Molecular Typing of *Toxoplasma Gondii* Isolated from Infertile Men and Its Effect on the Reproduction

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Abstract

Seminal fluid and blood samples were collated from sixty 20-60 years aged and attended Teba Center for Children and ICSI/ in Babylon Province during the period from 1st June 2016 to 1st February 2017. The same number of samples were also taken from 60 age matched apparently healthy individuals to act as a control group. Anti IgG levels were measured in the sera of both groups to detect the presence of *Toxoplasma* infection, while seminal fluid samples were examined to detect infertility. *Toxoplasma gondii* genotype was applied by using nested PCR to detect SAG2 gene. Gene sequencing technique infection was performed for detection occurrence of mutation in the mitochondria of the sperm. Results confirmed that the age group (20-40years) was significantly more prone P<0.05 to infective with *T. gondii* where the percentage of seropositivty was 65% (39 patients) while it was 35% (21 individuals) in the apparently healthy control group. Concerning the residence, there was a highly significant difference (P < 0.006) where the percentage of seropositivty was 68% in idiveduals living in rural areas while it was 32% among those living in the urban areas. Genotyping showed that presence in two strains of *Toxoplasma* which has been found in males infected with Toxoplasmosis. Type I was found in 10 persons (17%) while type II was found in 50 persons (83%) and the difference between two strains was significant (P<0.05). Oligospermia has recorded the highest number of positive cases among patients (92%; 55 cases), in comparison with negative cases (8%; 5 cases), Asthenospermia showed significant P<0.05 decrease (35%;

21 cases) in comparison with negative (65%; 39 cases). Mutation occurs with Oligospermia of both of types I and II of *T. gondii* strains in 2 cases only, which was found on DN2 gene with a highly significant P<0.01 difference of positive cases (3%; 2 cases), and negative cases (97%; 58 cases). The mutation in single-nucleotide polymorphisms SNP G4580A site, showed that G was converted to A that was recognized at nt 4580 in the ND2 region. This evolution was experiential in oligozoospermic samples (code 010830). This SNP is a synonymous substitution that occurred in the third position of methionine codon, changing the codon from ATG to ATA.

KeyWords: Infertile men, *Toxoplasma gondii*, mitochondrial DNA, ND2Gene, Oligospermia, mutation.

التنميط الجزيئي للمقوسة الكونيدية المعزولة من الرجال الذين يعانون العقم ومدى تأثيره على الإنجاب

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الخلاصة

عينات السائل المنوي والدم تم جمعها من ستين من الرجال المرضى الذين كانوا يعانون العقم والمصابين بداء المقوسات والذين تتراوح أعمارهم ما بين 20-60 سنة والتي أخذت من مركز طيبة للأطفال والحقن المجهري / بابل خلال الفترة من 1 حزيران 2016 إلى شهر 1 تشرين الأول 2017. تم أخذ نفس العدد العينات أيضا من 60 من الأفراد الأصحاء كمجموعة سيطرة. تم فحص مصول كلا المجموعتين للتحري عن إصاباتهم بالمقوسة الكونيدية، في حين تم فحص عينات السائل المنوي للتحري عن حدوث العقم. وكذلك تم تطبيق التنيمط الجيني للمقوسة الكونيدية باستخدام تفاعل البلمرة المتداخل nPCR للكشف عن جين SAG2. وأيضاً إجراء تقنية التتابع الجيني للكشف عن حدوث طفرة في التتابع الجيني لميتوكوندريا الحيوانات المنوية. وأكدت النتائج أن الفئة العمرية (20-40) كانت أكثر أستعداداً للأصابة بشكل معنوي P <0.05 بين المجموعة المدروسة حيث بلغت النسبة المئوية للايجابية المصلية للأضداد النوعية للمقوسة الكونيدية 65٪ في حين بلغت مجموعة السيطرة 35٪، وكان هناك فرق معنوي عالي بين سكان الريف 68٪ والحضر 32٪، P < (0.006) وفقا للإقامة. كما كان هناك فرق معنوي عالي جدا P < 0.001 بين النوع الثاني 50 (83٪)، وفقط 10 (17٪) من النوع الأول، P = 0.001. لسلالات المقوسة الكونيدية . وقد سجلت قلة النطافOligospermia أكبر عدد بين حالات

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المرضى 55 (92٪)، مقارنة مع الحالات السالبة 5 (8٪)، في حين أظهرت مجموعة وهن النطافAsthenospermia بعدم وجود فرق معنوي 21 (35٪) مقارنة مع الحالات االسالبة 39 (65٪)، P <0.05. وقد حددت الدراسة ان هنالك حدوث طفرة مع حالة قلة النطافOligospermia من كلا نوعي السلالات الأول والثاني للمقوسة الكونيدية في 2 من الحالات فقط، والتي حددت على الجين DN2 مع فارق معنوي كبير P <0.01 2 (3٪)، بالمقارنة مع مجموعة السيطرة 58 (97٪). وكانت الطفرة في الموقع G4580A، وأظهرت الدراسة تحول الكوانين G إلى الأدنين A هذه الطفرة التي تم إثباتها في الموقع 4580 على الجين ND2. هذا التحول كان في عينات oligozoospermic (رمز 010830). للنيكليوتايدات المفردة تعددة الأشكال SNP والتغير الذي حصل في المركز الثالث من كودون ميثيونين، وتحول الكودون من ATG إلى ATA.

الكلمات المفتاحية: الرجال العقيمين، دنا المايتوكوندريا، المقوسة الكونيدية، الحمض النووي، جين ND2، قلة النطاف، طفرة وراثية.

Introduction

Every moment there are new information regarding toxoplasmosis. It; has been found that the infection with this disease is linked with many other illnesses that affecting humans, including deadly diseases, like the caner (1). There have been some many studies about this disease and it is estimated about one third of the world population was infected (2). Mohammed (3) found that 50 of breast cancer, followed by lung, cervical and colon cancer among cancer patients are infected with toxoplasmosis in Iraq. On the other hand, many studies in the world and Iraq in particular, have shown that toxoplasmosis causes a recurrent abortion among pregnant women (4). With the progress of research in the fields of molecular biology it has been found that this parasite, which is transmitted through sexual contact causes serious complications (5). Recent studies conducted in the field of molecular biology has proven the transmission of the parasite through sexual contact leads to a new field of research about the parasite to infect the reproductive organs and causes proliferative device occurs great damage that cannot be cured (6). The symptoms in men infected with cases of toxoplasmosis could cause to permanent infertility (6). Of these symptoms in males are the **Oligospermia** and **Asthenospermia**. In a previous study, we have shown that the incidence of toxoplasmosis is one of the causes that lead to infertility (7). Occurrence of mutations, particularly in those

cases in the relay for nucleotides sequence of DNA of the mitochondrial sperms (8). This study aimed to prove if infection with *T. gondii* has an effect on men infertility.

Materials and Methods

Sixty seminal fluids and whole blood samples were collected from infertile male patients who were suffering from toxoplasmosis. Each sample was stored in a sterile container sealed with plastic cover, and incubated at -20 C to conduct the necessary analysis. Sixty seminal fluid samples and sera of apparently healthy individuals were also collected in the current study to serve as a control group the age of the participants was ranging between 20 and 60 years. Macroscopic and microscopic examination of seminal fluid were done to determine the sperm count and motility. Enzyme liked immunosorbent (ELISA) was applied for detection the Anti- *Toxoplasma* IgG antibodies parameter (ElISA TOXO IgG Biotik, SUA). DNA isolation kit (QIAGEN) was used to extract genetic DNA of *T. gondii* from blood of infected infertile men. SAG2 gene, genotyping was used for nested PCR using DNA Isolation Kit Genotyping for the nested PCR genetic markers. Amplification of Mitochondrial DNA; extraction and isolation of mtDNA were carried out by using mitochondrial isolation kit (Biovision). DNA Sequencing by Bio System Big Dye TM termination V 3.1 cycle sequencing kit (Biovision) was used to perform automated DNA sequencing. The mitochondrial DNA samples of different fertility groups were sequenced for ND2 gene.

Statistical analysis

SPSS Microsoft Office Excel program was used for statistical analysis of data. The values were represented as mean ± SEM (standard error of mean). The comparison of the numeric data for healthy control and patient groups was clone by using the Paired t-test (11).

Results

The distribution of seropsitity rate of toxoplasmosis among age groups among infertile men in comparison with control group was shown in Table 1, the seropsitity rate in the age group (41- 60 years), was significantly ($P< 0.05$) higher (65%) than that of the age group (20- 40 years) which was 35%. Concerning the link between the seropositivity and residency, the results showed that 41 subjects (68%) were living in the rural areas while 19 subjects (32%) were living in the urban areas and the difference between the two groups was statistically

significant (P < 0.01). Genotyping showed the presence of two strains which have been found in males infected with toxoplasmosis. Type I was found in 10 persons (17%) while type II was found in 50 persons (83%) and the difference between the two strains was significant (P< 0.05) (Table 1).

Table 1: Distribution of seropositivity of anti-*Toxoplasma gondii* IgG antibodies according to age, residency and genotypes

Parameters for Patients		N	%	Binomial (Z) Test (P-Value)
Age groups / Year	20 – 40	21	35%	P = 0.027
	41 – 60	39	65%	
	Total	60	100%	
Residency	Rural	41	68%	P = 0.006
	Urban	19	32%	
	Total	60	100%	
Toxoplasmosis genotypes	Type I	10	17%	P = 0.00
	Type II	50	83%	
	Total	60	100%	
Oligospermia	Positive	55	92%	P = 0.00
	Negative	5	8%	
	Total	60	100%	
Asthenospermia	Positive	21	35%	P = 0.027
	Negative	39	65%	
	Total	60	100%	
Mutation	Positive	2	3%	P = 0.001
	Negative	58	97%	
	Total	60	100%	

Table 2, shows that a highly significant difference regarding the levels of anti- *Toxoplasma* IgG antibody, among patients were infected with two types of *Toxoplasma* strains and it turns out that type I was 10 (1.3650±0.635), and type II was 50(1.3199±0.580) in contrast with the control group 60(0.5640±0.201), P<0.01HS. And ten motility of sperms was a greatly momentous dissimilar, between type I became 10(8.00±8.097) and type II, 50(10.76±6.784) of *T. gondii* infection of infertile men P<0.0, HS.

Table 2: Estimation of Anti- Toxoplasma IgG antibody, among toxoplasmosis patients

Parameters	Studied groups	N	Mean	Std. Deviation	Std. Error	P-value	
						ANOVA test	LSD test
Age / Year	A.H. Control	60	40.37	7.863	1.015	P = 0.269 Non sign. (P>0.05)	P^1=0.335
	Genotype I	10	42.80	4.984	1.576		P^2=0.133
	Genotype II	50	42.50	7.095	1.003		P^3=0.907
	Total	120					
Serum Anti-Toxoplasmosis IgG Ab.	A.H. Control	60	0.5640	0.201	0.026	P = 0.00 Highly sign. (P<0.01)	P^1=0.001
	Genotype I	10	1.3650	0.635	0.201		P^2=0.001
	Genotype II	50	1.3199	0.580	0.082		P^3=0.767
	Total	120					
Sperm motility%	A.H. Control	60	74.85	11.663	1.506	P = 0.00 Highly sign. (P<0.01)	P^1=0.001
	Genotype I	10	8.00	8.097	2.560		P^2=0.001
	Genotype II	50	10.76	6.784	0.959		P^3=0.411
	Total	120					

Not: P^1= **A.H. Control Vs Genotype I,** P^2= **A.H. Control Vs Genotype II &** P^3= **Genotype I Vs Genotype II.**

As noted in Table 3, it has been identified that two cases of malformation which is Oligospermia was 8 (80%) out of 10 cases from type I and 47 (97%) out of 50 cases from type II. While the Asthenospermia was 3(30%) out of 10 cases was type I and 18 (36) out of 50 cases was type II. Also there were mutation in each of the Oligospermia and Asthenospermia from both of types I, 1 (10%) out of 10 and II, 1 (2%) out of *T. gondii* infection which had non-significant different P>0.05.

Table 3: Detection of Oligospermia and Asthenospermia and genotypes of *Toxoplasma gondii*

Parameters			Toxoplasmosis genotypes		Chi-Square Test (P-value)
			Type I (N= 10)	Type II (N= 50)	
Oligospermia	Negative	N	2	3	P = 0.144
		%	20%	6%	
	Positive	N	8	47	
		%	80%	94%	
Asthenospermia	Negative	N	7	32	P = 0.717
		%	70%	64%	
	Positive	N	3	18	
		%	30%	36%	
Mutation	Negative	N	9	49	P = 0.198
		%	90%	98%	
	Positive	N	1	1	
		%	10%	2%	

Detection the genotype of *Toxoplasma gondii*

Primers had been selected for amplification of 5′ and 3′ ends of the SAG2 locus separately. The size of fragment that splatted was 241 bp and 221bp. The splitting process was performed by application of two types of endonucleases enzymes. Sau3A1 which digest the 3rdallele (Type III) at 5′ end, while *Hhal* enzyme acts to digest the 2nd allele (Type II) in which the splitting takes place at 3′. If the fragmentation or splitting does not occur by any of these two enzymes it will be indication for the presence of type I strain. It has been identified a clonally (Type II) in 50(83%) and nine cases of type I 19(17%) were found. Sixty samples were taken from all categories and after the completion of the reaction stage within the thermal polymer (Thermo-cycler) were deported reaction solution electrically (agarose gel) with concentration of 2.5% with the use of Volumetric Guide DNA ladder (M) 100bp size (Figures 1).

Detection of mitochondrial DNA

In order to performe to amplify the ND2 genes from the extracted DNA samples, 47 samples showed the proper PCR product size (1125 bp) with ND2 gene (Figures 1)

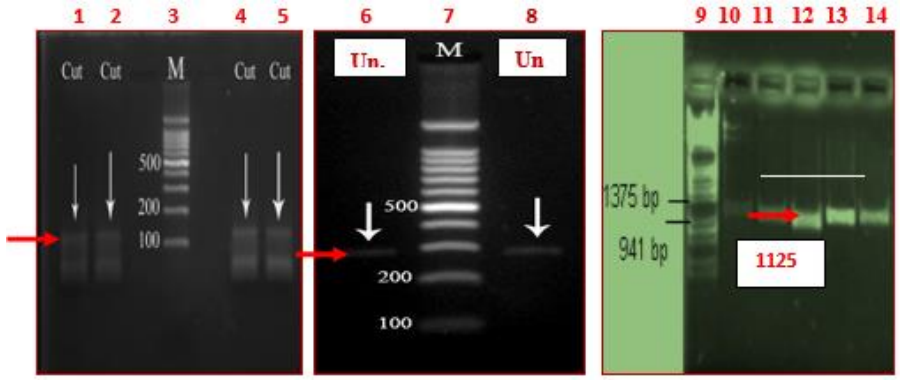

Figure 1: Lane 1, 2, 4 and 5: The cut 3' end of SAG2 with *HhaI*, in Locus (193 bp), (Type II).

Lane 3& 7: Molecular weight markers correspond to 100 bp ladder (fermintus).

Lane 6 & 8: The uncut 5' end of SAG2 (type I).

Lane 12: oligozoospermic of mitochondrial sperm in Locus 1125bp.

At 2.5% agarose gel with Ethidium bromide (0.5µg/ml), and

The Electrical current is equal to 60 volte at 30 minutes.

Sequencing of ND2 gene:

For the application of Bio System Big Dye TM termination V 3.1 cycle sequencing kit, twenty four *T. gondii* strain type II infected infertile patients and 4 type I strain *T. gondii* infected patient samples they were sequenced for the detection ND2 gene (Ha Thuc Ai Hien, *et al*, 2016), (14). The resulting sequences were compared with the reference sequence that was obtained from the NCBI database. To edit the sequences and determine the nature of mutations, a computer sequencing program TM was used. It was revealed that the base substitutions were pleased at 4514 and 4580 nucleotides (nts) as shown in (Table 4).

SNP G4580A

A G to A conversion was observed at nt 4580 in the ND2 region, and this evolution was experiential in one oligozoospermic (sample code 010830) sample (Fig 4). This SNP is a synonymous substitution that teak place which resulted in the change of the codon from ATG to ATA. (Figure 2):

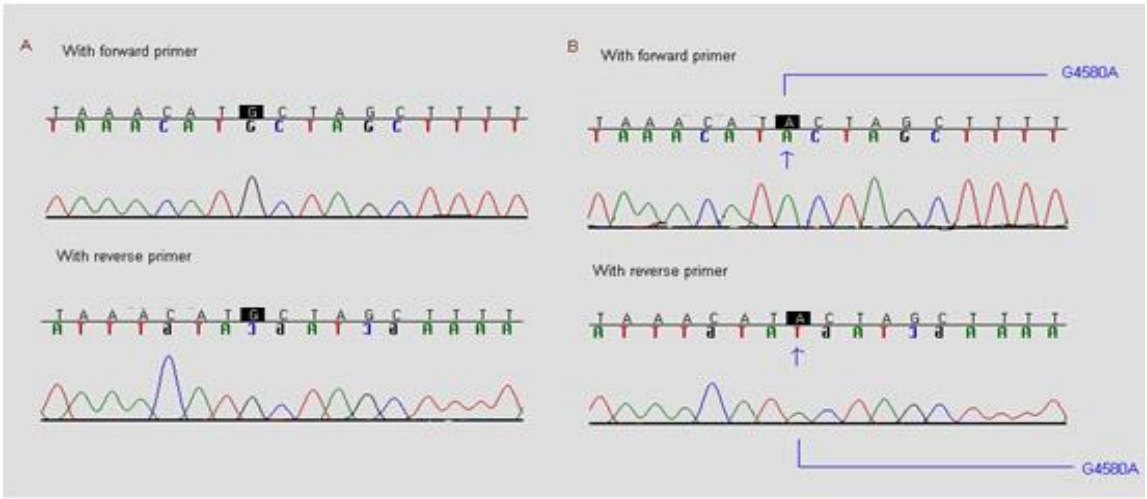

Figure2: A: Chromatogram of a control sample at nt 4580 in the *ND2* region, and B show the G4580A transition in an oligozoospermic (010830)

Analysis of Codon Usage in Synonymous Mutants in Mitochondrial DNA

The MEGA version 2.1 computer program was applied to resolve the regularity of codon using for each mitochondrial gene (Hu, *et al,* 2016), (10) the amino acid and their rate of recurrence change were shown to be strong minded Analyses of codon usage were in equal mutants from this study and from (Hu, *et al,* 2016), Table 4.

Table 4: DNA sequence analysis and mutations in different groups of seminal fluid samples in ND2 gene

Cases	Gene	Mutation	Codon change	Amino Acid change	Codon Frequency Change
Fertile	ND2	G4580A*	ATG→ATA	Silent	0.2- 2.01
Fertile	ND2	A4514G	GCA→GCG	Silent	1.66-0.59
Oligosperma	ND2	G4580A*	ATG→ATA	Silent	0.2- 1.2

*** Indicates the mutations of different individuals of seminal fluid samples.**

Discussion

Toxoplasmosis has become a life-threatening infection and many human diseases have accompanied this infection. It was found that toxoplasmosis is associated with more serious illnesses such as cancer (12, 13). This study has been conducted for the first time in Iraq. The results showed that the highest seropositivity rate was found in the age groups 20-40 years in comparison with the other age groups. These results are consistent with the results of Wilking *et al* (14), who assessed of seropositivity of *T.oxoplasma gondii* among adults. in Germany. The present study has shown that there are two types of *T. oxoplasma gondii* strains distributed among infertile males, type I and type II. The rate of type II was significantly higher (83%), than type I (17%). This result is in agreement with the results of the study which has been conducted by Al- Azawi (4), who which identified the genotyping of *T. gondii* strains and found that 48 men (93.3%) showed type II and while 9 men (15.25%) have been identified with type I, in Baghdad city. This study demonstrated that *T. gondii* had clear impact on the fertility of as evidenced by the identification of both; Oligospermia and Asthenospermia. Oligospermia refer to semen with a low number of sperms and is a common finding in male infertility. Asthenospermia is the condition in which sperms has poor motility. Normally, at least 50% of sperm should be motile. The present study indicated that the

Oligospermia was the most common incidence (in 39 men out of 60; 65%) in for infertile men. In this study, it has been observed that these cases were of unnatural causes by the toxoplasmosis and this agreed with Lekshmi, (15) and Ouladsahebmadarek *et al*, (16) they reported that the proteinase released *T. gondii* Produce anti-sperm antibodies. Mutation occurs with Oligospermia of both type I and II of *T. gondii* strains in 2 cases only, which was determined on DN2, and was experiential in oligozoospermic samples (code 010830). This SNP is a synonymous substitution that occurred in the third position of methionine codon, changing the codon from ATG to ATA of mitochondrial DNA of sperms**.** This mutation was seen in infected men with toxoplasmosis (9). Also the results in agreement with those of Wu, *et al*, (16). Who demonstrated that the ability of *T. gondii* to lower human fertilizing capacity via induction of sperms mitochondrial membrane loss to reduction of sperms ability to fertilize the ovum and it was observed that sperms lose their mitochondrial membrane potential and decrease their motility which was also affected there is need to explore molecular mechanism of *T. gondii* which induces reproduction dysfunction in the future (17) this result agreed with Eslamirad (18). This study has made clear that *T. gondii* is very dangerous parasite and it is causing complecated medical conditions, including infertility in men.

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