1].

Congenital toxoplasmosis occurs predominantly in women who acquire their

primary infection during gestation. However, in few rare cases congenital transmission occurred in chronically infected women which because of their immunocompromised state infection was reactivated [2].

The incidence of congenital transmission rises with the gestational age [3]. However, severe clinical signs in the infected infant are more frequently observed in babies of women who acquired infection in early stages of their pregnancy [4]. Congenital toxoplasmosis may result in miscarriage, stillbirth, serious and

progressive visual, hearing, motor, cognitive, and other problems in an infected baby [5]

Detection of infection in pregnant women is critical for clinical management of the mother and her fetus [6]. Serological tests are used as a routine practice to determine the immunological status of the patient by detecting and quantifying antibodies specific for toxoplasma in the serum of patients to determine whether the infection is acquired during gestation or before conception [5].

Conventional methods are usually not misleading, but are limited in prenatal cases or in immunocompromised patients. Molecular methods are used in addition to conventional serological methods for the diagnosis of toxoplasmosis [7]. Polymerase chain reaction technique (PCR) is a molecular technique which has been successfully assessed in the diagnosis of pre-natal congenital toxoplasmosis in various biological samples such as amniotic fluid and blood [8]. PCR is an efficient *in vitro* enzymatic amplification method that allows specific amplification of DNA from minute amounts of starting material in a short time [9].

T. gondii can invade and replicate within any nucleated cell of vertebrate hosts, including humans. Invasion by *T. gondii* tachyzoites is mediated by the sequential regulated release of specialized secretory organelles of the parasite including the micronemes, rhoptries, and dense granules [10, 11, 12].

As an obligate intracellular parasite, *T. gondii* must invade host cells to survive and replicate. During cell invasion, two subcellular organelles, micronemes and rhoptries contents are sequentially discharge at the apical end of the parasite to mediate invasion [13]. Protease inhibitor studies revealed that cysteine proteases are involved in the maturation of microneme and rhoptry proteins and the biogenesis of some subcellular organelles [14] [15].

Five cathepsin proteases are encoded in the genome of *T. gondii*, cathepsin L like protein (TgCPL), cathepsin B like protein (TgCPB), and three cathepsin C like proteins (TgCPC1,2, and 3). Recent studies revealed that cathepsins have crucial role in microneme and rhoptry

protein maturation, host cell invasion, parasite replication and nutrient acquisition [13].

T. gondii cathepsin L is expressed within the Vacuolar Compartment (VAC) of tachyzoites and bradyzoites. Its function in the VAC is not known, but since the VAC resembles a lysosome or lytic vacuole, *T. gondii* cathepsin L is proposed to function in protein degradation within this compartment and also associated with the residual body in the parasitophorous vacuole after cell division, where it could contribute to the destruction of mother cell organelles that are not needed in the daughter cells [15].

T. gondii cathepsin B expression and activity have been detected in tachyzoites. It remains unclear whether *T. gondii* cathepsin B is expressed in bradyzoites, since its transcript was not detected by RT-PCR in cysts recovered from mouse brains, but was shown by microarray analysis to increase during bradyzoite differentiation *in vitro* [16]. *T. gondii* cathepsin B localizes the rhoptries as revealed by immunoelectron and immunofluorescence studies, also detected in the residual body. Parasites treated with a cathepsin inhibitor showed impaired invasion, altered rhoptry morphology, and delayed maturation of *T. gondii* rhoptry protein 2, implicating *T. gondii* cathepsin B in rhoptry proteins maturation and parasite invasion [17].

The present study was aimed to investigate the prevalence of toxoplasmosis among women in Erbil, and to study the role of cathepsin B and cathepsin L genes in the pathogenesis of toxoplasmosis as well as their role as risk factors for abnormal pregnancy outcome.

Materials and Methodologies

Subjects

A cross sectional study was carried out in Erbil city in which 230 women in their reproductive age who attended Maternity Teaching Hospital and Nazdar Bamarni primary health care center from October 2018 to march 2019 were enrolled. An informative close end questionnaire including Age, residency, socioeconomic level, history and number of abortion, history and number of abnormal

parity, gestational age, was obtained through direct interview. The participants were apparently healthy and women with autoimmune diseases such as rheumatoid arthritis, SLE, and those with UTI (vaginal discharge, burning during urination, dysuria), were excluded from the study.

Sample collection

A volume of 5ml of intravenous blood was collected from all screened women. Serum was separated from half of each sample and kept at -20°C until used, while the other half of the sample was placed in a sterilized EDTA tube and stored at -20°C for amplification by conventional PCR.

Ethical considerations

The study was approved by the Research Ethical Committee of the College of Medicine, Hawler Medical University, Erbil. A verbal consent was obtained from each participant before collection of blood samples.

Detection of anti-Toxoplasma IgG and IgM

The samples were screened for Toxoplasmosis serologically with the use of cobas[®] 6000 analyzer series for anti-toxoplasma IgG and IgM antibodies (Roche diagnostics, Japan) according to the manufacturer's instructions.

Polymerase chain reaction (PCR)

DNA extraction

DNA was extracted from the whole blood samples using DNA extraction kit (GeNet Bio-Global Gene, South Korea) according to the manufacture protocol. Purity of the DNA was assessed by nanodrop spectrophotometer and kept at -20°C until Polymerase Chain Reaction (PCR) was performed.

Amplification procedure

Conventional PCR was performed with the use of two specific primers (Macrogen, Inc. South Korea) for *T. gondii* Cathepsin B gene 5'-TCTTCCGCTCTTGCTATTACGG-3' (F) 5'-GCTGATGGTGCAGGACTGAT-3' (R) with amplicon length of 92 base pair (bp) and 5'-ACATGGAGGCGACCAACAAA-3' (F) 5'-

GAAGGTCCCCGAAACACGAT-3' (R) with an amplicon length of 295 base pair (bp) for *T. gondii* Cathepsin L gene. In amplification processes, the PCR micro tube was involved 2.5 μl of each primer, 5 μl of DNA sample and 12.5 μl master mix, then completed with nuclease-free deionized distilled water to a final volume of 25 μl . Master mix (Promega, USA) is composed of Taq DNA polymerase, reaction buffer (pH=8.5), 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP and 3mM MgCl₂. The process of polymerase chain reaction was done by PCR Thermal cycler (Corbett Life Science Pty., Ltd. Germany), and the amplification protocol consisted of 40 cycles of initial denaturation at 95°C for 5 minutes followed by 40 cycles at 95°C for 30 seconds, Annealing 61°C for 30 seconds, extension 72°C for 30 seconds for Cathepsin B gene. While for Cathepsin L gene the amplification process composed of 40 cycles of initial denaturation at 95°C for 5 minutes followed by 40 cycles at 95°C for 30 seconds, Annealing 59°C for 30 seconds, extension 72°C for 30 seconds. The entire procedure was done according to [12] unless otherwise modification.

Statistical analysis

The Statistical Package for Social Science (SPSS version 21.0) was used for data entry and analysis. The student t-test and Chi – square were used to compare the data. P value of ≤ 0.05 was considered as statistically significant.

Results

A total of 230 women agreed to be involved in the study. The age of the participants ranged between 17 to 47 years with a mean age of 28.23 ± 6.35 years. More than half of the participants 138 (60%) had antibodies for *toxoplasma gondii*. Anti-toxoplasma IgG and IgM were seropositive in 105 (45.7%) and 18 (7.8%) women, respectively, only 15(6.5%) of women who were seropositive for toxoplasmosis carrying both anti-toxoplasma IgG and IgM as expressed in Figure 1.

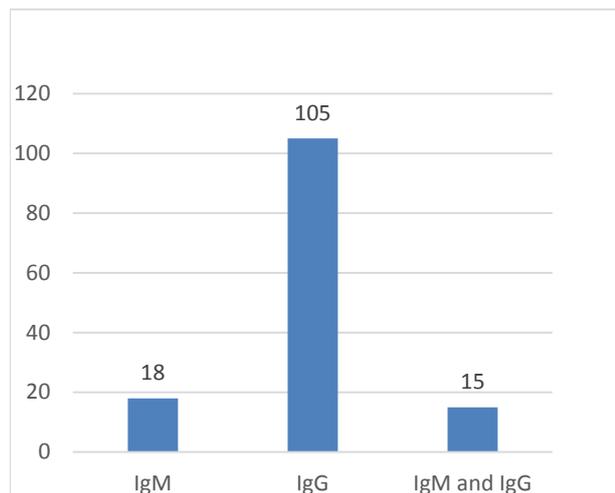


Figure 1: Prevalence of anti- toxoplasma IgM and IgG antibodies among 230 women of Erbil city.

According to educational level, no significant ($P > 0.05$) association of toxoplasmosis and educational level was observed (Table 1).

Table 1: Sero-prevalence of toxoplasmosis among women according to educational level

Education	No. of examined samples	IgM positive samples	IgG positive samples	IgM and IgG positive samples
Primary	53	5 (2.2%)	19 (8.3%)	2 (0.9%)
Secondary	82	5 (2.2%)	44 (19.1%)	4 (1.7%)
University	79	6 (2.6%)	36 (15.7%)	8 (3.5%)
Illiterate	16	2 (0.9%)	6 (2.6%)	1 (0.4%)
Total	230	18 (7.8%)	105 (45.7%)	15 (6.5%)
P- value		0.954	0.795	0.37

As it can be seen in Table 2, significant ($P=0.000$) association of toxoplasmosis and history of abortion was observed on screening for both anti-toxoplasma IgG and IgM antibodies (3.5% vs 3.0 %) by cobas® 6000 analyzer series in women with and without history of abortion, respectively.

Regarding the socioeconomic level of the patients, the results revealed highest rate of anti-*Toxoplasma gondii* IgG (41 %), IgM (7 %), and both IgG and IgM (4.8 %) among women with medium socioeconomic level, however, statistically no significant association was observed between toxoplasmosis and socioeconomic level ($P=0.295$, $P=0.547$, $P=0.444$) as revealed in (Table 3).

Table 2: Seroprevalence of toxoplasmosis among women according to history of abortion

History of abortion	No. of examined samples	IgM positive samples	IgG positive samples	IgG and IgM positive samples
Yes	85	13 (5.7%)	49 (21.4%)	8 (3.5%)
No	145	5 (2.25%)	56 (24.3%)	7 (3%)
Total	230	18 (7.8%)	105 (45.7%)	15 (6.5%)
p- value		0.838	0	0

Table 3: Seroprevalence of toxoplasmosis among women according to socioeconomic level

Socioeconomic level	No. of examined samples	IgM positive samples	IgG positive samples	IgM and IgG positive samples
Low	20	2 (0.9%)	10 (4.3%)	3 (1.3%)
Medium	204	16 (7%)	94 (41%)	11 (4.8%)
High	6	0 (0%)	1 (0.45)	1 (0.4%)
Total	230	18 (7.8%)	105 (65.7%)	15 (6.5%)
P- value		0.547	0.295	0.444

As it can be seen in Table (4) the highest rate of toxoplasmosis (IgG, 24.3 %; IgM, 3.0 %; IgG and IgM, 5.2 %) was observed among women aged between 21-30 years old. Even if the statistical analysis revealed no significant differences in the levels of IgG ($P=0.865$), IgM ($P=0.884$), both IgG and IgM ($P=0.905$) antibodies among the studied age groups

Table 4: Age distribution of anti-toxoplasma antibodies among women in Erbil

Age	No. of examined samples	IgM	IgG	IgM and IgG
		Seropositive samples	Seropositive samples	Seropositive samples
≤20	24	3 (1.3%)	11 (4.8%)	0 (0%)
21-30	131	7 (3%)	56 (24.3%)	12 (5.2%)
31-40	65	7 (3%)	33 (14.3%)	1 (0.4%)
≥41	10	1 (0.4%)	5 (2.2%)	2 (0.9%)
Total	230	18 (7.8%)	105 (45.7%)	15 (6.5%)
P- value		0.884	0.865	0.905

Figure 2 shows the prevalence of toxoplasmosis among 40 samples tested by PCR, 22 of them showed positive result for Cathepsin B and 29 of them showed positive result for Cathepsin L. Beside, Table 5 shows the results of molecular detection of toxoplasmosis in blood of abortive and non-abortive women that showed positive results for anti- *Toxoplasma* IgM and IgG in the serological testing. Cathepsin B and Cathepsin

L genes were positive in 14 (56%) and 19 (76%) of abortive women, respectively. versus 8 (53.3 %) and 10 (66.7%) for Cathepsin B and Cathepsin L, respectively in non-abortive women.

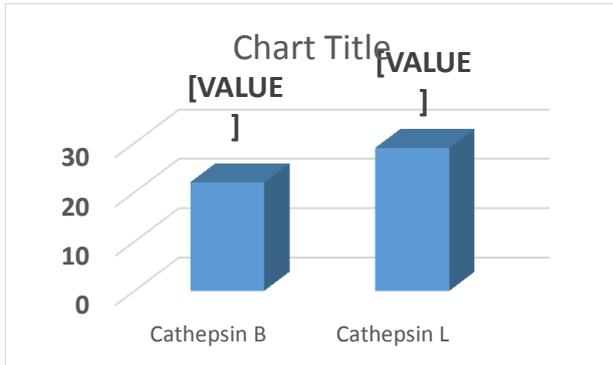


Figure 2: the prevalence of toxoplasmosis among 40 samples by PCR targeting Cathepsin B and Cathepsin L of *T. gondii*.

Table 5: Molecular detection of Cathepsin B and Cathepsin L genes of *T. gondii* among 40 samples by PCR in term of history of abortion

History of abortion	No. of examined samples	Cathepsin B	Cathepsin L
yes	25	14 (56%)	19 (76%)
No	15	8 (53.3%)	10 (66.7%)
Total	40	22 (55%)	29 (72.5%)
P- value		0.597	0.752
P- value		0.029	

Out of 40 women who were tested for toxoplasmosis by PCR, 36 of them had babies. 12 of them delivered babies with low birth weight and 24 of them delivered babies with normal birth weights (Table 6). When tested by PCR 5 (13.8%) and 7 (19.45) showed positive results for Cathepsin B and Cathepsin L respectively in women who delivered babies with low birth weight. Versus 11 (30.5%) and 13 (36.1%) for Cathepsin B and Cathepsin L respectively in women who delivered babies with normal baby weight. However statistically, no significant association ($P=0.488$; $P=0.658$) of toxoplasmosis and birth weight outcome was detected by PCR targeting both genes.

Table 6: Association of maternal toxoplasmosis and baby birth weight in 36 samples by PCR targeting Cathepsin B and Cathepsin L genes.

Baby birth weight	No. of examined samples	Cathepsin B	Cathepsin L
Low birth weight	12	5 (13.8%)	7 (19.4%)
Normal birth weight	24	11 (30.5%)	13 (36.1%)
Total	36	16 (44.3%)	20 (55.5%)
P- value		0.488	0.658

* Low: birth weight ≤ 2499 mg
Normal: birth weight 2500 – 4000 mg (18).

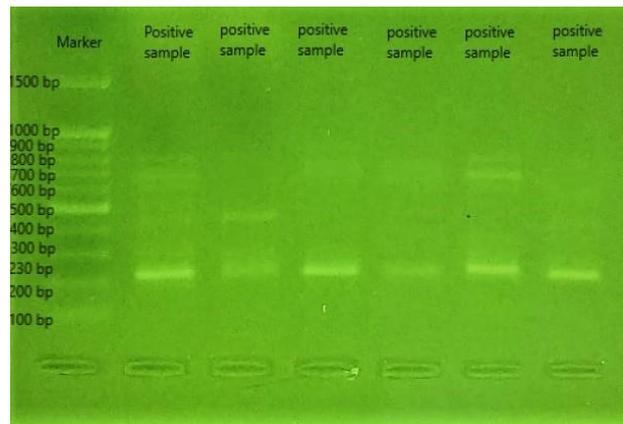


Figure 3: PCR amplification of Cathepsin L gene of *Toxoplasma gondii* genome on 2% agarose gel (at 80V for 1:20 hour) Lane 1. Marker, Mol. wt. marker (100 bp ladder) (Promega, USA), lane 2, 3, 4, 5, 6, and 7 are positive samples.

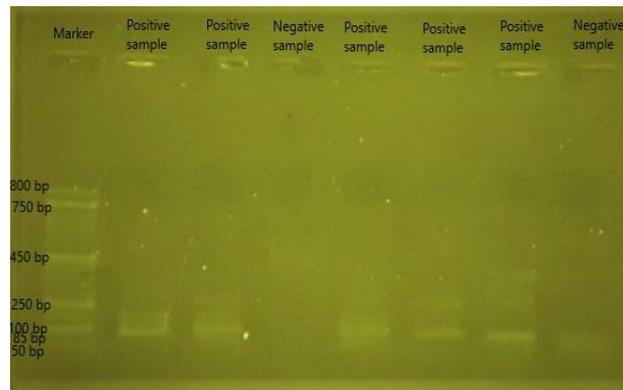


Figure 4: PCR amplification of Cathepsin B gene of *Toxoplasma gondii* genome on 2% agarose gel (at 80V for 1:20 hour). Lane 1. Marker, Mol. wt. marker (50 bp ladder) (Promega, USA), lane 2, 3, 5, 6, and 7 are positive samples. Lane 4 and 8 are negative samples.

Discussion

Toxoplasmosis is an infection caused by an intracellular parasite named *Toxoplasma gondii*. In general, toxoplasmosis causes mild

symptoms in non-pregnant women. But, it may cause severe damages if the organism is contracted during pregnancy as it may result in congenital toxoplasmosis with severe pathological effects to the fetus [19].

In the present study serological screening of toxoplasmosis among 230 pregnant and non-pregnant women with and without history of abortion, revealed that 18 (7.8%) of the samples were positive for IgM, 105 (45.7%) for IgG and 15 of the cases (6.5%) were positive for both IgG and IgM. Comparably, our finding was similar to that observed by previous studies, carried out in Erbil. Bakre [20] reported that sero-prevalence rate of toxoplasmosis was 8 (5.3%) and 23 (15.3%) for IgM and IgG, respectively among women who attended Mammon Dabax Health Center in Erbil over the period from January to September 2015. While Hamad [21] observed 9.13% and 37.5% positive cases for IgM and IgG, respectively. In Garmian region the infection rate was found to be 65(26 %) cases for IgG and 50 (20%) cases for IgM among a sample of women with normal delivery and those with miscarriage by ELISA [22]. In a study carried out in al-Najaf, sero-prevalence rates of anti-toxoplasma IgG and anti-toxoplasma IgM were 30.7% and 11.9 % among women at childbearing age [23].

Furthermore, the infection rate also reported in some surrounding countries as indicated in Iran, 5(0.63%) and 341 (42.7%) of women were obtained to have acute and chronic infections, respectively as detected by Rasti *et al.* [24]. Another study in Saudi Arabia reported that the prevalence of anti- *T. gondii* IgG and IgM antibodies was 32.5% and 6.4%, respectively with the use of ELISA [25].

Women of low income group could be at risk of repeated infections attributable to the unhygienic environment in which they reside [26]. However, in the present study no significant association of anti-toxoplasma antibodies and socioeconomic level was observed.

It is assumed that determination of encysted forms of toxoplasma in chronically infected uteri and their rupture in subsequent placentation may lead to infection of the fetus

in the first trimester and recurrent abortions. However, there is insufficient data to link *T. gondii* infection as a cause of recurrent or habitual abortions Borkakoty *et al.* (2016) [27]. In the present study no significant association of toxoplasmosis and history of abortion was detected on screening for both anti-toxoplasma IgG (21.4 % vs 24.3 %) and IgM (5.7 % vs 2.25 %) antibodies in women with and without history of abortion (Table 2) a study that was made in Ahvaz, Southwest of Iran shows similar results to our study, after testing the sera of 130 abortive and 130 non-abortive women by ELISA no statistical difference ($P > 0.05\%$) was detected between toxoplasma infection and abortion [28].

Regarding the age of the participants, our study observed highest rate of anti-toxoplasma (IgG, 24.3%; IgM, 3%) antibodies were among women with ages ranged between 21-30 years old. However, statistical analysis revealed no significant association. A study that was made in India estimated that the prevalence of toxoplasmosis increased steadily with ages ranged between 18 to ≥ 40 years old [29]. Another study that was carried out in Iran concluded that the highest rate of toxoplasmosis was observed in women at ages of high reproductive activities (> 20 years old) and the reason seems to be in the change in nutritional pattern and home responsibilities after marriage that increases the risk of exposure to *T. gondii* parasite [30].

Molecular detection of toxoplasmosis by PCR revealed variable reactions. Among 40 samples of abortive and non-abortive women who tested by PCR, targeting genes of cathepsin B and cathepsin L, no significant difference between both targets was observed in term of history of abortion. However, using cathepsin L gene as targets for PCR seems to be more efficient for molecular detection of toxoplasmosis.

Birth weight is the single most predictive factor of mortality in the first few months of life, and is an important indicator of his health [31]. Low birth weight refers to weight of new born infants below 2500 grams [18]. WHO estimated that about 25 million low birth weight babies are born each year, nearly 95%

of them in developing countries. Neonatal death among infants weighing 1500–2500 grams is 20 times higher than among infants of normal weight [32]. In our study no significant ($P= 0.446$) association was observed between toxoplasmosis and baby birth weight.

Conclusion

Sero-prevalence of toxoplasmosis is relatively high in Erbil and cathepsin L gene is an efficient target for PCR could be used as risk factor for abnormal pregnancy outcome.

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