Genotyping of *Toxoplasma gondii* isolates from human being in Wasit province / Iraq

Noaman N. A'aiz
Dept. of Microbiology and Parasitology / College of Vet. Medicine / Univ. of Al-Qadisiyha / Al-Qadisiyha, Iraq.
Email: noaman.a'aiz@qu.edu.iq

الخلاصة

الخلفية: يعتبر طفيلي مقوسة كوندي (*Toxoplasma gondii*) من الأوالي المجبرة التطفل داخل الخلايا والتي تصيب معظم حيوانات الدم الحار بالإضافة الى الأنسان. لم يعرف وجود بيانات حقيقية حول الانماط الجينية في الانسان في العراق، لذلك جاء هدف الدراسة لتحديد الانماط الجينية لطفيلي مقوسة كوندي المعزولة من الانسان في محافظة واسط/ العراق.

طرق العمل: تم اجراء الفحص المصلي لـ 508 نموذج سريري (500 دم و 8 نسيج مشيمة) مأخوذ من النساء وذلك بأستخدام فحص الممتز المناعي المرتبط بالأنزيم (ELISA) كفحص أولي ثم فحصت النماذج الموجبة مصليا بعد ذلك بواسطة تقنية تفاعل سلسلة البلمرة – الوقت الحقيقي (RT-PCR) من خلال مضاعفة الجين *B1* لتأكيد الاصابة بالطفيلي، ثم فحصت النماذج الموجبة للفحص اعلاة بأستخدام تقنية تفاعل سلسلة البلمرة المتداخل- تقييد طول الجزء المتعدد الاشكال (nested PCR-RFLP) للجين *SAG2* .

النتائج: تم تأكيد الاصابة بطفيلي *T. gondii* لخمسة عشر(15) نموذج فقط، وأظهر فحص التنميط الجيني ان 6.6% (15:1) و 13.3% (15:2) و 80% (15:12) من العزلات المفحوصة مثلت الانماط الجينية I و III و II على التوالي.

الأستنتاج: أن النمط الجيني II هو النمط السائد في الانسان في محافظة واسط / العراق.

الكلمات المفتاحية: مقوسة ، كوندي ، التنميط الجيني، تفاعل سلسلة البلمرة المتداخل، تقييد طول الجزء المتعدد الاشكال

Abstract

Background: *Toxoplasma gondii* is an obligate intracellular protozoan parasite that infects almost all-warm blooded animals and human beings. No really data is known of genetic diversity of *T. gondii* in human in Iraq. The aim of this study was to determine the genotypes of *T. gondii* isolates from human in Wasit province, east Iraq.

Methods: A total of 508 clinical specimens (blood 500 and placenta 8) of women were initially examined serologically by ELISA, and further tested by RT-PCR technique through *B1* gene amplification to confirm the infection with *T. gondii*. Then the positive DNA samples were assayed for genetic characterizations depend upon nested PCR-RFLP of *SAG2* gene.

Results: Out of 508 tested samples only 15 were confirmed positive *T. gondii* DNA. The genotyping assay revealed that 6.6% (1/15), 13.3% (2/15) and 80% (12/15) of examined isolates represent the genotypes of I, III and II respectively.

Conclusion: The type II appeared is a dominant picture in human being in Wasit province / Iraq.

Key words: *Toxoplasma gondii*, Genotyping, RFLP, strain, human, Iraq

Background

Toxoplasma gondii is an intracellular protozoan that can cause significant morbidity and mortality in human beings and animals (1). The infection is mainly acquired by ingestion of undercooked or raw meat containing viable tissue cysts or by ingestion of food and water that is contaminated with oocyst shed by cats (2).

Previously, it was described that *T. gondii* has a clonal population structure consisting of three genetic lineages, i.e., type I, type II and type III (3).

Type I strains are highly virulent to mice. The type II and type III lineages are widespread throughout all continents and dominate in North America, Europe and Africa meanwhile, type II strains are the most prevalent cause of human toxoplasmosis in both congenital infection and AIDS patients in North America and Europe (4, 5, 6).

In contrast, genetic characterization of isolates from human patients and animals in South America are genetically and biologically diverse (7), and serve toxoplasmosis in immunocompetent human patients is often associated with typical genotypes (8).

T. gondii prevalence in Iraq has been shows to be up to 48% (9, 10, 11). But no data are available related to the biological and molecular characteristics of isolates of *T. gondii* except one study exploiting six samples only (11), so the purpose of this study was to determine the genotypes of *T. gondii* infected the human being in Iraq.

Materials and Methods

A total of 508 clinical specimens (500 blood samples and 8 placenta tissues of aborted women) were obtained from toxoplasmosis suspected patients whom attended the gynecological and pediatrics Al-Kut hospital in Wasit province, Iraq, during the period from October 2013 to May 2014.

Regarding to the blood specimens the infection with *T. gondii* was detected initially serologically by ELISA, whereas the placenta specimens collected from positive toxoplasmosis aborted women. Then all specimens were subjected to RT-PCR to confirm the infection through amplification of *B1* gene, after that the genetic characterization were done according to nested PCR-RFLP method.

Genomic DNA extraction: Genomic DNA was extracted from frozen blood samples by using (Genomic DNA mini Extraction kit. Geneaid. USA). 200µl of blood placed in 1.5 ml eppindorf tube and 20µl proteinase K (10mg/ml) was added for cells lysis. Then genomic DNA extracted according to kit instructions. The purified DNA was eluted in elution buffer provided with kit and stored at -20°C, and the extracted DNA was checked by Nanodrop spectrophotometer .

Real-Time PCR:

Real-Time PCR based TaqMan probe was performed for rapid detection of *T. gondii* according to (12). Real-Time PCR TaqMan probe and primers were used for amplification of conserved region B1 gene in *T. gondii*. Primers were provided by (Bioneer Company. Korea) as forward primer (TCCCCTCTGCTGGCGAAAAGT), Reverse primer (TCCCCTCTGCTGGCGAAAAGT), and *B1* probe (FAM-TCTGTGCAACTTTG GTGTATTCGCAG-TAMRA). The Real-Time PCR amplification reaction was done by using (AccuPower® DualStarTM qPCR PreMix Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction (Table 1).

Table (1): The real-time PCR amplification reaction

qPCR master mix	Volume
Genomic DNA template	5µl
B1 Forward primer (20pmol)	1µl
B1 Reverse primer (20pmol)	1µl
B1 TaqMan probe (25pmol)	2 µl
DEPC water	11µl
Total volume	20µl

The qPCR master mix reaction components that mentioned above were added into AccuPower® DualStarTM qPCR PreMix tubes containing Taq DNA polymerases, dNTPs, 10X buffer for TaqMan probe amplification, then the tubes were centrifuged at 3000 rpm for 3 minutes, transferred to MiniOpticon Real-Time PCR system and applied the appropriate thermocycler conditions (Table 2):

Table (2): The RT-PCR construct reaction conditions

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5 minute	1
Denaturation	95 °C	15 sec	50
Annealing\ Extension	60 °C	1 minute	
Detection(scan)			

The resulting amplification products were analyzed by 2% agarose gel electrophoresis using marker (2000-100 bp) and positive control (Genkam / Germany).

Genotyping Method:

To determine the genotypes of *Toxoplasma gondii*, the positive samples was analyzed at the *SAG2* locus gene by using a nested PCR method that separately amplified the 5′ and 3′ ends of the locus according to (13). The 5′ end of the locus was amplified by standard PCR for 40 cycles with the primers *SAG2*.F4 (5′-GCTAC CTCGAACAGGAACAC-3′) and *SAG2*.R4 (5′-GCATCAACAGTCTTCGTTGC-3′) at an annealing temperature of 65°C. The second amplification of 40 cycles was performed with the internal primers *SAG2*.F (5′-GAAATGTTTCAGGTTGCTGC-3′) and *SAG2*.R2 (5′-GCAAGAGCGAACTTGAACAC-3′) by using 1 µl of the diluted PCR product as the template. The amplified fragments were purified with PCR Purification kit (Biobasic Inc. Canada) and digested with *Sau3*AI, (Biolab. UK). The restriction fragments were analyzed by agarose gel electrophoresis. The 3′end of the locus was similarly analyzed with the primers *SAG2*.F3 (5′-TCTGTTCTCCGAAGTGACTCC-3′) and *SAG2*.R3 (5′TCAAAGCGTGCATTATCGC-3′) for the initial amplifications and the internal primers *SAG2*.F2 (5′-ATTCTCATGCCTCCGCTTC-3′) and *SAG2*.R (5′-AACGTTTCACGAAGG CACAC-3′) for the second round of amplification at an annealing temperature of 63°C. The resulting amplification products were purified with PCR Purification kit (Biobasic Inc. Canada), digested with *Hha*I (Biolab. UK), and analyzed by 2% agarose gel electrophoresis (13).

Results

Eighty nine blood samples out of 500 suspected cases were positive by ELISA test and according to RT-PCR technique only 7 blood samples successful for amplification while all placental samples were successfully amplified.

The 15 (7 blood and 8 placenta tissues) *T.gondii* isolates were subjected to further analyze for genotyping detection by nested PCR-RFLP at *SAG2* locus. PCR reactions on 3' and 5' ends resulted in a 221bp and 241 bp amplified bands respectively (Fig. 1 and 2).

The digestion of 3' and 5' nested PCR products by both *Hha*I and *Sau3*AI restriction enzymes revealed that type II strain of *T. gondii* was represent 80% of isolates following by types III and I in rates13.3% 6.6% respectively (Fig. 3 and 4, Table 3). where the type I strain result when there is no digestion occur of both 3' and 5' amplification of nested-PCR products by both *Hha*I and *Sau3*AI restriction enzymes, but type II strain result when *Hha*I enzyme cleavage the 3' end product, while type III strain revealed

if the *Sau3*AI enzyme cleavage the 5' end product (13, 14).

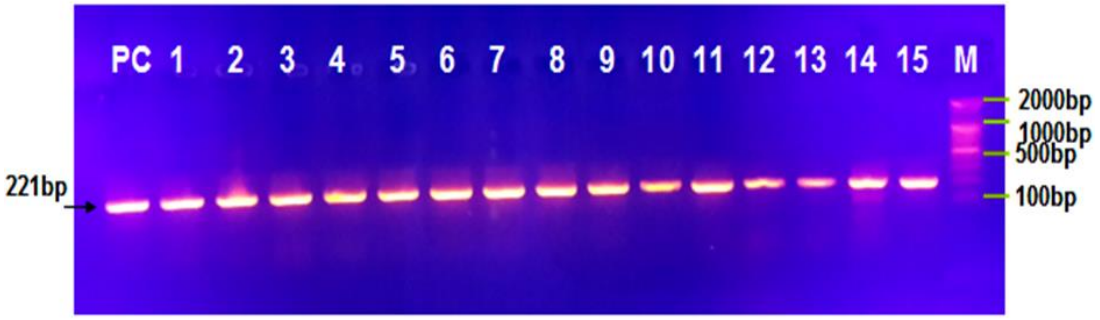

Figure 1: Agarose gel electrophoresis image show positive PCR product samples for SAG2 3' end locus region in Toxoplasma gondii positive samples. M= marker (2000-100bp), Lane (1-15) positive PCR product at 221bp, PC= positive control.

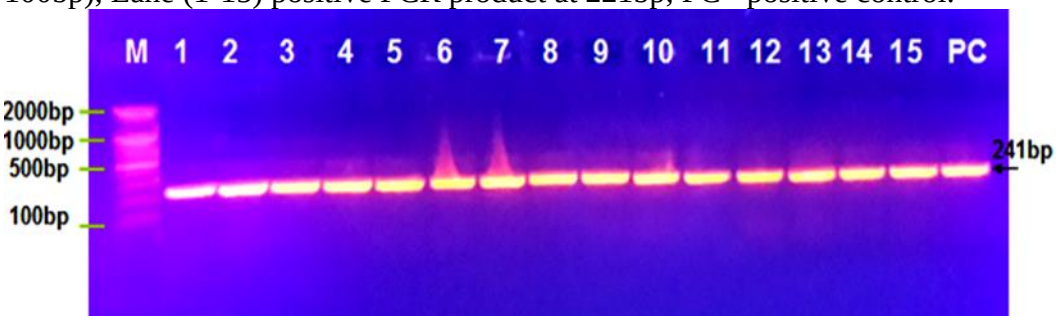

Figure 2: Agarose gel electrophoresis image show positive PCR product samples for *SAG2* 5' end locus region in *Toxoplasma gondii* positive samples. M= marker (2000-100bp), Lane (1-15) positive PCR product at 241bp, PC= positive control.

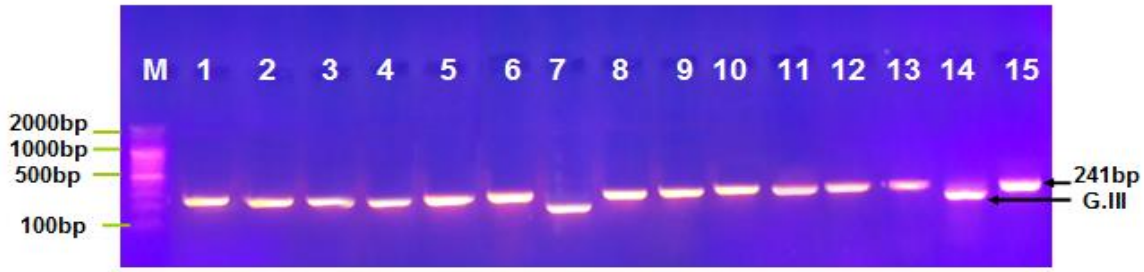

Figure 3: Agarose gel electrophoresis image show cleavage of 7 & 14 isolates only by *Sau*3AI restriction enzyme. M=marker (2000-100bp), Lane (7 and 14) positive Genotype III.

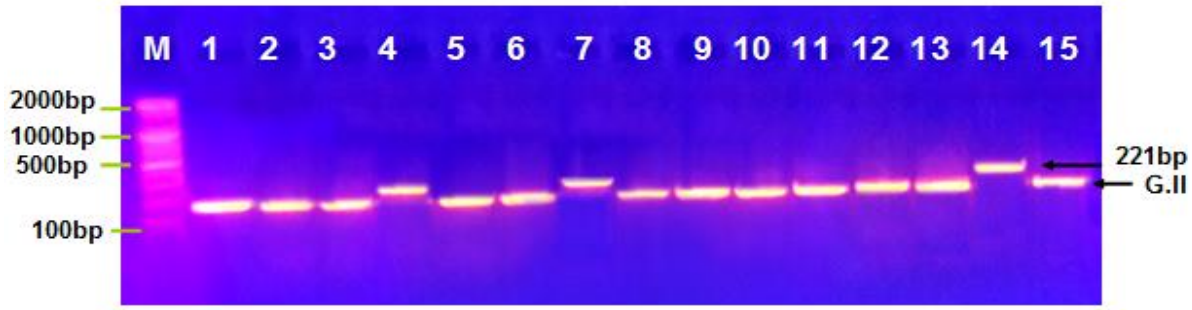

Figure 4: Agarose gel electrophoresis image show cleavage of all isolates by *Hha*I restriction enzyme except 4,7 & 14. M= marker (2000-100bp), Lane (1,2,3,5,6,8,9,10,11,12,13, and 15) positive Genotype II.

Table 3: The strain types according to cleavage of restriction enzymes.

No. of *T.gondii* isolates	3'end product cleavage by *Hha*I	5' end product cleavage by *Sau3*AI	Strain type
1	+	-	II
2	+	-	II
3	+	-	II
4	-	-	I
5	+	-	II
6	+	-	II
7	-	+	III
8	+	-	II
9	+	-	II
10	+	-	II
11	+	-	II
12	+	-	II
13	+	-	II
14	-	+	III
15	+	-	II

Discussion

Many previous studies were depending upon the nested PCR-RFLP method to determine the genotypes of the *T. gondii* at locus *SAG2* (13, 14, 15).

In the current study the result showed that all three genotypes are recorded but in various rates. The frequency of type II strain was 80% which the highest comparing with the frequencies of types I and III which were 6.6% and 13.3% respectively.

The highest frequency of type II is agreed with many previous studies like Howe, *et.al.* (13) who recorded that 81% of examined samples from different clinical conditions (AIDS, non AIDS immunosuppression and congenital infections) revealed positive result to type II strain, also Honere *et.al*., (16) and Behzadi *et.al*., (17) reported that type II is the common one in France and Iran which appeared in percentage of 76.7% and 85.7% respectively. This may be attributed to that type II is the most common in farm animals like sheep, goats and camels which are in contact with human through consumption to their products (18, 19, 20), in addition to that Hermann *et.al*., (21)mention that type II pattern is the most genotype identified in oocysts shed by cats in Germany.

Type III is found in 13.3% (2/15) of isolates, which is nearest to the result of (13) who recorded that 9% of examined isolates had the type III strain which mainly appeared in AIDS and immunosuppression patients. Ferreira *et.al*., (15) explain that 4% of AIDS patients with cerebral toxoplasmosis infected with type III, while Behzadi *et.al*., (17) not found type III in his isolates. The reason of such an observations could be due to the less virulent of type III strain (22, 23) which mostly appear in immunocompromised patients. In other hand type I strain observed in lowest rate of 6.6% (1/15).

Previously, the reports emphasized that type I is a highly virulent (22, 24) and most its infections showed in AIDS patients as well as ocular toxoplasmosis (25, 26). Behzadi *et.al.,* (17) and Ferreira *et. al.,* (15) recorded that 14.3% and 46% of isolates infected with type I strain in Iran and Brazil respectively. The variations among the results of our study and other studies may be attributing to the sample size, eating habits, geographical locations and life style.

Conclusion

The results of this study confirmed the predominant of type II strain of *T. gondii* in isolates from Iraqi patients and emphasized that this type is more prevalent than type III and type I.

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