Direct Amplification of B1 gene of *Toxoplasma gondii* DNA using Nested Polymerase Chain Reaction Following Microwave Treatment for Whole Blood Samples

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Summary

Infection with *Toxoplasma gondii* is a cause of fetal death since *T. gondii* can be transmitted to the fetus through the placenta (transplacental) from an infected mother or at vaginal delivery. Blood obtained from women and sheep to confirm their infection with toxoplasmosis by using Enzyme Linked Immunosorbant Assay test (ELISA) to ditective positive specific anti-*Toxoplasma* (IgM, IgG and IgM, or IgG) antibodies. This study used two methodes to extract DNA (the first one was a standard extraction commercial method (CM-PCR) of genomic DNA using a commercial kit (Promega, USA), and the second one was the direct heat DNA extraction using microwave oven (MW-PCR) for whole blood samples obtained from infected women and sheep. Then nested Polymerase Chain Reaction (n PCR) were used to amplify *Toxoplasma* B1 gene to detect *T. gondii* DNA in whole blood samples. The results indecated using of microwave treatment instead of commercial kit to extract DNA is low cost and short time,and complement serology for clinical studies and diagnostic purposes of toxoplasmosis.

Keywords: *Toxoplasma gondii*, Nested PCR, Microwave.

Introduction

Toxoplasmosis is caused by infection with *Toxoplasma gondii*, a single-cell protozoan that belongs to the family Coccidia. *T. gondii* is an obligatory intracellular protozoan with a heterogeneous life cycle in humans and other vertebrates (1). The prevalence of disease in Iraqi aborted women were 51.3% positive by serological test (ELISA) (2). Approximately one-third of humanity has been exposed to the parasite worldwide (3). Most human infections are asymptomatic and harmless in healthy person (4), while this parasitic disease can cause symptomatic and/or life-threatening conditions in congenital toxoplasmosis or immunosuppressed patients such as organ transplant recipients, cancer and HIV/AIDS patients (5). *T. 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 *i.e.* ingesting sporulated oocysts from cats or ingesting tissue cysts in raw or under cooked meat or tachyzoites in blood products or primary offal (viscera) of many different animals, tissue transplants and unpasteurized milk (6). Infection with *T. gondii* cause fetal death and in most of chronic latent infection there was cysts in skeletal muscle or brain (7). Furthermore, toxoplasmosis has been implicated in abortion, prematurity, stillbirth and early postnatal mortality and can also cause serious damage to different tissue organs but skeletal and cardiac muscles and central nervous system appear to be the most common sites of chronic infection (8).

Early diagnosis of toxoplasmosis in pregnant women allows early intervention and prevention of congenital disorders that usually lead to fetal death. The genomes of these parasites, like those of other eukaryotic cells, are composed of both nuclear and organellar DNA. *T. gondii* contains a nuclear genome of about 87Mb,a 6kb mitochondrial genome, and an episomal 35 kb plastid-like genome (9).The (PCR) is a potentially powerful method to complement culture and serologic testing for the diagnosis of active toxoplasmosis and is sensitive and highly specific in the detection of *T. gondii*, however, the exquisite sensitivity of PCR, by which a single tachyzoite can be detected in a clinical sample. 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 in vitro DNA amplification of a

repetitive B1 gene was established (10). B1 gene with unknown function that is 2214 bp DNA linear LOCUS AF179871 repeated 35 times in the genome of *T.gondii* (11). Astudy has used PCR after microwave treatment to detect *T. gondii* DNA in whole blood from pregnant women (12).

Materials and Methods

Blood smples acollected from sixty five Iraqi normal and aborted women were included in the present study. They were obtained from those who had been admitted to / or attended to the Eben AL-Balady Hospital and Kamal AL-Samarai Hospital as a health institution in Baghdad. Blood samples were also collected from infected sheep (25 sheep) from Baghdad city, the whole blood samples (4 ml) were divided in two ways, one sample of 2 ml of venous blood were collected in plan tube which left for 30 minutes at room temperature to clot, then centrifuged at 2000 rpm 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 serological test and other one (2 ml) collected in EDTA tube were stored at -20°C until used for molecular test. Statistical analysis was carried out using the computer program SPSS version 9.

ELISA determinations for human and sheep samples were performed with the kits:1. *Toxoplasma*-IgM; *Toxoplasma*-IgG: for human, Biocheck, Inc. Foster City.USA. 2. *Toxoplasma*- IgM; *Toxoplasma*-IgG: I d screen, France, for sheep. the manufacturer's instructions were followed for both kits. DNA was extracted from the whole blood samples using a commercial purification system (Wizard Genomic DNA purification kit, Promega, Madison,WI-USA) following the manufacturer's instructions. The extracted DNA was rehydrated by incubation the solution overnight at 4°C then DNA was kept in the deep freezer (-20°C) until used. DNA extraction by microwave treatment: Genomic DNA was isolated from human whole blood samples by a simple and rapid heat treatment procedure using microwave oven which enables direct PCR amplification to specific fragment from B1 gene in *T. gondii* genome on desiccated samples (13). Fifty µL of whole blood were allocated into thin wall tubes and were exposed to the maximum temperature setting in an 800 watt microwave oven [Kenwood,UK] for 2-3 minutes,or until the whole blood were desiccated. The tubes then were centrifuged briefly and the contents were applied for PCR amplification.

PCR was performed on all DNA samples (which extracted by standard commercial method and microwave treatment) to amplify a fragment from B1 gene in *T. gondii* genome.The primers used in the first round of the PCR (inner primer pair) were F1 (5-GGAACTGCATCCGTTCATGAG-3) and R1 (5-TCTTTAAAGCGTTCGTGGTC-3) which corresponded to nucleotides 694 -714 and 887-868, respectively. The primers used in the second round (outer primer pair) were F2 (5-TGCATAGGTTGCAGTCACTG-3), and R2 (5-GGCGACCAATGTGCGAATAGACC-3) which corresponded to nucleotides 757-776 and 853-831, respectively.

First round: Five microliters of teamplate 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,the final volume was completed by 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.

Second round: Three microliters of the PCR product from first-round 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, the final volume was completed by 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 PCR were 94°C for three minutes,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. The PCR products were subjected to electrophoresis on a1.5% agarose gel stained with ethidium bromide to detected 96bp fragment.

Results and Discussion

By the examination of serum samples from 65 normal and aborted women, there were 40 (61.54%) women infected with *T. gondii*, while 25 (38.46%) of women were ELISA negative (control group) (Table, 1). ELISA results for sheep: We examined 25 sheep serum sampels they showed 15 (60%) samples infected with *T.gondii* using ELISA test while 10 (40%) of sheep were ELISA negative (control group) as shown in (Table, 2).

Table, 1: ELISA results for human serum samples.

Type of cases (sheep)	Infected sheep no. (15)	Control no. (10)	Total	
			NO.	%
ELISA positive	15	0	15	60 %
ELISA negative	0	10	10	40 %
Statistical analysis	25**		25	100%

**Significant differences (P≤ 0.01).

Table, 2: ELISA result for sheep serum samples.

Type of cases (women)	Patients no. (40)	Control no. (25)	Total	
			NO.	%
ELISA positive	40	0	40	61.54%
ELISA negative	0	25	25	38.46%
Statistical Analysis	64.96**		65	100%

**Significant differences (P≤ 0.01).

A positive ELISA test results assigned that these women or sheep were infected at some time in their life and they carried the parasite. Negative ELISA test results appeared in the control group indicated that the women or sheep had not been infected with *T. gondii*, or sometimes they may be tested early in the course of the disease before their body had a chance to produce antibodies. If such women acquired primary infection during gestation they were at risk of transmitting the infection to their fetuses, so they will need to be tested in 2-3 weeks. PCR results for women and sheep using commercial kit: After examining serum samples for 65 normal and aborted women and by ELISA, It examind same samples (65) by nPCR test using commercial kit for extracted DNA from samples and the results that indicated there were 35(87.5%) of them positive for nPCR, while 5 (26.67%) were PCR negative. Control group (25) healthy women showed negative PCR results as mentiond in (Tabel, 3).

Tabel, 3: Nested PCR result for human blood sample

Type of cases (women)	Patients no. (40)		Control no. (25)	
	NO.	%	NO.	%
PCR positive	35	87.5%	0	00.0%
PCR negative	5	12.5%	25	100%
Statistical analysis	47.36 **			

** Significant differences (P≤ 0.01).

Tabel, 4: Nested PCR result for sheep blood samples.

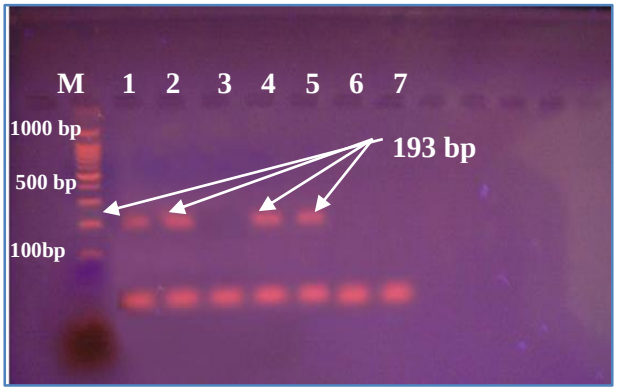
Type of cases (sheep)	Infected sheep no. (15)		Control no. (10)	
	NO.	%	NO.	%
PCR positive	11	73.33 %	0	0.00 %
PCR negative	4	26.67 %	10	100 %
Statistical analysis	11.81**			

** Significant differences (P≤ 0.01).

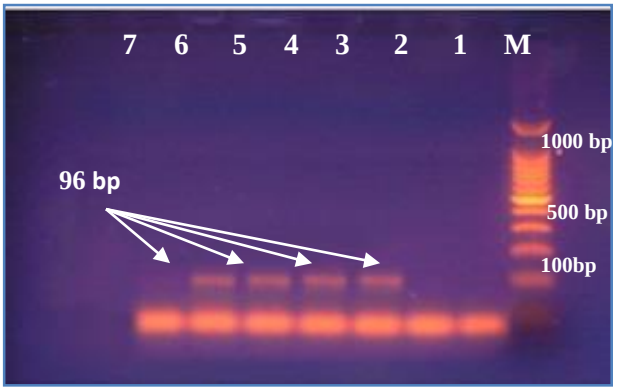
The results of nPCR test using commercial kit for extracted DNA from (25) sheep blood sampls showed that 11(73.33%) of them were nPCR positive, while 4(26.67%) of sheep sampls were nPCR negative. The control group 10 showed negative results for PCR test as mentioned in (Tabel, 4). The results of microwave-PCR (MW-PCR) for whole blood samples of women and sheep were exactly same to the results of commercial-PCR (CM-PCR) to the same samples. The result of nPCR (CM-PCR) and (MW-PCR) for (40) women blood samples mentioned that amplification of B1 gene fragment presence in 35 of them, so they were positive for nPCR test and that mean they were infected with *T.gondii*, and 5 of them showed no amplification of B1 gene fragment and they were negative nPCR and that mean they were free from *T.gondii* infection. Five women showed no evidence of infection by nPCR, while they detected the infection by anti-*Toxoplasma* IgM\ IgG antibody could be attributed to the presence of risedual immunoglobulins, since specific IgM antibodies can be detected in both the acute

and chronic phases of toxoplasmosis (14), at the same time anti-*Toxoplasma* IgG antibodies have been reported to persist for a long time, up to years (15). So serology is anadequate method because it depend on the level of antibody production which either fails or is significantly delayed, whereas nPCR depends on the presence of parasite genetic matereal.

The result of nPCR (MW) for sheep blood samples for 15 infected sheep tested by anti-*Toxoplasma* IgG and IgM antibodies ELISA, showed that 11 of them amplified fragmant of *T. gondii* B1 gene (193-96 bp) so they were positive nPCR, and 4 of them showed no amplifyed B1 gene fragmeant of *T. gondii* so they were negative nPCR. These results were identical to the results of nPCR (CM). Beside that there were no amplification of *T. gondii* B1 gene fragment in blood samples when used (MW) nPCR for all control group (25 women) and 10 sheep as in (Table, 3 and 4). Nested PCR amplification of the B1 gene of *T. gondii* using whole blood is a rapid, sensitive and specific diagnostic procedure and considered a valuable tool for establishing the diagnosis of *T. gondii* infection in human and sheep as comperaed with ELISA test. It has been evaluated the diagnostic value of PCR for the detection of *T. gondii* in blood samples from both immune-competent and compromised patients (16 and 17). In Conclusion, the results presented here suggest that it is possible to detect *T. gondii* DNA in body fluids such as venous blood by the MW-PCR and CM- PCR and that agree with (12 and 13). But MW-PCR technique is cheaper and much less time-consuming than the existing standard extraction commercial method. The results suggest that MW-PCR can be used routinely to complement serology for clinical studies and diagnostic purposes of toxoplasmosis. It is observed a correspondence of positive *T. gondii* DNA in whole blood samples of women and sheep between commercial-PCR (CM-PCR) and microwave-PCR (MW-PCR) (Fig. 1 and 2).



Figure, 1: First run of n PCR (amplification of 193bp fragment B1 gene of *T. gondii* DNA from blood sample of the infected women, sheep and control subjects). Lane-M is molecular marker (100 bp) Lanes 1-2 positive women (CM) and (MW) respectively, Lanes 3 negative women samples. Lanes 4-5 positive sheep (CM) and (MW) respectively, Lanes 6 negative sheep samples.Lanes 7 negative control samples at 193bp in the first round of n PCR.Running conditions: Agarose gel (1.5%), 5 volt/cm² for 2 hrs, stained with ethidium bromide.



Figure, 2: Second run of n PCR (amplification of 96 bp fragment of B1 gene *T. gondii* DNA product from first round n PCR from blood sample of the infected women, sheep and control subjects.Lane-M is molecular marker (100bp). Lanes 1 Negative Control. Lanes 2 negative human samples at 96bp. Lanes 3, 4 are positive women(CM) and (MW) respectively. Lanes 5, 6, are positive sheep (CM) and (MW) respectively. Lanes 7 are negative sheep samples at 96bp. Running conditions: Agarose gel (1.5%), 5 volt/cm² for 2 hrs. stained with ethidium bromide.

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التظخيم المباشر لجين B1 في المادة الوراثية لطفيلي المقوسات الكوندية بأستخدام تفاعل البلمرة المتسلسل متبوعا بالمعالجة الحرارية بواسطة المايكرويف لعينات الدم

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الخلاصة

أن الأصابة بطفيلي داء المقوسات الكوندية من الأسباب التي تؤدي الى موت الجنين أذ ان الطفيلي له القدرة على الأنتقال من الأم الحامل الى جنينها عن طريق المشيمة والتسبب بالأجهاض أو أنتقال الطفيلي أثناء الولادة. تم سحب عينات الدم من نساء وأغنام أكدت أصابتهم بداء المقوسات الكوندية بأستخدام الفحص المناعي الأليزا وذلك عن طريق تشخيص وجود الأجسام المناعية نوع (IgM, IgG/IgM, IgG). الهدف من الدراسة هو استخدام طريقتين لأستخلاص المادة الوراثية من عينات دم النساء والأغنام المؤكد مسبقا اصابتها بطفيلي داء القوسات الكوندية (الأولى هي الطريقة اللأعتيادية بأستخدام عدة تجارية نوع (Promega) الأمريكي أما الطريقة الثانية هي أستخدام الفرن الحراري (المايكرويف)، من ثم استخدام تفاعل البلمرة المتسلسل نوع المجموعة المتداخلة لتضخيم الجين (B1) الخاص بطفيلي المقوسات الكوندية لنفس عينات الدم لتشخيص وجود الأصابة بداء المقوسات الكوندية. أكدت النتائج ان بلأمكان استخدام الفرن الحراري المايكرويف بدل العدة التجارية لأستخلاص المادة الوراثية من عينات الدم، وبهذا يمكننا استخلاص المادة الوراثية بوقت قصير وبتكلفة قليلة جدا مقارنة بالعدة التجارية المكلفة ومكملا لنتائج الفحوصات المناعية لغرض دراسة وتشخيص داء المقوسات الكوندية.

الكلمات المفتاحية: المقوسات الكوندية، تفاعل البلمرة المتسلسل، الفرن الحراري.