Diagnosis of Toxoplasmosis among couples by immune detection and genetic evaluation of *Toxoplasma gondii*

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Abstract

In this study, 27 married patients were totally examined for the presence of antibodies against *Toxoplasma gondii* by using a standard commercial enzyme-linking immunosorbent assay (ELISA) and Polymerase Chain Reactin (PCR) technique was used evaluate the genome of this parasite. Of 27 whole blood of married samples, nested PCR was positive in 22 (77.77%) by got the 96 bp DNA fragment, 9(40.9%) of them appeared with IgM and 6 (27.27%) of no anti-Toxoplasma, while 7 (31.81%) was positive nPCR results revealed both IgM^+ and IgG^+ antibodies, and 5 (22.22%) were negative results obtained by ELISA and nPCR.

تشخيص داء المقوسات بين الأزواج بواسطة التحري المناعي والتقييم الوراثي لطفيلي *Toxoplasma gondii*

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الخلاصة

في هذه الدراسة تم فحص مجموعة مكونة من 27 زوجا من المرضى المتزوجين تحريا عن وجود الأجسام المضادة للطفيلي *Toxoplsma gondii* باستخدام الاختبار المناعي المرتبط بالإنزيم ELISA مع تقييم مجين هذا الطفيلي بواسطة تقنية تفاعل البلمرة المتسلسل (PCR). من مجموع 27 زوجا من عينات دم المتزوجين، كان لفحص الـ nested PCR لـ 22 عينة (77.77%) موجباً بعد الحصول على قطعة دنا بحجم 96 زوج قاعدي، وأعطت 9 عينات (40.9%) نتيجة إيجابية مع IgM و6(27.27%) ايجابي مع غياب الأجسام المضادة للمقوسة، بينما سجلت 7 عينات (31.81%) نتيجة إيجابية مع كل من nPCR ومع الضدين المتأزرين (IgM، IgG)، في حين ان خمسة عينات (22.22%) كانت النتائج فيها سلبية في كلا الاختبارين (الاختبار المناعي المرتبط بالإنزيم وتفاعل البلمرة المتسلسل).

Introduction

Toxoplasma gondii is a ubiquitous apicomplexan parasites of human and others worm-blooded animals which has been considered as the cause of the most prevalent parasitic zoonosis (1, 2) and is the causal agent of significant morbidity and mortality among human worldwide (3, 4, 5). Congenital toxoplasmosis (CT) contamination occurring early during pregnancy can lead to severe fetal damage. Clinical manifestations depend mainly on when the infection was acquired in utero. Infectivity is highest during the later stages of pregnancy; however, the earlier in gestation is an infection which occurs, the greater the likelihood of severe postnatal sequelae (6). Most cases of acquired *Toxoplasma* infection are asymptomatic and self-limited; hence many cases remain undiagnosed. The incubation period of acquired infection is estimated to be lasted or continued during 4-21days (7 days on average) (7). When symptomatic infection does occur, the only clinical findings may be focal lymphadenopathy. This involves a single site around the head and neck frequently. Acute infection is rarely accompanied by a mononucleosis-like syndrome characterized by fever, malaise, sore throat, headache and an atypical lymphocytosis on peripheral blood smear (8). The major objective cause is that one third of the world population has been infected with *Toxoplasma gondii*. The latent toxoplasmosis was recently considered as asymptomatic which has potentially been shown to have serious consequences for physical and psychical health (9). Serological studies showed a considerable difference in the

prevalence of Toxoplasma infection from 0-95% in different parts of the world and indeed between different population groups within the same country (10, 11, 12, 13). In Iraq, Juma and Salman 2011 (14) found that the infection of *T. gondii* in women was 19.17%. In Tikrit province in Iraq, Al-Doori 2010 (15) showed the presence of infection of about 49 to 95% and higher rate of infection can be seen among those who are 25 to 31 years old particularly women and their husbands. Seroprevalence of *T. gondii* infection among men rises with age and it did not vary greatly between sexes (16). The prevalence of *Toxoplasmosis* significantly increasesed with age and the highest seropositivity rate, 35.4% was found among pregnant women in the age group of 35 to 44 years old in Slovakia (17). The overall seroprevalence of Toxoplasmosis in South African was 29/160 (18.1%). Seroprevalence in males and females were 7/42 (16.7%) and 22/118 (18.6%), respectively and the difference was not statistically significant ($p>0.05$). The age distribution was 0.63% (1/160) for individuals of 20 years old and below, 10.6% (17/160) for those between 21 and 35 years old and 6.9% (11/160) for individuals who were 36 years old and above (18). The serologic evidence of toxoplasmosis in Ethiopia was found in 60% (39/65) of them. A large number of the seropositives were females (64.1%), while in male was 53.8% (19). The overall anti-*T.gondii* IgG prevalence in China was 12.3%, the seroprevalence was 10.5% in men versus 14.3% in women (20). This study used PCR to amplify *Toxoplasma* DNA from the B1 gene to detect *T.gondii* in whole blood of married patients with confirmed clinical toxoplasmosis and positive specific anti-*Toxoplasma* (IgG and IgM) antibodies.

Materials and methods

Whole blood samples were clinically and laboratory collected from 27 *Toxoplasma* infected patients. The samples were collected between November 2010 to May 2011 from a clinical laboratory in Ramadi Hospital.

- **IgG and IgM antibodies detection:** This assay was performed by two different approaches. One for detection of IgG and another one for detection of IgM specific antibodies against *T. gondii* antigens among the patient's serum (Biokit Diagnostics Company, Spain).

Detection of IgG and IgM titers in all samples were analyzed for *T. gondii* by the titer of IgG and IgM antibodies using ELISA kit as described by Biokit Diagnostics Company, Spain. The optical densities (OD) of the samples were measured at 450 nm, using the OD value of the blank well to correct all the OD reading from test wells (Biokit Diagnostics Company, Spain).

- **Isolation of DNA:** DNA also was extracted from samples using QIAmp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.
- **Detection of *T. gondii* by PCR:** Detection of *T. gondii* infections was determined by nested PCR amplification targeting the B1 gene (GenBank: AF146527) (21). The PCR mixture for the nested reaction consisted of 2X PCR master mix solution with a final concentration of 1.5 mM MgCl2, 200 µM of each dNTP and 2.5 U Taq DNA polymerase (Promega, USA); 10 pmol of each primer; and as a template, 2 µl of extracted and 5 µl of treated sample DNA for the first round and 2 µl of a 1:10 dilution of the products of the first amplification for the second round, in a final volume of 25 µl. PCR reactions were performed in a thermal cycler (Techn , UK). First step PCR was 5 minutes at 94ºC, followed by 35 cycles, each cycle consisting of 60 seconds at 94ºC, 30 seconds at the annealing temperature for each primer pair, and 60 seconds at 72ºC. The final cycle was followed by heating for 5 minutes at 72ºC. Primary PCR amplification was performed with outer primers B1F1 (5'-CCGTTGGTTCCGCCTCCTTC-3') and B1R1 (5'-GCAAAACAGCGGCAG CGTCT-3') at an annealing temperature of 54ºC. In the second round, the internal primers B1F2 (5'-CCGCCTCCTTCGTCC GTCGT-3') and B1R2 (5'-GTGGGGG CGGACCTCTCTTG-3') were used at an annealing temperature of 60ºC. Primers were synthesized by Eurofins Genomics India Pvt, Ltd (India).The PCR products were electrophoresed on 1.5% agarose gel in 1x TAE buffer and stained with ethidium bromide (0.5µg/ml). Under a transilluminator with a 100 bp DNA ladder (Promega, USA).

Results

The results of two tests (nPCR , ELISA) showed that 22 pairs were positive nPCR (77.77%), the PCR product of the positive result was 96 bp (Fig. 1), and the positive results of ELISA test 17 (62.96%) (Table 1). The couples 22 (77.77%) who recorded positive results in nPCR analysis were distributed on the patterns of the anti-Toxoplasma antibodies, it was found that 9(40.9%) of them appeared with IgM^+ pattern and no the patterns of IgG^+ and 6 (27.27%) of no anti-Toxoplasma antibodies. In addition, 7(31.81%) of a positive nPCR results revealed both

(IgM^+ and IgG^+) antibodies. Notable, these results showed low significant differences ($P \leq 0.05$) (Table 2 and Fig .1).

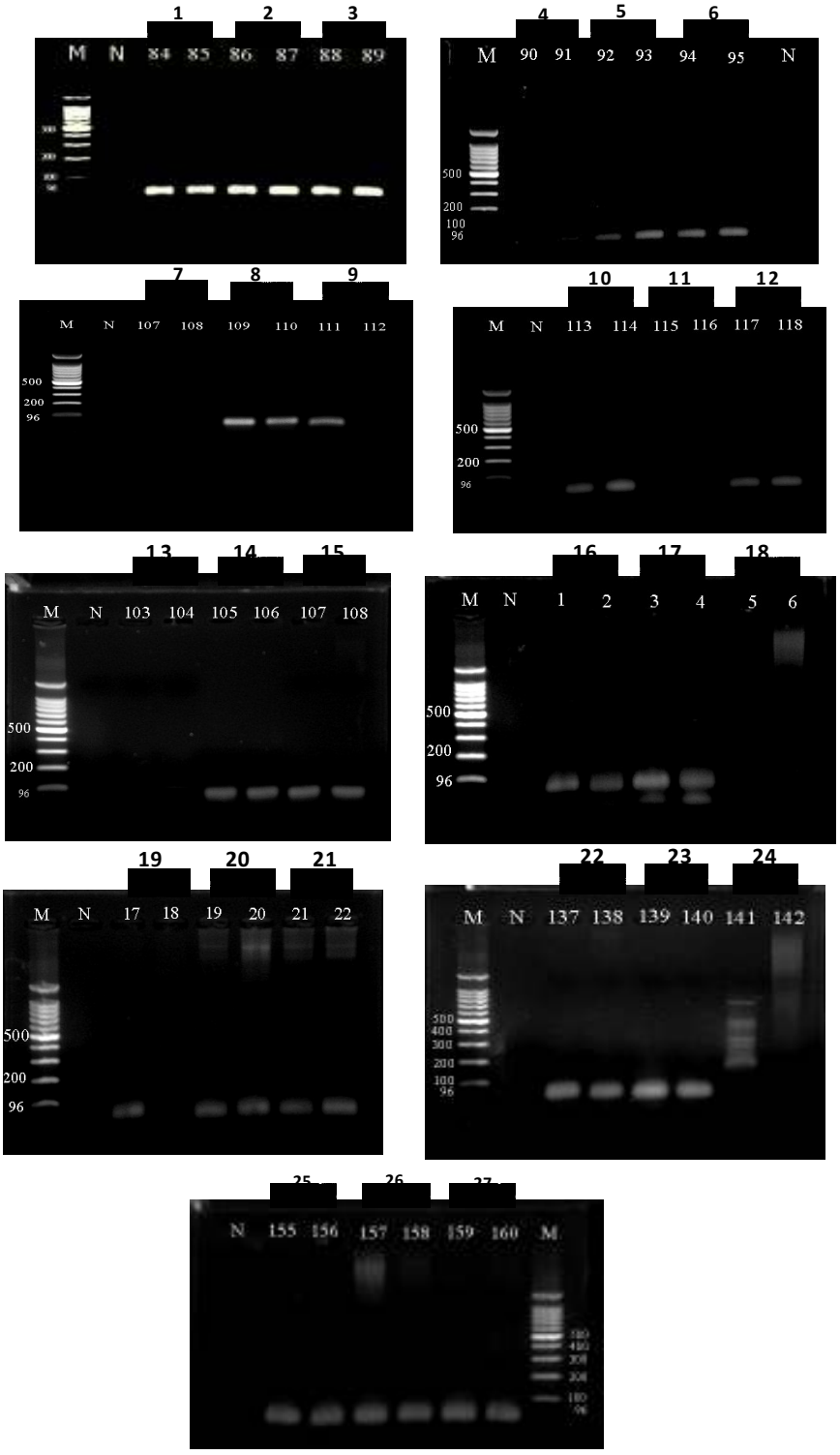

Fig. (1) DNA amplification by nested PCR for the 96 bp fragment from B1 gene. M: 100 bp DNA ladder, N: negative control, Numbers 1-27: No.s of couples

Table (1) Prevalence of Toxoplasmosis in couples by two techniques (nPCR, ELISA)

Test	Positive		Negative	
	No.	%	No.	%
nPCR	22	77.77	5	22.22
ELISA	17	62.96	10	37.03

Table (2) Nested-PCR analysis distributed by anti-*Toxoplasma* antibodies in couples

Pattern of antibody	Positive		Negative		Total	
	No.	%	No.	%	No.	%
IgM^+ ve	9	40.9	1	20	10	37.03
IgG^+ ve	0	0	0	0	0	0
IgM^+ ve & IgG^+ ve	7	31.81	0	0	7	25.92
No anti-*Toxoplasma* Abs.	6	27.27	4	80	10	37.03
Total	22	100	5	100	27	100
Statistical analysis	Cal. $\chi^2 = 7.126$; Tab. $\chi^2 = 7.815$; $P \leq 0.05$					

Discussion

Normally the diagnosis of congenital toxoplasmosis is based on serological demonstration of IgM antibody but may be specific IgM antibodies not present and antibody synthesis is delayed in infants, therefore we added PCR technique to increase the validity of results as was suggested earlier. It is mentioned that PCR for detection of *T. gondii* had high sensitivity and specificity (22). The serological testing had been one of the major diagnostic for toxoplasmosis, it had many limitations. It might fail to detect specific anti-*Toxoplasma* IgG or IgM during the active phase of *T.gondii* infection, therefore, the risk of congenital toxoplasmosis of a fetus might be undetected because the pregnant mother might test negative during the active phase of *T.gondii* infection, furthermore, the test might fail to detect *T.gondii* infection in certain immunocompromised patients due to the fact that the titers of specific anti-*Toxoplasma* IgG or IgM antibodies might fail to rise in this type of patient (23). In our study, there were negative results obtained by both PCR and ELISA 5 (18.51%) rule out an infection in the couples. These 5 couples continued their pregnancies and no congenitally infected newborn were delivered, similar results have been reported in previous studies (24). In our results, negative nPCR and positive IgM antibodies (20%) demonstrated that the acute phase of the disease had passed and they were in chronic phase, and residual IgM detected during prolonged periods of time, even after the end of the acute episode and consequent eradication of parasitemia, also, false positive IgM antibody test results have been reported previously (25, 26). Previous studies have documented that PCR could actually detected *T. gondii* in blood specimens of women before or during pregnancy (27, 28). Based on this, the presence of Toxoplasma DNA in the maternal blood probably indicates a recent infection or apparent parasitaemia, which is likely to be clinically significant. The clearance time for Toxoplasma DNA from the blood of patients with acute toxoplasmic lymphadenopathy was estimated to be 5.5-13 weeks (29). In this study, the presence of *Toxoplasma* DNA in the maternal blood and positive IgM antibody 9/27 (40.9%) probably indicates a recent infection or apparent parasitemia or active toxoplasmosis, which was likely to be clinically significant. However, PCR could not discriminated between latent or acute infection. On the other hand, couples who have experienced the birth of congenitally deformed children who recorded positive gene product in nPCR analysis, 7 (31.81%) were revealed to hold IgM^+ and IgG^+. This refers to the presence of latent infection and not taking the treatment because of the absence of cases of abortion during pregnancy, causing congenital birth of

deformed children. The findings lead to the interpretation that chronic toxoplasmosis infection is able to promote positive PCR results when the parasite is found circulating. The diagnosis of primary toxoplasmosis in pregnant women early in the first trimester is of utmost importance in order to offer them early therapy or other interventions to prevent congenital infection of fetuses (25, 26, 30, 31). Our conclusion, we found a relationship between husband and wife in terms of injury, disease toxoplasmosis, especially when determining the genome of the parasite Toxoplasma where we showed that all the couples infected with the existence of cases of abortion in most of the couples and exciting thing that coincides the occurrence of birth defects with positive results for antibodies Toxoplasma (IgM and IgG) and nPCR positive, suggesting sexual transmission probability.

References

1. Sanad, M. M. & Al-Ghabban, A. J. 2007. Serological survey of Toxoplasmosis among sheep and goats in Tabouk, Saudi Arabia. J. Egypt Soc. Parasitol., 37: 329-340.
2. Sabry, M. A. & Reda, W. W. 2008. Infection by cyst producing Protozoa among human and food producing animal in Egypt. J. Boil. Sci., 8: 889-895.
3. Dubey, J. P. 2007. *Toxoplasma gondii* in the model Apicomplexan- perspective and method, Weiss, L. D. & Kim, K. (Eds) Elsevier, London.
4. Chen, X. G. & Tan, F. 2009. *Toxoplasma gondii*: Past, present and future. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi., 27: 426-431.
5. Asgari, Q. D.; Mehrabani, D.; Moazzeni, M.; Akrami-Mohajeri, F.; Kalantari, M.; Motazedian, M. H. & Hatam, G. R. 2009. The Seroprevalence of ovine Toxoplasmosis in Fars Province . Southern Iran. Asian J. Anim. Adv., 4: 332-336.
6. Garcia-Meric, P.; Frack, J.; Dumon, H. & Piarroux, R. 2010. Management of congenital toxoplasmosis in France: current data. Presse. Med., 39: 530-538.
7. Bowie, W. R.; King, A. S.; Werker, D. H.; Isaac-Renton, J. L.; Bell, A. & Eng, S. B. 1997. Outbreak of Toxoplasmosis associated with municipal drinking water. The BC Toxoplasma Investigation Team. Lancet, 350: 173-177.
8. Lynfield, R. & Guerina, N. G. 1997. Toxoplasmosis. Pediatr. Rev., 18: 75-83.
9. Arling, T. A.; Yolken, R. H.; Lapidus, M.; Langenberg, P.; Dickerson, F. B.; Zimmerman, S. A.; Balis, T.; Cabassa, J. A.; Scrandis, D. A.; Tonelli, L. H. & Postolache, T. T. 2009. *Toxoplasma gondii* antibody titers and history of suicide attempts in patients with recurrent mood disorders. J. Nerv. Ment. Dis., 197: 905-908.
10. Asthana, S. P.; Macpherson, C. N.; Weiss, S. H. & Stephens, R. 2006. Seroprevalence of *Toxoplasma gondii* in pregnant women and cats in Grenada, West Indies. J. Parasitol., 92: 644-645.
11. Ghorbani, M.; Edrissian, G. H. & Assad, N. 1978. Serological survey of toxoplasmosis in northern part of Iran, Using in direct fluorescent antibody technique. Trans. R. Soc. Trop. Med. Hyg.,72: 369-371.
12. Abu-Zeid, Y. A. 2002. Serological evidence for remarkably variable prevalence rates of *Toxoplasma gondii* in children of major residential areas in United Arab Emirates. Acta Trop., 83: 63-69.
13. Fan, C. K.; Su, K. E. & Wu, G. H. 2002. Seroepidemiology of *Toxoplasma gondii* infection among two mountain aboriginal populations and South east Asian laborers in Taiwan. J. Parasitol., 88: 411-414.
14. Juma, A. S. & Salman, S. 2011. Correlation between apoptosis and *Toxoplasma* in abortion induction : Relevance of caspase 8. Int. J. Med. Sci., 3(6): 181-192.
15. Al-Doori, M. A. 2010. Epidemiological study of *Toxoplasma gondii* between couples in Tikrit city, and experimental trial about possibility of sexual transmission of infection in mice. M.Sc. Thesis, college of Education, University of Tikrit.

16. Montoya, J. G. & Remington, J. S. 2000. *Toxoplasma gondii*. In: Principles and Practice of Infectious Diseases, Mandell, G. L.; Bennett, J. E. & R. Donlin (Eds.), 5th Ed., Churchill Livingstone, Philadelphia, PP. 2858-2887.
17. Studenicova, C.; Bencaiova, G. & Holkova, R. 2006. Seroprevalence of *Toxoplama gondii* antibodies in a health population from Slovakia. Eur. J. Inter. Med., 17: 470-473.
18. Bessong, P. O. & Mathomu, L. M. 2010. Seroprevalence of HTLV1/2 , HSV1/2 and *Toxoplasma gondii* among chronic HIV-1 infected individuals in rural northeastern South African. Afr. J. Microbiol. Res., 4(23): 2587-2591.
19. Negash, T.; Tilahum, G. & Medhin, G. 2008. Seroprevalence of *Toxoplama gondii* in Nazaret Town, Ethiopia. East Afr. J. Pub. Heal., 5(3): 23-30.
20. Xiao, Y.; Yin, J.; Jiang, N.; Xiang, M.; Hao, L.; Lu, H.; Sang, H.; Liu, X.; Xu, H.; Ankarklev, J.; Lindh, J. & Chen, Q. 2010. Seroepidemiology of human *Toxoplasm gondii* infection in China. BMC Infect. Dis., 10: 4-13.
21. Burg, J. L.; Grover, C. M.; Pouletty, P. & Boothroyd, J. C. 1989. Direct and sensitive detection of pathogenic protozoan *Toxoplasma gondii*, by polymerase chain reaction. J. Clin. Microbiol., 27: 1787-1792.
22. Fuentes, I.; Rodriguez, M.; Domingo, C. J.; Castillo, F.; Juncosa, T. & Auvar, J. 1996. Urine sample used for congenital toxoplasmosis diagnosis by PCR. J. Clin. Mic., 48: 2368-2371.
23. Morussi, R.; Madalena, T. M.; Alves, D. & Azevedo, P. 2006. *Toxoplasma*- IgM and IgG-avidity in single samples from areas with a high infection rate can determine the risk of mother-to-child transmission. Rev. Inst. Med. Trop. S. Paulo., 48(2):4665-4676.
24. Montoya, J. G.; Liesenfeld, O.; Kinney, S.; Press, C. & Remington, J. S. 2002. VIDAS test for avidity of Toxoplasma-specific immunoglobulin G for confirmatory testing of pregnant women. J Clin. Microbiol., 40: 2504-2508.
25. Singh, S. 2003. Mother-to-child transmission and diagnosis of *Toxoplasma gondii* infection during pregnancy. Indian J. Med. Microbiol., 21: 69-76.
26. Emna, S.; Karim, A.; Mohammed, K. & Aida, B. 2006. Difficulty in dating primary infections by Toxoplasma gondii in pregnant women in Tunisia. Tunis Med., 84: 85-87.
27. Chabbert, E.; Lachaud, L.; Crobu, L. & Bastien, P. 2004. Comparison of two widely used PCR primer systems for detection of Toxoplasma in amniotic fluid, blood, and tissues. J. Clin. Microbiol., 42: 1719-1722.
28. Slawska, H.; Czuba, B.; Gola, J.; Mazurek, U.; Wloch, A.; Wilczok, T. & Kaminski, K. 2005. Diagnostic difficulties of Toxoplasma gondii infection in pregnant women. Is it possible to explain doubts by polymerase chain reaction? Ginekol Pol., 76: 536-542.
29. Guy, E. C. & Joynson, D. H. M. 1995. Potential of the polymerase chain reaction in the diagnosis of active Toxoplasma infection by detection of parasite in blood. J. Infect. Dis., 172: 319-322.
30. Akoijam, B. S.; Shashikant, S. & Kapoor, S. K. 2002. Seroprevalence of Toxoplasma infection among primigravid women attending antenatal clinic at a secondary level hospital in north India. J. Indian Med. Assoc., 100: 591-602.
31. Reis, M. M.; Tessaro, M. M. & D'Azevedo, P. A. 2006. Toxoplasma-IgM and IgG-avidity in single samples from areas with a high infection rate can determine the risk of mother-to-child transmission. Rev. Inst. Med. Trop. Sao Paulo, 48: 93-98.