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MOLECULAR STUDY OF TOXOPLASMOSIS IN ABORTED PLACENTA SAMPLES WITH NPCR METHOD IN ARDEBIL- IRAN

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ABSTRACT

Toxoplasma gondii is a protozoan parasite that infects up to a third of the world's population. Infection is mainly acquired by ingestion of food or water that is contaminated with oocysts shed by cats or by eating undercooked or raw meat containing tissue cysts. Primary maternal infection with toxoplasmosis during pregnancy is frequently associated with transplacental transmission to the fetus. Although *Toxoplasma gondii* infection generally shows subclinical courses in adults, it may have severe pathologic effects on the fetus. Infection in pregnant women may lead to abortion, stillbirth or other serious consequences in newborns. This study was conducted to test the utility of a polymerase chain reaction (PCR) assay to detect recent infections with *Toxoplasma* in abortive women. Two hundred placentas from aborted women in various gestational ages and with a history of abortion, admitted in Alavi Obstetric and Gynecological department of Ardabil were collected during 2013 to 2014. The placenta was tested by nested PCR, using fragments of the GRA6 gene. Using PCR method, 17 out of 200 (8.5%) samples of spontaneous aborted placenta were found to be positive for *T. Gondii.* A significant relationship was not observed between the presences of Toxoplasma, mother's

age, and history of abortion, gestational age and illnesses such as allergy, diabetes, hypertension and hypothyroidism.

The present findings reveals that *Toxoplasma gondii* may be one of the potential agents in causing significant rate of abortion in the Ardabil area and placenta analysis is important to improve the sensitivity of the diagnosis .

Keywords: Toxoplasma gondii, Abortion, Nested-PCR, Iran

INTRODUCTION

Toxoplasmosis is a parasitic infection disease which is caused by *Toxoplasma gondii* and courses with various clinical findings. Up to one-third of the human population in the world is chronically infected (1). Human infection generally occurs by ingestion of tissue cyst in raw or undercooked meat or by ingestion of parasite oocytes shed in feces of cat that contaminate water and food sources. Congenital transmission is the other route of infection (2).

Congenital toxoplasmosis generally occurs when a woman is newly infected with T. gondii during pregnancy (3). Congenital infection can lead to a wide variety of manifestations in the fetus and infant including spontaneous abortion, still-birth, a live infant with classic signs of congenital toxoplasmosis such as hydrocephalus or microcephalus, cerebral calcifications and retinochorioditis, an infant who fails to thrive or has CNS involvement or retinochoroiditis, or an apparently normal infant who develops retinochoroiditis or symptoms of CNS involvement later in life (3-4).Development of fetal infection and the severity of sequels that may develop are related to the gestational week (5-6). The incidence of congenital infection is 10-25% in the first trimester, 30-54% in the second trimester and 60-65% in the last trimester5-9. While the transplacental transmission appears to be less frequent in early pregnancy, the sequels occurring are more severe. The transmission rate is higher in latent period but the severity of the infection developing in fetus is mild. Mild symptoms may develop in fetus or they are asymptomatic in delivery (7-8-9).

It has been estimated that congenital toxoplasmosis affects 1 to 10 per 10,000 infants in Europe (10). In Iran the rate of abortion due to congenital toxoplasmosis is unclear in many areas where the rate of acquired toxoplasmosis is reasonably high. up to present, most of the decisions on infected fetuses were made based on serological findings in their mothers, which might have led to abortion of many uninfected fetuses.

The dense granule of T. gondii is a secretory vesicular organelle, which produces proteins that participate in the modification of the parasitophorous vacuole (PV) and PV membrane for the maintenance of intracellular parasitism in almost all nucleated host cells (11). GRA6 is considered a good marker of acute infection (12-13-14).

In the present study, the role of *Toxoplasma gondii* in abortion in human was evaluated by molecular method in Ardabil city (north-west of Iran).

MATERIALS AND METHODS

This study has been performed on two hundred women in various gestational ages and with a history of single or repeated abortion, admitted in Alavi Obstetric and Gynecological department of Ardabil in one year during 2013 to 2014.

a- Sample collection:

Around 20gr placenta from the patient was cut in a sterile form and kept at −20°C until further testing. Information collected on patients were age , abortion history and pregnancy levels.

b- DNA extraction and PCR detection :

T. gondii DNA was extracted from placenta of abortive women using the QIAamp DNA mini kit (Qiagen, France). Tissues were cut with maximum weight of 50 mg and placed in micro tube with the addition of 180 µl ALT buffer 20 µl proteinase k and fallowed with vortexing and incubated at 56°C until the tissue was completely lysed. Then 200 µl buffers AL was added to the sample and heated at 70°C for 10 min.200 µl ethanol (96 – 100%) was added to the samples and then 2 ml mini spin column was applied and centrifuged for 1 min at 6000 Xg. The mini spin column was placed in a clean 2ml collection tube and 500 µl buffer AW1 was added to it and it was centrifuged at 6000Xg for 1min and followed by placing another collection tube and adding 500 µl buffer AW2 and it was centrifuged at full speed for 3min and again spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 min spin column was placed again in microcentrifuge tube and 50 µl buffer AE was added to spin column and incubated at room temperature for 1 min and centrifuged at 6000Xg for 1 min. DNA samples was stored at -20°C .

Nested primer sets were used for amplifying fragments of the GRA6 gene as described by Khan et al. (2005), a modification of the GRA6 marker originally described by Fazaeli et al. (2000).

The external primers were GRA6-F1x (5-ATTTGTGTTTCCGAGCAGGT-3) and GRA6-R1 (GCACCTTCGCTTGTGGTT) producing an amplified product of 546 bp.

Internal primers were GRA6-F1 (TTTCCGAGCAGGTGACCT) and GRA6-R1x (TCGCCGAAGAGTTGACATAG) producing an amplified product of 351 bp.

The first25 μL of PCR reaction mixture contained outer primers at a final concentration of 50 pmol each, 2.5 mmol dNTPs, 1 μg of template, and 1.5 U recombinant taq DNA polymerase, in 1× PCR reaction buffer (50 mmol/L KCl and10 mmol/L Tris–HCl, 1.5 mmol/L MgCl2, and 0.1% triton X-100; Sinagen Co., Iran).

The first step of amplification was 5 min of denaturation at 94°C. This step was followed by 40 cycles, with one cycle consisting of 45 s at 94°C, 45 s at the annealing temperature (55°C) for each pair of primers, and 60 s at 72°C. The final cycle was followed by an extension step of 10 min at 72°C.

The nested PCR reaction was performed using 1 μL of the first PCR reaction in a mixture containing the inner primers at final concentration of 50 pmol each, 2.5 mmol/L dNTPs, and 1.5 U recombinant taq DNA polymerase in 1× PCR reaction buffer. Amplification was carried out at 94°C for 5 min (one cycle), then followed by 40 cycles each for denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s. The run was terminated with a final extension at 72°C for 10 min. The amplification products were detected by gel electrophoresis using 2% agarose gel in 1× Tris–borate–EDTA buffer. DNA bands were visualized in the presence of ultraviolet light, following the staining with 0.5% ethidium bromide.

RESULT

A total of 200 abortive women were recruited. Their age range was from 16 to 41 years with a mean of 30.5 years. The majority of these women (44 %) were between 20 and 30 year, Most of these pregnant women (53%) were in their first trimester of pregnancy and73. % has one or no child and 71% had no experience of miscarriage (Table 1). As observed in Figure 1, 8 cases (4%) had history of hypothyroidism, 9 cases (4.5%) diabetes, 13 cases allergy(6.5%) ,2 cases hypertension(4%) and 168 cases(85%) had history of no diseases except miscarriage (fig1).

Using PCR method, 17 out of 200 (8.5%) samples of spontaneous aborted placenta were found to be positive for *T. gondii* (Tab 1, Figure 2).

npcr results and data from questionnaires were analyzed employing Chi-square statistical test with 95% confidence interval using SPSS software version 16. A significant relationship was not observed between the presences of Toxoplasma,

mother's age, and history of abortion, gestational age and illnesses such as allergy, diabetes, hypertension and hypothyroidism.

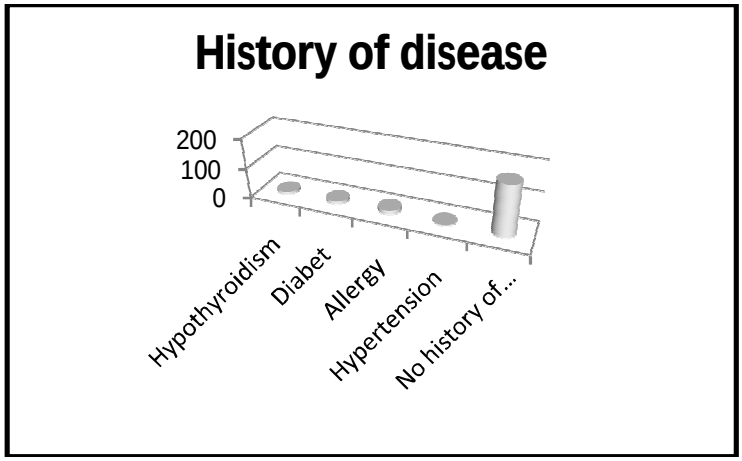

Fig 1: History of disease in abortive women

Table 1: Demographic characteristics of mothers who had abortion and results of diagnostic technique

		No	PCR	
			Positive	Negative
Age groups(yr)	≤20	43	4	37
	20-30	88	7	79
	>30	69	6	62
Gestational age	1st trimester	106	11	95
	2th trimester	62	8	54
	3th trimester	32	2	30
History of abortion	Yes	58	3	55
	No	142	18	124

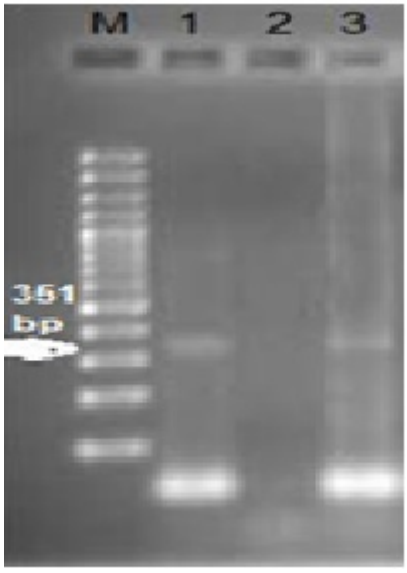

Fig. 2: Agarose gel electrophoresis of nested PCR products of tissue samples of placenta of abortive women with the internal GRA6 primers. Lane M molecular marker, lane 1 Toxoplasma gondii positive control, lane 2 negative control, lane 3 tissue from placenta of abortive women

DISCUSSION

In order to definitely assess or rule out the diagnosis of congenital toxoplasmosis, a whole set

of recommendations applies: i) prenatal diagnosis based on molecular testing of amniotic fluid and ultrasound examinations; ii) molecular testing of placenta and cord blood, comparative mother–child serologic tests and a clinical examination at birth; iii) neurologic and ophthalmologic examinations and a serologic survey during the first year of life (17). One third of mothers who acquire a primary *T.gondii* infection during pregnancy transmit the infection to their fetuses (18).

Transplacental infection of the fetus occurs in 12% of cases in which the mothers acquire infection during the first trimester (19).

Recently, the value of PCR examination of placenta was compared to their inoculation into mice (Bessières et al, 2009). The sensitivity and specificity of PCR on placenta were 52% and 99% respectively, which was equal to that obtained by the mouse inoculation whereas the combination of PCR and mouse inoculation of placenta raised the sensitivity to57% (20). Placental examination by PCR and mouse inoculation was reported to be an efficient tool for the early diagnosis of CT in neonates (21).

Placenta from infected women were analyzed to detect T. gondii by PCR, and the results were compared to those of the other samples collected at birth as cord blood and newborn blood. Ninety-four placenta, were analyzed by in vitro culture, mouse inoculation, and PCR . The PCR sensitivity was higher than that of cell culture and mouse inoculation but the false-positive PCR results were also higher (22).

Suzuki et al confirmed IgM by detection of the Toxoplasma SAG1 gene in placenta and concluded that PCR using the placental tissue might be useful for the rapid diagnosis of congenital toxoplasmosis by detection of the Toxoplasma SAG1 gene by PCR in placenta. (23).

The dense granules (GRA) are parasitic organelles involved in cell invasion and in the intracellular survival of the parasite. GRA proteins are expressed by the three stages of *T. gondii*: the tachyzoite, bradyzoite (24), and sporozoite (25) stages. GRA6 is a GRA antigen of 32 kDa described for the first time by Lecordier et al. (38). In extracellular parasites, GRA6 exists in dense secretor granules mostly as soluble proteins. Like the other GRA proteins, GRA6 is involved in host cell invasion. GRA6 is a glycine-rich protein and behaves like an integral membrane

protein within the parasitophorous vacuole (26, 27).

Rate of infection in pregnant women in Iran is relatively high. This rate in Zahedan, southeast of Iran, was reported to be 27% while in north of the country was 43.8% (28-29).

In Shiraz, Southern Iran, the seroprevalence of toxoplasmosis among pregnant women was reported to be 77.2% (30).

The rate of *Toxoplasma* infection in aborted placenta in Shiraz in the other study was 14.4 %(31).

Study by Hoveyda showed in out of 65 delivery product 15.48% was positive for Toxoplasma gondii by PCR teqnique in Isfahan, Iran (32).

A study on seroprevalence of toxoplasmosis in Kosovo in pregnant women demonstrated that 1.2% of women acquired toxoplasmosis during their pregnancy (33).

In other studies transmission rate to the children in the Finland was 40 %(34).

Direct detection of T. gondii in clinical samples confirms the parasite presence leading to the diagnosis of primary, reactivated or chronic toxoplasmosis (35).

In this study, the rate of *Toxoplasma* infection in aborted placenta was 8.5%. This is lower than the rates that have been reported for abortion due to toxoplasmosis in other studies in Iran (31, 32). Prevalence of Toxoplasmosis in region, type of sample, method of sampling, and size of sample may all affect the outcome of the experiment.

In the present research similar to the results of previous studies (36, 37, 38, 39), there was no significant association between mothers' age, history of abortion, gestational age, hypertension and existence of toxoplasma.

CONCLUSION

Our findings show that toxoplasmosis acts as a warning and main risk factor for increase the possibility of abortion in pregnant woman in this region of Iran and placenta analysis is important to improve the sensitivity of the diagnosis at birth, especially when the prenatal diagnosis was negative or not performed.

REFERENCES

[1] Montaya JG, Liesenfeld O. Toxoplasmosis. Lancet .2004; 363: 1965-76.

[2] Jones JL, Dubey JP. Waterborne toxoplasmosis--recent developments. Exp Parasitol. 2010; 124 (1): 10-25.

[3] Remington JS, McLeod R, Thulliez P, Desmonts G.Toxoplasmosis. In: Remington JS, Klein JO, Wilson CB, Baker CJ. Infectious Diseases of the Fetus and Newborn Infant. Philadelphia: Elsevier Saunders; 2006: 947–1091.

[4]McAuley J, Boyer KM, Patel D, Mets M, Swisher C, Roizen N, Wolters C, Stein L, Stein M, Schey W, Remington J, Meier P, Johnson D, Heydeman P, Holfels E, Withers S, Mack D, Brown C, Patton D, McLeod R . Early and longitudinal evaluations of treated infants and children and untreated historical patients with congenital toxoplasmosis: the Chicago Collaborative Treatment Trial. Clin Infect Dis.1994; 18: 38–72.

[5] Kaye A. Toxoplasmosis: diagnosis, treatment, and prevention in congenitally exposed infants. Journal of Pediatric Health Care 2011; 25: 355-64.

[6] Dunn D, Wallon M, Peyron F, Mother-to-child transmission of toxoplasmosis: risk estimates for clinical counseling. Lancet. 1999;353:1829-33. .

[7] Raeber PA, Berger R, Biedermann K, La prévention de la toxoplasmose congénitale en Suisse. Texte de consensus du Groupe de travail "Toxoplasmose congénitale" de l'Office fédéral de la santé publique [Prevention of congenital toxoplasmosis in Switzerland. Consensus report of the study group "Congenital toxoplasmosis" of the federal public health office]. Schweiz Med Wochenschr Suppl. 1995; 65: 113-20.

[8] Hohlfeld P, Daffos F, Thulliez P, Aufrant C, Courvreur J, MacAleese J, et al. Fetal toxoplasmosis: outcome of pregnancy and infant follow-up after in utero treatment. J Pediatr. 1989; 115: 765-9.

[9] Romand S, Chosson M, Franck J, Wallon M, Kieffer F, Kaiser K, et al. Usefulness of quantitative polymerase chain reaction in amniotic fluid as earlyprognostic marker of fetal infection with *Toxoplasma gondii*. Am J Obstet Gynecol. 2004; 190: 797-802.

[10] Gilbert RE. Epidemiology of infection in pregnant women. In: Petersen E, Amboise-Thomas P. Congenital Toxoplasmosis: Scientific Background, Clinical Management and Control. Paris: Springer-Verlag ;2000.p. 237–249.

[11] Nam HW.GRA Proteins of Toxoplasma gondii: Maintenance of Host-Parasite Interactions across the Parasitophorous Vacuolar Membrane. Korean J Parasitol. 2009, 47: 29-37.

[12] Gatkowska J[1], Hiszczynska-Sawicka E, Kur J, Holec L, Dlugonska H.. *Toxoplasma gondii*: an evaluation of diagnostic value of recombinant antigens in a murine model. Exp. Parasitol.2006; 114:220–227.

[13] Golkar M[1], Azadmanesh K, Khalili G, Khoshkholgh-Sima B, Babaie J, Mercier C, Brenier-Pinchart MP, Fricker-Hidalgo H, Pelloux H, Cesbron-Delauw MF.. Serodiagnosis of recently acquired *Toxoplasma gondii* infection in pregnant women using enzyme-linked

immunosorbent assays with a recombinant dense granule GRA6 protein. Diagn. Microbiol. Infect. Dis.2008; 61:31–39.

[14] Redlich A[1], Müller WA .Serodiagnosis of acute toxoplasmosis using a recombinant form of the dense granule antigen GRA6 in an enzymelinked immunosorbent assay. Parasitol. Res.1998; 84:700–706.

[15] Khan A, Su C, German M, Storch GA, Clifford DB, Sibley L D. Genotyping of Toxoplasma gondii strains from immunocompromised patients reveals high prevalence of type I strains. Journal of Clinical Microbiology.2005; 43: 5881–5887.

[16] Fazaeli A, Carter PE, Darde ML, Pennington TH. Molecular typing of *Toxoplasma gondii* strains by *GRA6* gene sequence analysis. *Int J Parasitol.2000; 30:* 637–642.

[17] Sterkers Y, Ribot J, Albaba S, Issert E, Bastien P, Pratlong F, Pratlong F. Diagnosis of congenital toxoplasmosis by polymerase chain reaction on neonatal peripheral blood. Diagnostic Microbiol Infect Disease.2011; 71:174–176.

[18] Fricker-Hidalgo H, Pelloux H, Muet F, Racinet C, Bost M, Goullier-Fleuret A, Ambrois Thomas P. Prenatal diagnosis of congenital toxoplasmosis: Comparative value of fetal blood and amniotic fluid using serological tech- niques and cultures Prenat Diagn.1997; 17(9): 831-35.

[19] Desmont, G., Daffos, F., Forestier, F., Capella-Pavlovsky, M., Thulliez, P.,Chartier, M. Prenatal diagnosis of congenital toxoplasmosis.1985. *Lancet* ;325(8427):500-504.

[20] Bessières MH, Berrebi A, Cassaing S, Fillaux J, Cambus JP, Berry A . Diagnosis of congenital toxoplasmosis: prenatal and neonatal evaluation of methods used in Toulouse University Hospital and incidence of congenital toxoplasmosis. Mem Inst Oswaldo Cruz.2009; 104:389–392.

[21] Robert-Gangneux F, Dupretz P, Yvenou C, Quinio D, Poulain P, Guiguen C. Clinical relevance of placenta examination for the diagnosis of congenital toxoplasmosis. Pediatr Infect Dis J .2010;29:33–38

[22] Fricker-Hidalgo H, Pelloux H, Racinet C, Grefenstette I, Bost-Bru C, Goullier-Fleuret A, Ambroise-Thomas P. Detection of Toxoplasma gondii in 94 placentae from infected women by polymerase chain reaction, in vivo, and in vitro cultures. Placenta.1998; 19,545–549.

[23] Suzuki Y, Takemoto O, Arai H, Goto M, Yamada J, Morimoto K, Nakayama M. Futaqi y. Yano A. A case of congenital toxoplasmosis confirmed by detection of Toxoplasma gondii in placenta. No To Hattatsu.1998; 30:411–416.

[24] Lecordier L, Moleon-Borodowsky I, Dubremetz JF, Tourvieille B, Mercier C,

Deslée D, Capron A, Cesbron-Delauw MF.. Characterization of a dense granule antigen of *Toxoplasma gondii* (GRA6) associated to the network of the parasitophorous vacuole. Mol. Biochem. Parasitol. **1995;70:**85–94.

[25] Tilley M[1], Fichera ME, Jerome ME, Roos DS, White MW.**.** *Toxoplasma gondii* sporozoites form a transient parasitophorous vacuole that is impermeable and contains only a subset of dense-granule proteins. Infect. Immun.1997; 65:4598–4605.

[26] Labruyere E[1], Lingnau M, Mercier C, Sibley LD. Differential membrane targeting of the secretory proteins GRA4 and GRA6 within the parasitophorous vacuole formed by *Toxoplasma gondii*. Mol. Biochem. Parasitol.1999; 102:311–324.

[27] Mercier C[1], Adjogble KD, Däubener W, Delauw MF. Dense granules: are they key organelles to help understand the parasitophorous vacuole of all apicomplexa parasites? Int. J. Parasitol.2005; 35**:**829–849.

[28] Sharifi-Mood B, Hashemi-Shahri M, Salehi M, Naderi M, Naser-Poor T .Seroepi-demiology of Toxoplasma infection in the pregnant women in Zahedan, Southeast of Iran. J Res Health Sci.2006; 6:1-3.

[29] Saeedi M, Veghari GR, Marjani A. Seroepidemiologic evaluation of anti-Toxoplasma antibodies among women in north of Iran. Pak J Biol Sci.2007; 10(14):2359.

[30] Alleyassin F, Moatari A, Zare FThe prevalence of Toxoplasma gondii antibodies in pregnant women in Shiraz. Iranian J Med Sci . 1990; 1(15):17-13.

[31] Asgari Q, Fekri M, Monabati A, Kalantary M, Mohammadpour I, Motazedian MH,Sarkari, B. Molecular genotyping of Toxoplasma gondii in human spontaneous Aborted fetuses in Shiraz, southern Iran. Iranian J Publ Health.2013; 42(6): 620-625.

[32] Hoveida L, Doudi M , Setorki M.Evaluation of toxoplasma gondii abortion cases by PCR teqnique in Isfahan, Iran. IJBPAS. 2014; 3(4): 524-531.

[33] Dentico P, Volpe A, Putoto G, Ramadani N, Bertinato L, Berisha M. Toxoplasmosis in Kosovo pregnant women . *New Microbiologica* .2011; 34(2),203-7.

[34] Lappalainen, M., M. Koskiniemi, V. Hiilesmaa et al. Outcome of children after maternal primary

Toxoplasma infection during pregnancy with emphasis on avidity of specific IgG. *Pediatr Infect Dis J.1995;* 14: 354–361.

[35] Bastien P.Molecular diagnosis of toxoplasmosis. Trans R Soc Trop Med Hyg. 2002; 96: 205-215.

[36] Akhlaghi L, Ghasemi A, Hadighi R, Tabatabaie F. Study of seroprevalence and risk factors for Toxoplasma gondii among pregnant women in Karaj township of Alborz province. J Entomol Zool . 2014; 2 (6): 217-219.

[37] Akhlaghi L ,Shirbazou2 S, Maleki F, Keyghobadi A ,Tabaraei Y, Tabatabaie F. Seroepidemiology of Toxoplasma Infection in Pregnant Women in Qom Province, Iran. (2010).Life Sci J. 2013;10(7s): 322-325.

[38] Tabatabaie F,Mafi M, Golestani M, Shahmohammad N, Mafi H, Maleki F. Seroprevalence of and rick factors for toxoplasma gondii among pregnant women in Abyek township of Gazvin province,Iran. (2013) Asian J Pharm Clin Res.2015; 8(1): 1-3.

[39] Hajsoleimani F, Ataeian A, Nourian AA, Mazloomzadeh S. Seroprevalence of Toxoplasma gondii in Pregnant Women and Bioassay of IgM Positive Cases in Zanjan, Northwest of Iran. Iranian J Parasito.2012; 7(2):82-86.

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