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Diagnostic study of *Toxoplasma gondii* in students of Thi-Qar university-Iraq by Real-Time PCR

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Abstract

Toxoplasma gondii is a unique intracellular parasite, which infect a large proportion of the world, population, but clinically un commonly causes significant disease. The present study was performed for the first time in Thi-Qar province to estimate the prevalence of toxoplasmosis among university students . Venous blood samples were collected from 319 (111 males and 208 females) apparently healthy students ,they have ages between (18-42) years attended from different colleges of Thi-Qar university-Iraq, during the period from October 2013 to April 2014. Enzyme linked Immunosorbant Assay (ELISA), was used to evaluate the presence of anti-*Toxoplasma* IgM and IgG antibodies and detection of *B1* gene of *T.gondii* DNA by Real-Time Polymerase Chain Reaction(RT-PCR). The results indicated that 70 (21.94%) of students were exposed positive for anti-*Toxoplasma* antibodies,17 (5.33%) of them had IgM,38 (11.91%) had IgG, and 15 (4.70%) had both IgM and IgG. Statistically, significant difference between them. The results showed significant difference between males and females in rate of positive for anti- *Toxoplasma* antibodies, which recorded 24 (7.52%) and 46 (14.42%) respectively . Besides the serological diagnosis of *T.gondii* Real-Time PCR (RT-PCR) technique was used to confirm the infection with *T.gondii* by detection B1 gene of *T.gondii* DNA in the blood of students. out of 319 students only 6.26% showed positive toxoplasmosis among those 8(2.50%)were males and 12(3.76%) were females. The positive result in RT-PCR analysis were distributed on the patterns of the anti-*Toxoplasma* antibodies by ELISA test , it was no found any positive blood samples with IgM,IgG and both IgM and IgG respectively whereas 20 positive cases of no anti –*Toxoplasma* antibodies. Real-Time PCR test in blood of students has advantages in detection of recent or active toxoplasmosis.

Keywords: Toxoplasma gondii, Real-Time PCR, B1 gene, University students

دراسة تشخيصية لطفيلي المقوسة الكونديه *Toxoplasma gondii* في طلبة جامعة ذي قار –العراق باستخدام تفاعل سلسلة البلمرة ذو الوقت الحقيقي

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الخلاصه

المقوسه الكونديه هو طفيلي يعيش داخل الخلايا حيث انه يصيب عدد كبير من سكان العالم لكن ليس من الشائع ان يسبب امراض خطيرة.الدراسه الحاليه انجزت لاول مره في محافظة ذي قار لمعرفة انتشار داء المقوسات في طلبة الجامعه. جمعت عينات الدم الوريدي من319 (111 ذكور و208 اناث) طالب تتراوح اعمارهم 18–42 سنه من مختلف الكليات لجامعة ذي قار خلال المده من شهر تشرين الاول 2013 الى نهاية شهر نيسان2014 ،قد

استخدم فحص الادمصاص المناعي المرتبط بالانزيم (ELISA) لتقييم وجود الاجسام المضاده للمقوس نوع IgM وIgG واستخدمت تقنية تفاعل سلسلة البلمره ذو الوقت الحقيقي للكشف عن الجين B1 في الحامض النووي DNA لطفيلي المقوسة الكوندية .اظهرت النتائج 70من الطلبه يحملون الاجسام المضاده للمقوسه الكونديه وبنسبة (21,94%) منهم 17حالة موجبة للضد المناعيIgM وبنسبة(5,33%) و38 حالة موجبة للضد المناعي IgG وبنسبة (11,91%) و15حالة موجبة لكلا الضدين IgM وIgG وبنسبة (4,70%).احصائيا يوجد فرق معنوي بين انواع الاجسام المضاده.بينت الدراسه وجود فرق معنوي بين الذكور والاناث في المعدل الكلي لكن لايوجد فرق معنوي بينهما من حيث انواع الاجسام المضاده حيث سجلت24 حالة موجبة من الذكور وبنسبة (7,52%) و 46 حالة موجبة من الاناث وبنسبة (14,42%) يحملون الاجسام المضاده للطفيلي استخدمت الدراسة بالاضافة الى التشخيص السيرولوجي للمقوسه الكوندية تقنية تفاعل سلسلة البلمره ذو الوقت الحقيقي للكشف عن جين B1 في الحامض النووي DNA لطفيلي المقوسة الكوندية في دم الطلبة . اجري الفحص على319 عينة دم كانت هناك 20 حالة موجبة فقط لداء المقوسات وبنسبة 6,26% بينهم 2,5% ذكور و 3,76% اناث.النتائج الموجبه التي شخصت بواسطة تقنية Real–Time PCR قد وزعت حسب انواع الاجسام المضادة فلم تسجل الدراسة اي حالة موجبة مع IgM و IgG و كلا من IgG وIgM بينما سجلت 20 حاله موجبة في العينات السالبة للاضداد.من مميزات تقنية Real–Time PCR انها سجلت الاصابة الحديثة لداء المقوسات في دم الطلبة.

الكلمات المفتاحية : طفيلي المقوسات الكوندية ، تفاعل سلسلة البلمرة ذو الوقت الحقيقي ،الجين b1، طلبة الجامعة .

Introduction

Toxoplasmosis is caused by an obligate intracellular tissue protozoan parasite *Toxoplasma gondii*, which is able to infect humans as well as other warm blooded domestic and wild animals. The infection has a world-wide distribution with approximately one-third of the world population estimated to be exposed to this parasite (Dubey and Jones, 2008). Toxoplasmosis is a widespread zoonotic disease caused by *T.gondii*. It has economic relevance to both veterinary and human medicine (Hill *et al* .,2005).In sheep and goats infection not only results in significant reproductive looses ,but also has implication for public health since consumption of infected meat and milk can facilitate zoonotic transmission (Bisson *et al.*,2000). *T. gondii* has a wide variety of hosts, as almost all warm blooded animals can be infected. Sexual replication of the parasite occurs only in domestic cats and wild felidae (definite hosts), while asexual replication occurs in both intermediate and final hosts (Frenkel, 1970; Tenter *et al*., 2000). Oocysts are passed in the feces of cats and become infectious within 21 days of being shed .Tachyzoites survive and multiply only in an intra - cellular location while tissue cysts containing few or many bradyzoites occur in the tissues of infected animals within a week of infection(Lainson, 1958) Ingestion of tissue cysts in infected meat or oocysts from soil, food, or water contaminated with cat feces are the two major routes of transmission(Montoya and Remington, 2008).Rarely, transmission of T.gondii occur through blood transfusions and organ transplantations (Singh,2003).In immunocompetent individuals, 90% of T. gondii infections are asymptomatic (Kravetz and Fedeman, 2005).Symptomatic infections usually cause low grade fever, malaise, headache and cervical Lymphadenopathy. Severe manifestations such as encephalitis, myocarditis, hepatitis and pneumonia are rare but can complicate acute toxoplasmosis (Kravetz and Fedeman., 2002) and may even lead to death in immunocompromised patients(Singh ,2003). The diagnosis of T.gondii infection may be established by serologic tests, molecular methods, histological demonstration of the parasite , a toxoplasmin skin test and by isolation of the organism (Remington et al .,2001). Molecular methods rely on PCR for the specific detection or analysis of T. gondii DNA. These methods have proved to be simple, sensitive, reproducible and cost-effective, and have been applied to a variety of clinical samples from animals and humans (Contini et al.,2005; Bastien et al.,2007).Real-time PCR has been used to amplify and quantify DNA from the *T. gondii B1* gene (Costa *et al*.,2000). Real-time PCR utilizes the 59 nuclease activity of Taq DNA polymerase (Holland *et al*., 1991) to cleave a non extendible, fluorescence-labeled hybridization probe during the extension phase of PCR.The fluorescence of the intact probe is quenched by a second fluorescent dye, usually 6-carboxy-tetramethyl- rhodamine (TAMRA). This study, we describe the development of a real-time quantitative PCR for the detection of T. gondii. The use of this methodology will facilitate the diagnosis of T. gondii in clinical laboratories.

Material and Methods

Serological test

The sera of all cases were tested for the presence of specific IgM and IgG anti-*Toxoplasma* antibodies via ELISA kits (BioChik Diagnostics Company, USA) according to the manufacture'sinstructions

Isolation of genomic DNA from whole blood

DNA was extracted from the whole blood samples of the study groups using acommercial purification system (Reagent Genomic DNA extraction kit (.Invitrogen. USA))following the manufacture's instruction for DNA purification from blood. Purified DNAmolecules were stored at -80○C, after estimation of DNA concentrationand purity, The extracted genomic DNA from whole blood samples was checked by using Nanodrop spectrophotometer (THERMO.USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm).

Real-Time PCR

Real-Time PCR basedTaqMan probe was performed for rapid detection of T. gondii according to method described by Meihuilin et al. (Fernanda et al.,2010). Real-Time PCR TaqMan probe and primers were used for amplification of conserved region B1 gene in T. gondii .These primers were provided by (Bioneer Company. Korea) as showed in table (1).

Table (1): Real-Time PCR TaqMan probe and primers

Primer		Sequence	Product size
B1 primer	F	TCCCCTCTGCTGGCGAAAAGT	94bp
	R	AGCGTTCGTGGTCAACTATCGATTG	
B1 probe		5-6FAM-TCTGTGCAACTTTGGTGTATTCGCAG -TAMRA-3	

The Real-Time PCR amplification reaction was done by using (AccuPower® DualStarTM qPCR PreMixBioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction as following table (2):

Table (2): The qPCR master mix

RT-PCR master mix	Volume
DNA template	5µL
Forward B1 gene primer (20pmol)	2.5µL
Reverse B1 gene primer (20pmol)	2,5µL
TaqMan B1 gene probe (20pmol)	2.5µL
DEPC water	37.5 µL
Total	50µL

These qPCR master mix reaction components that mentioned in table above were added into AccuPower® DualStarTM qPCR PreMixtubes which containing Taq DNA polymerases, dNTPs, 10X buffer for TaqMan probe amplification. Then tubes placed Exispin vortex centrifuge at 3000rpm for 3 minutes, after that transferred into MiniOpticon Real-Time PCR system and applied the following thermocycler conditions as the following table (3):

Table (3): Thermocycler conditions

Step	Condition	Cycle
Pre-Denaturation	95 °C 5 min	1
Denaturation	95 °C 20 sec	45
Annealing/Extension	60 °C 30 sec	
Detection (Scan)		

Statistical analysis

Data were analyzed with chi-square and P value < 0.05 was considered statistically significant**.**

Results

The present study carried out on 319 apparently healthy students from Thi-Qar university that included in this study,111 male students and 208 female students ,to elucidate *Toxoplasma gondii* infection by using Enzyme Linked Immunosorbent Assay (ELISA),as well as Real Time Polymerase Chain Reaction(Real time-PCR). Results presented in this study showed that overall the prevalence of toxoplasmosis were 70(21.94%) among those 24(7.52.%) were males and 46(14.42%) were females by using ELISA test while the overall prevalence of toxoplasmosis were 20(6.26%) among those 8(2.50%)were males and 12(3.76%) were females by using Real time quantitative PCR, as shown in table(4).

Table (4) percentage distribution of students from Thi-Qar university infected with toxoplasmosis according to test type

Test type	Males			Females			Total		
		+ve			+ve			+ve	
	No.	No.	%	No.	No.	%	No.	No.	%
ELISA test	111	24	7.52	208	46	14.42	319	70	21.94
Real-timePCR	111	8	2.5	208	12	3.76	319	20	6.26

ELISA IgM and IgG tests for toxoplasmosis seroprevalence

The current study tried to estimate the actual percentage of toxoplasmosis in students from different colleges by using specific tests ELISA IgM and IgG. Out of 319 students 17(5.33%) were had acute toxoplasmosis characterized by the presence of positive IgM antibodies,38(11.91%) of samples had chronic toxoplasmosis characterized by the presence of positive IgG antibodies only , while 15(4.70%) of samples had both acute and chronic toxoplasmosis characterized by the presence positive of both IgM and IgG antibodies .The statistical analysis showed significant differences between them ($p<0.05$),table(5).

Table (5) percentage distribution of students from Thi-Qar university infected with toxoplasmosis according to ELISA IgM and IgG tests

Test / Class	ELISA test						Chi-sq. X^2
	Positive		Negative		Total		
	No.	%	No.	%	No.	%	
IgM	17	5.33	302	94.67	319	100	25.02**
IgG	38	11.91	281	88.09	319	100	58.03**
IgM&IgG	15	4.70	304	95.30	319	100	82.03**
Chi- Squire	X^2=13.93*		P<0.05				

Detection of *T.gondii* gene by B1 gene Real-Time quantitative PCR

Besides the serological diagnosis of *T.gondii* Real-Time PCR (RT-PCR) technique was used to confirm the infection with *T.gondii* by detection of *T.gondii* DNA in the blood of students .*Toxoplasma gondii* DNA was successfully extracted and analyzed by RT-PCR. The study revealed that out of 319 students only 20 (6.26%) showed positive toxoplasmosis by RT-PCR technique among those 8(2.50%) were male and 12(3.76%) were female. Statistically, no significant difference between them($p<0.05$) . A typical amplification plot (change in fluorescent signal versus cycle numbers) with a CT of :28-37 was obtained .The cycle threshold value (CT), indicative of the quantity of target gene at which the fluorescence exceed a preset threshold was determined , where the negative samples show as undetermined by amplification plot as shown in figure (1).

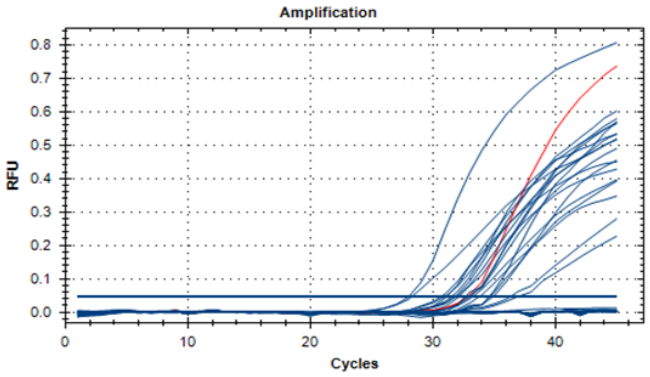

Figure (1): Real-Time amplification plot of *B1* gene in *T.gondii* from blood samples of students .Where, Blue plots: 20 positive samples, Red plot: Positive control (DNA *Toxoplasma gondii*)

Correlation between real time PCR analysis and ELISA results

Students 20(6.26) who recorded positive results in RT-PCR analysis were distributed on the patterns of the anti-*Toxoplasma* antibodies by ELISA test , it was no found any positive blood samples with IgM,IgG and both IgM and IgG respectively whereas 20 positive cases of no anti –*Toxoplasma* antibodies . as shown in table (6), figure (2).

Table (6) Correlation between real time PCR analysis and ELISA results

Real-time -PCR / ELISA-test	Real-time PCR +ve		Real-time PCR -ve		Total	
	No.	%	No.	%	No.	%
ELISA +ve	0	0	70	21.94	70	21.94
ELISA-ve	20	6.27	229	71.79	249	78.06
Total	20	6.27	299	93.73	319	100

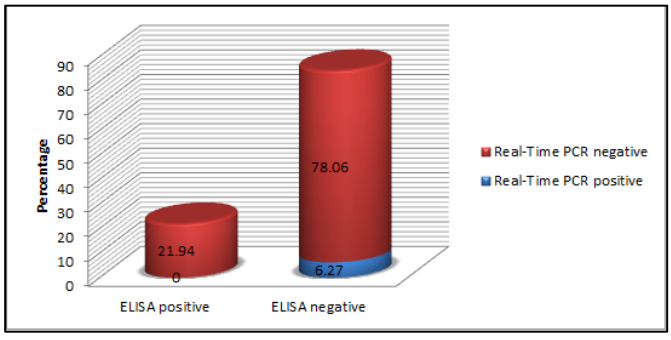

Figure (2) Correlation between real time PCR analysis and ELISA result

Discussion

Besides the serological diagnosis of *T.gondii* Real-Time PCR (RT-PCR) technique was used to confirm the infection with *T.gondii* by detection *B1* gene of *T.gondii* DNA in the blood of students. *Toxoplasma gondii* DNA was successfully extracted and analyzed by RT-PCR. The diagnostic value of PCR for the detection of *T.gondii* in blood samples has been evaluated from both immunecompetent and immunecompromised patients (Ho-Yen *et al*., 1992; Bou *et al*., 1999; Kompalic-Cristo *et al*., 2007).Several PCR-based techniques have been developed as alternative diagnostic measurements for *T.gondii* infection. These techniques make use of the most conserved gene sequences among different strains of *T.gondii* (Ellis, 1998), including the *B1*gene repetitive sequence, theP30 (SAG1) gene and ribosomal DNA. The use of the *B1* gene for *T.gondii* detection originated with Burg *et al*.(1989).The B1 gene ,although of unknown function ,is mostly exploited in a variety of diagnosis and epidemiological studies thanks to its specificity and sensitivity (Ivovic *et al* .,2o12; Tlamcan *et al*.,2013).The cycle threshold value (C_T) for positive samples in Real-Time PCR ranged from C_T28 to C_T 37. The C_T of a reaction is determined mainly by the amount of template present at the start of the amplification reaction.If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background.Thus, the reaction will have a low, or early, C_T. In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background. Thus, the reaction will have a high, or late, C_T. This relationship forms the basis for the quantitative aspect of real-time PCR (Carr and Moore, 2012). The study revealed that out of 319 students only 6.26% showed positive toxoplasmosis among those 8(2.50%) were male and 12(3.76%) were female. Statistically, no significant difference between them($p<0.05$) .The positive result was higher in this study than those recorded by Gunel *et a*l.(2012) in Turkey ,who demonstrated that rate was(1.3%) and with Chiang *et al*.(2012) in Taiwan, who recorded no active parasitemia was detected by real-time PCR assay, while rate was lower in this study than those recorded by Wallon *et al*.(2010),who recorded that the rate infection of toxoplasmosis was(69%,) other studies in Iraq(Al-Abudy,2014; Abbas et al.,2014 and Al-nasrawi et al.,2014) who showed that rate was(38.0%,17,7%,16%) of aborted women .The explanation of these differences stated by other researcher may be resulted to the use of only healthy students for both sexes in this study gave findings that may therefore differ from findings in other population. Students 20(6.26) who recorded positive results in RT-PCR analysis were distributed on the patterns of the anti-Toxoplasma antibodies , it was no found any positive case with IgM,IgG and both IgM and IgG respectively whereas 20 positive cases of no anti –Toxoplasma antibodies .This result was in- line with the result obtained by Chiang et al.(2012) who showed that out of 1783 blood from healthy blood donors were tested for the presence of T.gondii antibodies and DNA using ELISA and RT-PCR respectively ,5(0.28%) 166(9.3%),tested positive for anti-Toxoplasma IgM and IgG respectively .No active parasitemia of positive ELISA result was detected by real-time PCR assay and other study by Pignanelli.(2011) who revealed that no active parasitemia of anti- Toxoplasma IgG was detected by real-time PCR assay. Also, these results was agreed with a number studies has already shown that a positive PCR result is not always accompanied by positive serology indicating local synthesis of antibodies (Talabani et al.,2009,Al-nasrawi et al.,2014). Current diagnosis of toxoplasmosis dependent on serological detection it may fail to detect specific anti-Toxoplasma IgG or IgM during the active phase of T. gondii infection, because these antibodies may not be produced until after several weeks of parasitemia. Therefore, in this study we used highly specific molecular as Real-Time PCR based TaqMan probe and primers to amplify the T. gondii B1 gene for detection of Toxoplasma gondii (Lin et al.,2000).

Conclusion

We have developed a rapid, sensitive, and quantitative real-time PCR for detection of *T.gondii*. The advantages of this technique for the diagnosis of toxoplasmosis in a clinical laboratory are discussed.

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