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RESEARCH ARTICLE

Detection of *Toxoplasma gondii* by Polymerase Chain Reaction in Recurrent Abortive Women

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ABSTRACT

Polymerase chain reaction was applied for the detection DNA of the pathogenic protozoan *Toxoplasma gondii* based on 35-fold-repetitive gene (the *B1* gene) as a target. Blood from (65) recurrent abortive women who were positive for (ELISA) and (25) apparently healthy pregnant women with no history of abortion as control group, were taken to extract DNA from it and to detect the *B1* gene if present. The *B1* gene was present and conserved in all *T. gondii* strains and to detect this gene from purified DNA samples, a two-stage PCR assay (nested PCR) was conducted employing oligonucleotide specific primers. PCR result indicated that 39(60%) recurrent abortive women had positive results while ELISA recovered 65(100%) positive result. This outcome revealed a significant difference between patients and control in both methods. PCR technique regarded a very useful method for diagnosis of toxoplasmosis inrecurrent abortive women because positive PCR result appeared to be a helpful indicator of active form of the disease.

INTRODUCTION

Toxoplasmosis is a zoonotic disease caused by protozoan parasite, *T. gondii* which can infect all mammals and birds species throughout the world. Approximately half of the world's population is predisposed to this parasitic infection (Remington and Mcleod, 1992). All animal species act as intermediate hosts, except feline species which acts as a definitive and intermediate host (Innes, 1997; Jenum and Stray-Pedersen, 1998). *Toxoplasma gondii* infection in humans may occur vertically by tachyzoites that are passed to the fetus via the placenta, or horizontal transmission which may involve three life- cycle stages tachyzoites, bradyzoites , and oocysts i.e. ingesting tissue cysts in raw or under cooked meat or ingesting sporulated oocysts from cats or tachyzoites in blood products or primary offal (viscera) of many different animals, tissue transplants and unpasteurized milk (Dubey, 2004). Toxoplasmosis is generally asymptomatic; however, this parasitic disease can cause symptomatic and/or life-threatening conditions in congenital toxoplasmosis or immunosuppressed patients (Herold *et al*., 2009). Transplacental transmission of *T. gondii* from an infected pregnant woman to the unborn, results in fetal damage to a degree depending on the gestational age (Kopecky *et al*., 2001).

Infection with *T. gondii* can cause significant morbidity and mortality in the developing fetus and when reactivated in the immunocompromised individual so rapid and specific detection of the parasite within the host is required for accurate diagnosis and treatment, since serologic detection of a host response is inadequate because one of the diagnostic problems is that Toxoplasma specific IgM may occasionally persist for several years (Skinner *et al*., 1989) which may lead to misinterpretation. The polymerase chain reaction (PCR) has been used as an alternative to serology by amplification of *Toxoplasma* DNA sequences present in various clinical samples, such as amniotic fluid (Jenum *et al*., 1998), cerebrospinal fluid (Cingolani *et al*, 1996), tissues (Held *et al*., 2000), aqueous humor (Mahalakshmi *et al*., 2006), and human blood (Kompalic-Cristo *et al*., 2007). In 1989, the first PCR based assay for the direct detection of the presence of a single tachyzoite of *T.gondii* on the basis of invitro DNA amplification of a sequence with in the 35- fold repetitive B1 gene was established (Burg *et al*., 1989). *B1* gene with unknown function that is repeated 35 times in the genome of *T.gondii* (Jalal and Nord, 2004).

Aim of study: Detection of *T.gondii* DNA in sample of Iraqi women with recurrent abortion.

Subject, Materials and methods

Study Groups: This study included 90 women allocated to 65 patient with recurrent abortion and 25 pregnant apparently healthy women with no history of abortion as control group matching with age which range from (19-42) years. Both subject groups were obtained from those who had been admitted and / or attended to Eben AL-Balady and Kamal AL-Samarai hospitals in Baghdad\ Iraq.

Blood Sample

Sample of (5 ml) of venous blood was drawn from each subject of this study, and was divided into two parts, first part of blood (3 ml) placed in a sterilized plain tube and left to stand for 30 minutes at room temperature to clot, then centrifuged at 2000rpm for 10 minutes for serum collection which was aspirated by using micropipette and dispensed into sterile tube and stored in -20°C until used for Enzyme Linked Immunosorbant Assay (ELISA) test. A second part (2ml) was collected in EDTA tube which used for isolation of genomic DNA from whole blood.

Serological Diagnosis

Serologic determinations were performed with the kits *(Toxoplasma*-IgM; *Toxoplasma*-IgG: Biocheck, Inc. Foster City. USA.) and the manufacturer's instructions were followed.

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DNA extraction: DNA was extracted from the whole blood samples using a commercial purification system (Wizard Genomic DNA purification kit, Promega, Madison, WI) following the manufacturer's instructions. Alternatively, the DNA was rehydrated by incubation the solution overnight at 4°C then DNA was keep in the deep freezer (-20°C)until used.

Amplification and Detection of *T. gondii* DNA by Nested PCR.

Nested PCR was performed on all DNA samples to amplify a fragment from *B1* gene, which is present in 35 copies and is conserved in the *T. gondii* genome. The primer used in the first round of the PCR (inner primer pair) are F1(5-GGAACTGCATCCGTTC ATGAG-3), and R1(5-TCTTTAAAGCGTTCGTGGTC-3), which correspond to nucleotides 694-714 and 887-868, respectively. The primer used in the second round (outer primer pair) are F2(5-TGCATAGGTTGCAGTCACTG-3), and R2(5-GGCGACCA ATGTGCGAATAGACC-3), which correspond to nucleotides 757-776 and 853-831, respectively. Five microliters of template DNA were added to a final volume of 50 μL of PCR mixture consisting of 25 μL master mix, 2 μL (F1) and (R1) concentration [10 pmol] from each primer,1 μL MgCL2,15 μL nuclease free water. The amplification was performed in the GenAmp 9700 PCR System (Applied Biosystems, Foster City, CA). The cycling conditions for first round PCRs were 94°C for three minutes, followed by 40 cycles at 94°C for 30 seconds, 50°C for 45 seconds, and 72°C for one minute, and a final extension at 72°C for 10 minutes. Ten microliters of the PCR product were subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide to detected 193bp fragment. Three microliters of the first-round product were used as template for the second-round PCR in a total volume of 50 μL of PCR mixture consisting of 25 μL master mix, 2 μL (F2) and (R2) concentration [10 pmol] from each primer,1 µL MgCL2,17 µL nuclease free water. The PCR mixtures without DNA and with DNase-free water were used as negative controls to monitor for cross-contaminations. The cycling conditions for second round PCRs were 94°C for three minutes, followed by 40 cycles at 94°C for 30 seconds, 50°C for 45 seconds, and 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. Ten microliters of the PCR product were subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide to detected 96bp fragment.

RESULTS AND DISCUSSION

In this study result of distribution study groups according to number of previous miscarriage showed significant difference between patients and control subjects as shown in Table (1). Patient women with two miscarriages or more (53.8%) demonstrated a high ratio of miscarriage than those who had one (46.2%). These may be due to the persistence of infection with toxoplasmosis or that those women become pregnant within a short period after the first miscarriage while cell mediated immunity was still active which lead to the further miscarriage.

Table 1. Previous miscarriage distribution in patients and controls.

Previous Miscarriages no.	Patients (No. = 65)		Controls (No. = 25)	
	N.	%	N.	%
Non	0	0	25	100.0
1	30	46.2	0	0.0
≥ 2	35	53.8	0	0.0
Total	65	100.0	25	100.0

Serological test Result

Serological testing has been one of the major diagnostic techniques for toxoplasmosis. According to miscarriage state the patients (65) and control subjects (25) were distributed as shown in Table (2).Which represent comparison between patients and control subjects using Chi-square analysis revealed a significant difference.

Table 2. Frequency distribution of *T. gondii* antibodies by ELISA in patients and control (90 total no.)

Type of cases	ELIZA +ve		ELIZA -ve	
	NO.	%	NO.	%
Women with miscarriage (patients)	65	100	0	00.0
Women without miscarriage (control)	0	00.0	25	100

Antibody Pattern detection by ELISA for 65 sample of women sera had been tested for specific IgG and IgM antibodies, showed that, 17 (26.2%),10 (15.4%) ,38 (58.4%) of the patients were IgM^{+}– IgG^{-}, IgM^{+}–IgG^{+} and IgM^{-}–IgG^{+}, respectively, while results of the 25 sera samples of apparently healthy women were negative (100 %) for anti-*T. gondii* antibodies. Statistical analysis showed a significant difference in pattern of antibody distribution between patients and control subjects as mentioned in Table (3).

Table 3. Pattern of antibody detection by ELISA in Patients and Control (90 total no.)

Pattern of Antibody	Patients No. 65		Control No. 25	
	No.	%	No.	%
IgM^{+} -IgG^{-}	17	26.2	0	00.0
IgM^{+} -IgG^{+}	10	15.4	0	00.0
IgM^{-} -IgG^{+}	38	58.4	0	00.0
IgM^{-} -IgG^{-}	0	00.0	25	100
Total	65	100.0	25	100.0

In the current study the presence of IgM 17(26.2%) in abortive women sera detected the acute form of the disease. However, low level of IgM antibodies is able to persist for many months, even years after acute infection. Such IgM antibodies are called residuals and their presence does not indicate recent infection this finding is in agreement with Leite *et al.* (2008). The positive IgG was found in 38(58.4%) abortive women sera assign that these women were infected at some time in their life and they are now immune to the disease. However, when IgG were present in the blood indicated that toxoplasmosis cysts were already present in the tissues (Dupon *et al.,* 1995). But positive IgM - IgG in 10(15.4%) abortive women are difficult to interpret in the first serum samples without any previous results. So we required more specific and rapid technique to remove the pregnant women's anxiety associated with further test, and to decrease unnecessary miscarriages (Montoya and Remington, 2008). Negative results appeared in control group indicated that the women has not been infected with *T. gondii,* or sometimes they may tested early in the course of the disease before their body had a chance to produce antibodies. If such women acquire primary infection during gestation or the results were not actually correct they became at risk of transmitting the infection to their fetuses, so they need to be tested in 2-3 weeks (Gollub *et al.,* 2008).

Molecular Result

Positive nPCR results depend on amplification of two DNA fragments (193bp and 96bp) of *B1* gene by subjected PCR product to electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The outcome of nPCR analysis were exhibited that 39(60%) of women with recurrent abortion had positive result while 26(40%) were negative. Whereas 8(32%) of healthy women had positive result as shown in Table (4) and Figure (1, 2). In comparing between molecular and serological analysis, it was found that only 60%(39/65) patient women appeared positive by nPCR, that means the latter method does not depend on an immune response or the titer of antibodies and it appears to be a reliable, rapid and sensitive assay for detecting *Toxoplasma* as parasite, so a positive PCR result appeared to be a helpful indicator of active form of the disease. Routine serologic diagnosis of toxoplasmosis provides high sensitivity, but specificity varies depending on the test used and false-positive IgM antibody test results have been reported by other

investigators (Wilson *et al*., 1997). In this study *Toxoplasma* DNA was detected in 8/25 (32%) of seronegative samples explain by representing a state of immunodeficiency, those individuals which are not able to produce specific antibodies, or alternatively, it could correspond to a very recent infection at the time of serological test, leading to an insufficient production of immunoglobulin not detected by serology. The diagnostic value of PCR for the detection of *T. gondii* in blood samples has been evaluated from both immune-competent and compromised patients (Kompalic-Cristo *et al.*, 2007). All women that are actually positive PCR have a recent infection, apparent parasitemia or active toxoplasmosis, which is likely to be clinically significant and they are at risk of transmitting the infection to their fetuses (Gollub *et al.,* 2008).

Table 4. PCR Analysis to Patients and Controls (Total Number 90).

PCR analysis	Patient No.(65)		Control No. (25)		Total	
	NO.	%	NO.	%	NO.	%
PCR +ve	39	60	8	32	47	52.2
PCR –ve	26	40	17	68	43	47.8
Total	65	100.0	25	100.0	90	100.0

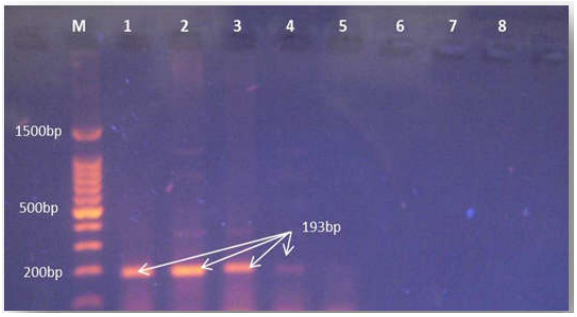

Figure 1. First round of nPC Ramplification of (193bp) *B1* gene of *T. gondii* DNA from blood of abortive women and control. Lane-M =marker (100 bp). Lanes 1-4 positive samples. Lanes 5-8 negative samples. Running conditions: Agarose gel (1.5%), 60V. for 2 hrs, stained with ethidium bromide.

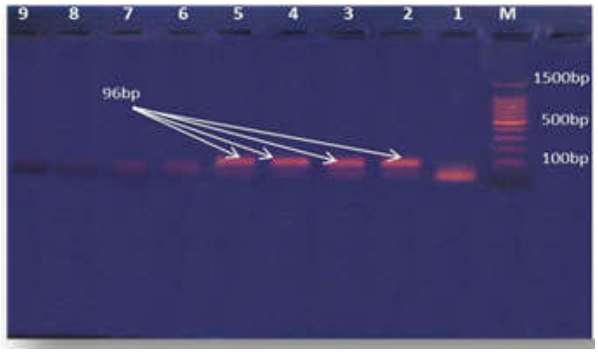

Figure 2. Second run of nPC Ramplification of (96 bp) of *B1* gene of *T. gondii* DNA from blood of abortive women and control. Lane-M=marker(100 bp). Lanes 2-5 are positive samples. Lane 1=negative control. Lanes 6-9 are negative samples. Running conditions: Agarose gel (1.5%), 60V.for 2 hrs, stained with ethidium bromide.

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