Detection of Toxoplasma Parasitemia by PCR: Does it Correlate with IgG and IgM Antibody Titers?

Behzad Haghpanah[1], Mansoor Salehi[2], Shahram Sadri[1]

[1]Department of Parasitology and Mycology, [2]Department of Genetics and Molecular Biology, Medical School, Isfahan University of Medical Sciences, Isfahan, Iran

ABSTRACT

Background: Toxoplasmosis is a zoonotic disease with high seroprevalence worldwide. Several immunological methods have been described for diagnosis of toxoplasmosis. **Objective:** To determine the parasitemia period in patients infected with toxoplasma using PCR and comparing serological data with molecular results. **Methods:** 154 serum samples from patients with toxoplasmosis were examined. Presence of parasite DNA was evaluated using PCR method. IgG and IgM antibody titers were measured using IFA test. **Results:** Of 154 studied samples, 28 were positive for IgM and 60 were positive for IgG with titers higher than 1/400. PCR was performed on those samples having either IgG or IgM titers. Samples with IgM titers lower than 1/800 and higher than 1/3200 had no detectable level of parasite DNA. Parasitemia was detected in cases with IgG titer of 1/100 to 1/200. All samples with no detectable IgM and with IgG titers higher than 1/400 were negative when tested by PCR. **Conclusion**: IgM specific antibody titer between 1/800 and1/3200 represents a window opportunity in treatment of patients with toxoplasmosis. Absence of parasite DNA in patients with higher IgM antibody titer is explained by the effector mechanism of antibody for clearance of the parasite.

Keywords: Toxoplasmosis, Parasitemia, PCR

*Corresponding author: Dr. Behzad Haghpanah, Department of Parasitology and Mycology, Medical School, Isfahan University of Medical Sciences, Isfahan, Iran. P.O. Box: 81744-176, Tel: (+) 98 311 7922418, e-mail: haghpanah@med.mui.ac.ir

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite in the Apicomplexa phylum. It causes toxoplasmosis in many vertebrates including humans and therefore has great medical and veterinary importance (1). Because of immune system activity, parasitemia will soon be eliminated from blood and the parasite cysts will localize in different tissues (2). Clinical interference and treatment in toxoplasmosis, especially in some clinical conditions such as pregnancy and immune deficiency, clearly have positive effects on the prognosis of the disease (3). Considering the point that pharmacotherapy of toxoplasmosis is only effective in the active parasitemia phase of the disease, therefore exact and early detection of infection is of great importance in adequate treatment and follow up of the patient (4, 5). In serological methods, pattern of increase and decrease of anti-toxoplasma antibodies, especially IgG and IgM, are routinely used for toxoplasma detection. These techniques are simple, quick and cheap but clearly are not efficient in active toxoplasmosis detection. There also might be ambiguity with interpretation of the results of serology tests (6). It is important to note that in toxoplasmosis acute and current infection is always accompanied by parasitemia. Therefore the simplest way for effective detection of active infection, that needs treatment and clinical follow up, would be the detection of parasitemia in the patients (7). Many scientists applied different methods, such as inoculation in laboratory animals, tissue cultures, and search for circulating antigen to detect toxoplasmosis parasitemia.

One of the newest methods for parasitemia detection is based on polymerase chain reaction (PCR). James (1996), Angle (1997), Burney (1999), Dasilva (2000) and Hafid et al. (2001) demonstrated that PCR has high sensitivity and specificity in toxoplasmosis detection and parasitemia determination (8, 9, 10, 11, 12). In 1995 Guy et al. applied PCR and demonstrated that in toxoplasma infected patients, parasitemia is present from the second week to the 17^{th} weeks since the start of the infection. His study clarified the uncertainty about the duration of parasitemia (13). In this study we tried to detect parasitemia, by PCR, and simultaneously measure levels of IgG and IgM antibodies using IFAT method in toxoplasmosis.

MATERIALS AND METHODS

Sample Collection. Serum samples were collected from 154 clinically and laboratory confirmed toxoplasma infected patients. The samples were collected during a six months period from clinical laboratories in Isfahan. Collected samples were divided into two groups and stored at -20ºC till required.

Antibody Measurement. IgG and IgM specific antibodies against T.gondii were measured on freshly collected serum samples, using IFAT method. For this study we have used Flu.TOXO KIT provided by Bahar Afshan Co. (IRAN) and anti human IgM and IgG, FITC labelled provided by DAKO (Denmark). IgM titre was examined from 1/10 to 1/12800 dilutions and IgG from 1/100 to 1/12800 dilutions.

Parasitemia Detection. PCR technique was applied to determine the presence of parasite nucleic acid in the collected serum samples. For this reason, after setting up DNA extraction and optimization of PCR, sensitivity of the technique was evaluated using control samples. Then PCR was applied on the collected samples.

DNA Extraction. To extract DNA from both patient samples and the prepared positive controls (containing tachyzoites) a modified phenol/chloroform protocol specific for DNA extraction from serum and plasma (CNAPS) was applied (14). First, 1ml 10× SDS/PK (5ml SDS; Sigma 10%, 25mg Proteinase K; Fermentas) was added to 9ml TE-9 (500mM Tris pH 9; Merck, 20mM EDTA; Merck, 10mM NaCl; Merck). Next 380µl of serum sample was added to 380µl 1× SDS/PK solution and incubated at 55ºC overnight. This was then added to 760µl PC-8 solution (62.5ml Aquaphenol pH 8; Roch, 10ml ddH_2O, 0.625ml 0.5M EDTA pH 8.5; Merck, 50ml chloroform; Roch) and vortexed vigorously. The tube was then centrifuged at 2500 rpm for 10min, and the upper aqueous phase was transferred to a new tube and the phenol extraction step was repeated once more on the supernatant. Next 1µl of 20µg/ml glycogen solution; 0.3ml of 7.5M Ammonium acetate and 1ml of cold absolute ethanol was added to the tube and mixed. This was then centrifuged at 6000 rpm for 20min, and the supernatant was removed. The DNA pellet was then washed with 70% ethanol, centrifuged (3000 rpm, 5min.) and after removing the 70% ethanol the pellet was dried and resuspended in 80µl of ddH_2O. The extracted DNA was then stored at -20ºC until required.

PCR Assay. PCR was performed to amplify gene B1 as explained by Burge at al., (15) with minor modifications. After optimization the following reaction mixture was prepared in a final volume of 50µl: 5µl 10×PCR buffer; 3µl of $MgCl_2$ (50mM); 1µl of 10mM each dNTP, 1µl of 25pmol of primer 1 and 1µl of 25pmol primer 2, and 0.2µl of 5u/µl Taq DNA polymerase, 1µl of DNA sample (up to 200ng) and deionized water up to 50µl.

The reaction was then overlayered with 50µl of Mineral oil and the following PCR cycles were performed in a Hybide (Omnigene) thermocycler; One cycle of initial denaturation at 95 °C for 5 min, followed by 40 cycle of: denaturation at 95°C for 1 min, annealing at 56 °C for 45 sec and extension at 72°C for 45 sec. This was followed by 1 cycle of final extension at 72 °C for 10 min. 16µl of the PCR product was then mixed with 4µl of loading dye (0.2% Bromphenol Blue, 20% sucrose, 1% SDS and 5mM EDTA) and electrophoresed on a 2.5% agarose gel using 0.5× TE buffer (Tris-base 0.89M, Boric acid 0.89M, 40ml 0.02M EDTA; pH8.4). After electrophoresis, the bands on the gel were visualised using Ethidium bromide and UV irradiation. To determine the sensitivity of PCR for detection of Toxoplasma parasitemia, we performed PCR on positive controls containing 2, 5, 10, 15, 20, and up to 15,000 tachyzoites.

For amplification of positive controls by PCR, DNA was extracted from peritoneal fluid of an infected mouse (containing tachyzoites) and a negative control with no DNA, was also used. Experiments were performed in duplicate.

RESULTS

Serologic Test. In this study 154 serum samples from toxoplasma infected patients were examined. IgG and IgM titres of all samples were measured using IFAT test. For interpretation of IFAT results, IgG titres over 1/200 and IgM titres over 1/100 were considered positive.

Of 154 examined samples, 65 were negative for IgM and their IgG titres were also lower than 1/200, indicating no previous exposure to the parasite. Sixteen out of

these 65 samples were examined by PCR, which were negative for Toxoplasma DNA.

Out of 109 examined samples 84 had enough levels of specific antibodies against T. gondii to be considered positive. Sixty samples had IgG titres higher than 1/400, indicating previous exposure to the parasite. Results of IFAT test are summarized in tables 1 and 2.

Table 1. Results of IgG antibody titer and PCR amplification of 109 positive examined samples

IgG titre	Number PCR%		Total
	PCR Negative	PCR Positive	
1/100	10 (9.6%)	4 (80%)	14 (12.8%)
1/200	21 (20.2%)	1 (20%)	22 (20.2%)
1/400	26 (25%)	-	26 (23.9%)
1/800	26 (25%)	-	26 (23.9%)
1/1600	11 (10.6%)	-	11 (10.1%)
1/3200	7 (6.7%)	-	7 (6.4%)
1/6400	3 (2.9%)	-	3 (2.8%)
Total:	104 (100%)	5 (100%)	109 (100%)

Table 2. Results of IgM antibody titer and PCR amplification of 109 positive examined samples

IgM titer	Number PCR%		Total
	PCR Negative	PCR Positive	
Negative	77 (74%)	-	77 (70.6%)
1/10	4 (3.8%)	-	4 (3.7%)
1/100	7 (6.7%)	-	7 (6.4%)
1/200	6 (5.8%)	-	6 (5.5%)
1/400	5 (4.8%)	-	5 (4.6%)
1/800	3 (2.9%)	1(20%)	4 (3.7%)
1/1600		2 (40%)	2 (1.8%)
1/3200		2 (40%)	2 (1.8%)
1/6400	2 (19%)	-	2 (1.8%)
Total:	104 (100%)	5 (100%)	109 (100%)

Positive controls, containing toxoplasma gondii DNA, were amplified using a pair of specific primers for B1 gene. The PCR product was a 194bp band (Figure 1). After PCR optimization we were able to detect the presence of as low as 3-5 tachyzoites in a volume of 380µl serum. Considering that each parasite contains 0.2 pg DNA, the sensitivity of PCR was expected to be 1pg/380µl.

Of the collected samples, 109 were IgG and IgM positive. PCR was performed on these samples to detect the presence of the parasite. Only 5 samples with positive IgM titres ranging from 1/800 to 1/3200 were positive when PCR test was applied. All IgM negative samples were also PCR negative and therefore had no parasitemia. The relationship between negative and positive PCR results (i.e. presence or absence of parasitemia) and the level of IgG and IgM specific antibodies against T. gondii in the serum of Toxoplasma infected patients are presented in tables 1 and 2.

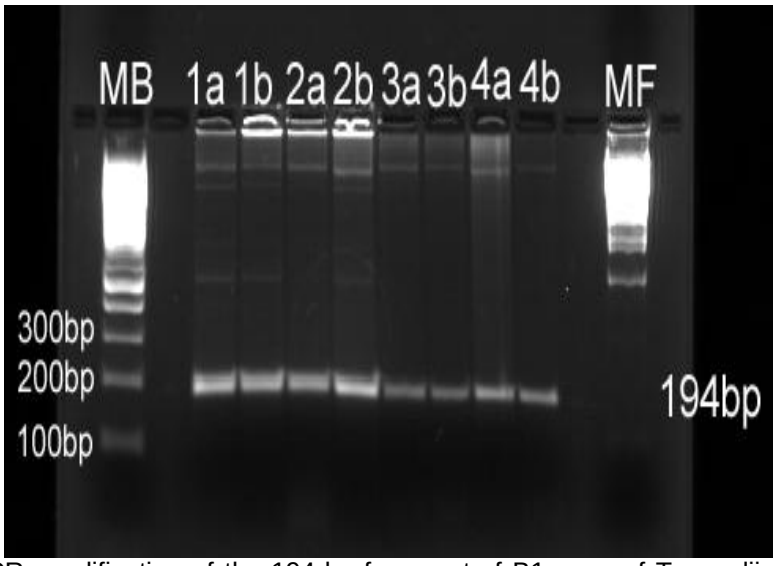

Figure 1. PCR amplification of the 194 bp fragment of B1 gene of T. gondii genome. DNA was extracted from positive controls containing tachyzoites of the parasite. Lanes 1, 2, 3 and 4 contained 50, 20, 10 and 5 tachyzoites per 380µl, respectively. Each test had one repeat (a and b). Lanes Mb and MF are size markers.

DISCUSSION

The aim of this study was to determine parasitemia period in patients infected by toxoplasma and also to evaluate the possible relationship between the presence of parasitemia and the level of specific IgG and IgM antibodies in patients serum. In 60 samples the IgG titre was higher than 1/400, which was an indication for previous exposure to the parasite. As mentioned above sensitivity of PCR in our study was 1pg/380µl. This is in concordance with other studies (12). Negative PCR results for these patients demonstrate that the acute phase of the disease has passed and they were in chronic phase. In positive sera, IgM titre over 1/100 were observed only in 28 samples. Nine of these 28 IgM positive samples had IgM titres equal or lower than 1/800 and IgG titres over 1/800. In these 9 samples, the serological results (reduction in IgM titre and increase in IgG titre) indicated that the infection has passed the acute phase. Interestingly, PCR test demonstrated that all of these 9 samples were negative for the presence of parasite DNA and parasitemia.

In fact these patients were in the chronic phase of the infection. In these patients because of conquest of immune system over the parasite, no parasitemia was present and therefore PCR results were negative. In the rest of IgM positive samples, twelve had IgM titers lower than 1/400 and mostly around 1/100. In this group, IgG levels were mostly low and were less than 1/200. For interpretation of serological results of these 12 samples (low titre of IgM and no IgG), it can be concluded that toxoplasma infection was in its early acute phase in which, tachyzoites were starting intracellular proliferation in the reticuloendothelial system and circulating white blood cells. However, tachyzoite lysis by specific antibody and complement dependent reactions have not yet happened at a level to cause the genetic components of the parasite to be released in blood in a measurable amount, therefore PCR results in these samples were negative. Seven of the IgM positive samples had titers higher than 1/800 in which only 5 were PCR positive. These 5 samples definitely had parasitemia. In all of these 5 samples, with positive PCR, IgG titers were lower than 1/200, which indicates IgM activation phase for tachyzoite lysis in the acute phase of the disease when tachyzoites are in their highest destructive activity. IgM titers of these 5 samples were from 1/800 to 1/3200. The 1/800 titer was border lined for the presence of parasitemia, because in this IgM titre, PCR tests were negative in some samples and therefore no parasitemia existed. Statistical analysis of the serological and PCR results demonstrated that PCR positive samples had high titers of IgM and low titers of IgG, therefore a relationship between the presence of parasitemia and titers of specific antibodies against T. gondii has been determined. IgG titers for PCR positive samples were 1/100 in 80% of the samples and 1/200 in the rest. Anti-parasite IgM titers for PCR positive samples were 1/800 in 20%, 1/1600 in 40% and 1/3200 in 40% of the samples. Using t-test and Pearson and Chi-square test for statistical analysis, we demonstrated that toxoplasma parasitemia is present with IgM titers ranging form 1/800 to 1/3200 and low or negative titers of IgG. In IgM titers higher than 1/3200 parasitemia would not be present.

Results of this study are in accordance with the reports of others that parasitemia stage reduces at the end of the acute phase of toxoplasma infection. Physiologically, the level of specific antibodies against T. gondii remains high only in a limited time period. The exceptions to this are some of rare cases with persistent IgM rise, and also false positive results that normally are seen in serological techniques (5, 6).

In a study in 1997, Angel et al. evaluated different clinical stages of toxoplasmosis by application of blotting technique. Nine out of 17 patients with Toxoplasmosis lymphadenopathy had positive dot blot, indicating the presence of parasitemia (9). Although all these 17 patients had positive serological results, using molecular techniques showed that parasitemia and acute infection were present only in 67% of them. This is in accordance with our results which demonstrated that positive serological results in patients do not necessarily correspond to acute and active phase of infection.

In conclusion, the results of our study demonstrated that parasitemia can be present in patients with increasing IgM titre and therefore drug therapy is effective when serological results show that antibody titre is increasing. Clearly, pharmacotherapy in other phases of infection other than the mid acute phase has no effect and therefore has no clinical value. Based on our results, presence of parasitemia is mostly prob-

able when IgM titres range from 1/800 to 1/3200 and therefore complete drug therapy in this window period is necessary.

ACKNOWLEDGEMENTS

This work was funded by grant number 81202 from Isfahan University of Medical Sciences.

REFERENCES

1. Carruthers VB. Host cell invasion by the opportunistic pathogen Toxoplasma gondii. Acta Trop.2002;81:111-22.
2. Kasper L. Toxoplasma infection. In : Brauwald E, Fauci A, Kasper D. Harrisons Principle of Internal Medicine. 15 ed. New York, McGraw Hill Medical Publishing Division.2001:1222-1227.
3. Frenkel JK, Escajadillo A. Cyst rupture as a pathogenic mechanism of toxoplasmic encephalitis. Am J Trop Med Hyg.1987;36:517-22.
4. Dubey JP. Toxoplasmosis. In : Collier L, Balows A, Sussman M. Microbiology and microbial infections. 9th ed. London, Arnold press.1998: 303- 318.
5. Holliman RE. Toxoplasmosis. in: Cook GC. Mansons Tropical disease. 12ed. Philadelphia,Saunders press,1996:1246-1253.
6. Montoya J, Remington JS. Toxoplasma gondii. In : Mandell G, Beenett J, Dolin R. Principle and practice of infectious diseases. 5ed. Philadelphia, Churchill Livingstone press. 2000 : 2858-2881 .
7. Ho-Yen DO, Joss AW, Balfour AH, Smyth ET, Baird D, Chatterton JM. Use of the polymerase chain reaction to detect Toxoplasma gondii in human blood samples. J Clin Pathol.1992;45:910-3.
8. James GS, Sintchenko VG, Dickeson DJ, Gilbert GL. Comparison of cell culture, mouse inoculation, and PCR for detection of Toxoplasma gondii: effects of storage conditions on sensitivity. J Clin Microbiol.1996;34:1572-5.
9. Angel SO, Matrajt M, Margarit J, Nigro M, Illescas E, Pszenny V et al. Screening for active toxoplasmosis in patients by DNA hybridization with the ABGTg7 probe in blood samples. J Clin Microbiol.1997;35:591-5.
10. Burney DP, Lappin MR, Spilker M, McReynolds L. Detection of Toxoplasma gondii parasitemia in experimentally inoculated cats. J Parasitol.1999;85:947-51.
11. da Silva AV, Langoni H. The detection of Toxoplasma gondii by comparing cytology, histopathology, bioassay in mice, and the polymerase chain reaction (PCR). Vet Parasitol.2001;97:191-8.
12. Hafid J, Flori P, Raberin H, Tran Manh Sung R. Comparison of PCR, capture ELISA and immunoblotting for detection of Toxoplasma gondii in infected mice. J Med Microbiol.2001;50:1100-4.
13. Guy EC, Joynson DH. Potential of the polymerase chain reaction in the diagnosis of active Toxoplasma infection by detection of parasite in blood. J Infect Dis.1995;172:319-22.
14. Sambrook J, Russell DW. Molecular cloning : a laboratory manual.3 ed . Cold Spring Harbor Laboratory Press, New York, 2001: 5.1, 8.1.
15. Burg JL, Grover CM, Pouletty P, Boothroyd JC. Direct and sensitive detection of a pathogenic protozoan, Toxoplasma gondii, by polymerase chain reaction. J Clin Microbiol.1989;27:1787-92.