Genotyping of *Toxoplasma gondii* Isolated from Aborted Iraqi Women

Nazar Sh. Mohammed (MSc)[1], **Akeel H A Al-A'ssie** (PhD)[2] **and Ihsan M Al-saqur**(PhD)[3]

Abstract

Background: Toxoplasmosis is a worldwide-distributed infection which is mostly asymptomatic but can cause serious health problems in congenitally-infected newborns.

Objectives: Genotyping of *Toxoplasma gondii* and its distribution among aborted Iraqi pregnant and its relationship with sero-prevalence of alpha-fetoprotein positivity among toxoplasmosis cases.

Martials and Methods: Ninety six blood and amniotic fluids samples were collected from aborted women that are suspected to have *Toxoplasma gondii* infection from 368 samples collected from Al-Sadir Hospital, Al-Habibia Hospital, Al-Ilwea Hospital, Baghdad for delivery Hospital, Al Khark Hospital, and Educational Laboratories of medicine City, during the period from the 1st May 2014 till the end of June 2015.The results of aborted women investigations were compared with those of 79 apparently healthy controls volunteers pregnant.

Results: This study revealed that there is a highly significant increment in genotyping of *Toxoplasma* Type II, [9(15.25%)], type I and III [2(3.38)]. While the distribution the genotypes of *Toxoplasma* strains according to age groups showed that the highest frequency (55.6%) of Type I was among those at age (15-25) years, while Type II was predominant (39.6%) among the age group (36-45) years. For Type III, it was observed in very low percentage among aborted (only 2 cases out of 96). The results showed a highly significant difference in IgM level [48(93.8%)] among type II, [9(100%)] Type I, and [2(100%)] Type III in comparison with healthy control ($P<0.01$). Moreover, the current results revealed a highly significant different in Alpha-fetoprotein AFP positivity among [8(88.9**%**)], [46(95.8%)] and [2(100.0%)] for Type 1, II and III respectively in comparison with [16(43.2%)] of healthy control While level of AFP ($P<0.01$).

Conclusions: In view of the current results it could be concluded that type II strain was the most common strain among the Genotypes. The genotype I the most deadly type, while type III, which is an entero-zoonotic parasite recorded for the first time in Iraq.

Key Words: Toxoplasmosis, Genotyping, Alpha- fetoprotein, Aborted women.

Corresponding Author: nazarnazar909@yahoo.com

Received: 30th September 2015

Accepted: 4th October 2015

[1]Department of Technical Analytical - College of Health and Medical Technology - Middle Technical University - Baghdad - Iraq.

[2]Department of Biology - College of Science - University of Tikrit - Iraq.

[3]Department of Biology - College of Sciences - University of Baghdad - Baghdad – Iraq.

Introduction

Toxoplasmosis is zoonotic disease caused by opportunistic protozoan *Toxoplasma gondii*. It has been well documented that Toxoplasmosis is of crucial importance especially for pregnant women, and immunocompromised patients. In addition to the risks of gestational complications and congenital infections; it has been suggested that Toxoplasmosis has some unfavorable effects on reproductive capacity in females.

Toxoplasma gondii acquired during pregnancy can spread to the fetus and results in premature birth, permanent neurological damage and visual impairment [1]. Scientists reported if primary maternal *Toxoplasma* infection is diagnosed promptly, chemotherapy can reduce both transmission of the parasite to offspring and morbidity and mortality associated with the congenital infection [2].

Generally, laboratory diagnosis of *Toxoplasma i*nfection can be established by isolation of the parasite from blood or body fluids, demonstration of the parasite in tissues, or detection of *T. gondii*-specific immunoglobulin's [3]. Diagnosis of toxoplasmosis in immune-competent individuals' *e.g*. pregnant women is mainly based on serological tests since more than 90% of pregnant women present no specific clinical signs of the infection [4].

On the other hand, definitive diagnosis in immune-compromised individuals is mostly undertaken by direct detection of the parasite by means of PCR, histology, cell culture and mouse inoculation [5], detection of specific nucleic acid sequences with DNA probes [4].

Materials and Methods

From the 15th of May 2014 till the 30th of April 2015, 364 samples of both amniotic fluids and sera were collected from aborted Iraqi women attended Baghdad Province Hospitals, such as Ibn-Albalady Hospital, Al-Ilweia Hospital, Fatimat-Alzahra'a Hospital, Baghdad Teaching Hospital [Medical City Hospital], and Al-Shafaa Hospital for delivery. The design of this study was based on testing the sera of aborted Iraqi women were suspected to have with Toxoplasmosis and by screening tests of IgM, IgG and alpha-fetoprotein assayed. The detection of Genotypes by splitting process was performed by application of two types of endonucleases enzyme Sau3A1 which digest the 3rdallele (Type III) at 5′ end, meanwhile Hhal enzyme acts to digest the 2nd allele (Type II) in which the splitting takes place at 3′. If the fragmentation or splitting doesn't occur by any of these two enzymes it will referred to presence of Type I strain.

Mice inoculation and Tachyzoites' Isolation

Amniotic fluids samples were washed for several times. Streptomycin and penicillin were added to the least suspension of the washed cells before inter peritoneal mice inoculation. A period of 6-8 days of incubation period is required for tachyzoite transformation before collection of peritoneal fluids and its examination for assurance of Tachyzoite presence [6].

DNA extraction

Extraction of the DNA from the peritoneal exudate of mice by manner of a ready kit of blood, 96 pairs of DNA were extracted from peritoneal exudate samples according to the internal newsletter of blood extraction kit.

Amplification and detection of Bi gene of *T. gondii* by nested PCR

Nested PCR [nPCR] analysis was performed in 96 of the DNA samples to amplify the gene B1 by two steps which used a couple of different primers to

amplify each piece separately from the second piece [7].

A. Amplify the end - 5 of the site SAG2

The amplification of the end of the fifth end site in the same gene SAG2 steps nPCR was the same for Jane B1 except for primers used in the round initiator F4 and R4, in the first round and the degree of adaptation of 59 ° C. At the second amplification 3 µL of the PCR products from the first amplification reaction was used as a template for the second round under the same conditions with the exception of primers F and R2.

B. Amplify the end – 3 of the site of SAG2

The amplification using starters F3 and R3 warmly localization 46 ° C, and for the second amplification used 3 µl of the PCR products from the first amplification as a template for the second round of the same conditions interaction with the exception of primers F2 and R, It was electric relay on using gel agarose with a concentration of 2%.

C. Determine the genotype of T. gondii

Samples were analyzed at the *SAG2* locus by using a nested PCR approach that separately amplified the 5' and 3' ends of the locus (Prince *et al.*, 1990). The 5' end of the locus was amplified by standard PCR for 40 cycles with the primers SAG2.F4 (5' GCTACCTCGAACAGGAACAC 3') and SAG2.R2 (5' GCATCAACAGTCTTCGTTGC 3') at an annealing temperature of 65°C. The resulting amplification products were diluted 1/10 in water, and a second amplification of 40 cycles was performed with the internal primers SAG2.F (5' GAAATGTTTCAGGTTGCTGC 3') and SAG2.R2 (5' GCAAGAGCGAAC TTGAACAC 3') by using 1 ml of the diluted product as the template. The amplified fragments were purified with Quia Clean (Qiagen Inc.) and digested with *Sau*3AI, and 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' TCAAAGCGTGCA TTATCGC 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 quiaClean, digested with *Hha*I, and analyzed by agarose gel electrophoresis. The amplified products were digested with *Sau*3AI (5'-end products) and with *Hha*I (3' -end products). The PCR products and the restriction fragments were analyzed by 2% agarose gel electrophoresis. Restriction digestion of 5'-end-amplified products with *Sau*3AI distinguished the type III strain from types I and II strains and HhaI digestion of the 3'-end-amplified fragments differentiated types I and III strains from type II strains, [8]. In order to avoid possible contamination, several measures, such as

separate space to set up PCRs, filter tips, etc., were taken, as well a negative control (no DNA), and positive controls from different strains of *T. gondii* were used in order to locate any possible contamination [9]. These primers were selected to amplify the ends of the fifth and third site of SAG2 separately, and the size of each segment is 241bp and 221bp, respectively, and it was cuts using two types of enzymes Sau3AI which digested allele III (strain of type III) occurs cutting in the fifth end, and HhaI enzyme that works to digest the second allele (strain of type II) occurs cuts in the third end, but if they are not cutting any of the former enzymes it means that the strain of the first type I.

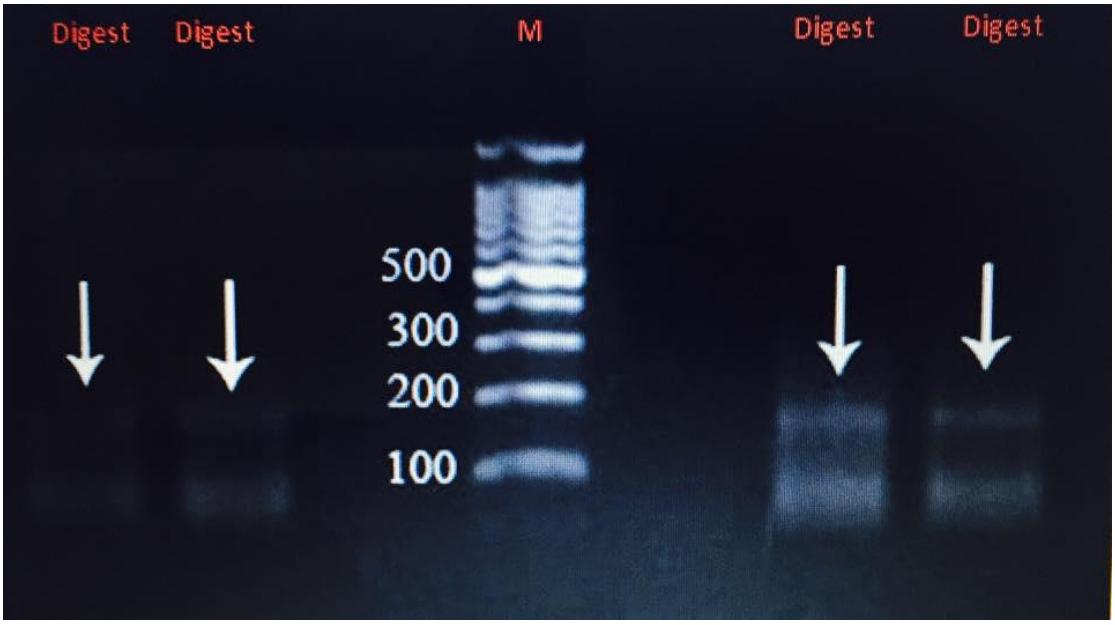

Figure (1): 3- Molecular weight markers correspond to 100 bp ladders (fermintus).1 and 2. The digested 3' end of SAG2 with HhaI.4 and 5The 5' end of SAG2 digested with Sau3AI. At 2.5% agarose gel with ethidium bromide (0.5µg/ml).

Results

According to (Table 1) the highest frequency (55.6%) of type I was among those at age (15-25) years, while Type II was predominant (39.6%) among the age group (36-45) years so as for Type III thought it was observed in very low percentage among aborted (only 2 cases out of 96).

It has been identified a clonal (Type II) in 48 (93.3%) and were found nine cases of type I [9(15.25%)], [8]. While that detected [2(3.38)] of the third type (Type III) and this case recorded for the first time in Iraq.

Table (1): Distribution of the studied groups according the types of *Toxoplasma* strains.

Age groups / Year		Types				Total	Chi-Square (P-value)
		Negative	I	II	III		
15 - 25	No.(%)	15(40.5%)	5(55.6%)	16(33.3%)	0	36(37.5%)	P=0.272 NS (P>0.05)
26 - 35	No.(%)	13(35.1%)	2(22.2%)	13(27.1%)	0	28(29.2%)	
36 - 46	No.(%)	9(24.3%)	2(22.2%)	19(39.6%)	2(100.0%)	32(33.3%)	
Total	No.(%)	37	9	48	2	96	
		100.0%	100.0%	100.0%	100.0%	100.0%	

It seems that Type II predominant (77.1%) among Rural meanwhile majority of Urban infected with Type II again as shown in (Table 2). There was an insignificant difference the Types of *Toxoplasma* strains according to their residency (P>0.05).

Table (2): Distribution of the genotypes of *Toxoplasma* strains according residency.

Residency		Types				Total	Chi-Square (P-value)
		Healthy control	I	II	III		
Rural	No.	21	7	37	1	66	P=0.195 NS (P>0.05)
	%	56.8%	77.8%	77.1%	50.0%	68.8%	
Urban	No.	16	2	11	1	30	
	%	43.2%	22.2%	22.9%	50.0%	31.3%	
Total	No.	37	9	48	2	96	
	%	100.0%	100.0%	100.0%	100.0%	100.0%	

It's clear that most pregnant aborted women complaining from single abortion (54.2%), while (45.2%) of abortion cases were of multi abortions type. Most of them (47.9%) infected with Type II, though (100%) occurred only among the two cases of Type III. However, no significant difference was noticed according the number of abortions (P>0.05) Table-3.

Table (3): Distribution of genotype of *Toxoplasma gondii* among abortion number.

Number of abortions		Types				Total	Chi-Square (P-value)
		Negative	I	II	III		
Single	No.(%)	20(54.1%)	7(77.8%)	25(52.1%)	0	52(54.2%)	P=0.215 NS (P>0.05)
Multi (2 - 4)	No. (%)	17(45.9%)	2(22.2%)	23(47.9%)	2(100%)	44(45.8%)	
Total	No.(%)	37(100 %)	9(100%)	48(100%)	2(100%)	96(100%)	

Table 4: revealed that among (69.8%) of positive IgM Anti-*T. gondii* cases, it was observed that (93.8%) of it were Type II with highly significant difference in comparison with other Types, (P<0.01).

Table (4): Relationship between genotypes and IgM antibody level in toxoplasmosis**.**

Anti-Toxoplasmosis IgM Ab.		Types				Total	Chi-Square (P-value)
		Healthy control	I	II	III		
Negative	No.(%)	26(70.3%)	0	3(6.3%)	0	29(30.2%)	P=0.00 HS (P<0.01)
Positive	No.(%)	11(29.7%)	9(100%)	45(93.8%)	2 100%	67(69.8%)	
Total	No.(%)	37(100%)	9(100%)	48(100%)	2(100%)	96(100%)	

It is noticed from table 5, that most positive cases for AFP (75%) were of Type II, (95.8%). However, the only two cases of Type III were positive with AFP.

Table (5): Relationship between genotypes and Alpha-fetoprotein AFP level in toxoplasmosis.

Alpha fetoprotein (AFP)		Types				Total	Chi-Square (P-value)
		Healthy control	I	II	III		
Negative	**No.(%)**	21(56.8%)	1(11.1%)	2(4.2%)	0	24(25.0%)	P=0.00 HS (P<0.01)
Positive	**No.(%)**	16(43.2%)	8(88.9%)	46(95.8%)	2(100%)	72(75.0%)	
Total	**No.(%)**	37(100%)	9(100%)	48(100%)	2(100%)	96(100%)	

Discussions

The results of this study was going well with the results of studies in other countries, which indicated that type II strains often associated with human toxoplasmosis and dominant among Iraqi female [7,8].

Some studies suggest a link between Type I spread in patients with congenital toxoplasmosis (which is consistent with these findings) and Toxoplasmosis network depending on the loci of SAG2 [6].

The genotyping of *T. gondii* in clinical infections is complicated by the chronic nature of the infection which features semi-textile bags underlying the absence of the parasite in the circulatory system. For this fact there is no accurate information about the parasite strains that cause infections is obvious clinically in humans (in most cases), but the recent attempts to develop serological based genotyping tests were promising but lack the ability to identify all those types as it is limited application, [7].

Thus, the first genotype I the most deadly type Williams and this means an increased risk of going through the placenta and produce serious symptoms of the fetus and newborn. This is true since explains the cases of dead births and birth defects that have appeared among women embryos obtained in this study, though they were in low frequency in comparison with type II.

These finding are quite difference from previous studies which have shown that type II are the most prevalent in animals and humans, and may be a high rate of spread of strains of type II in human Toxoplasmosis reflects simply source strains that lead to infect humans [6]. This congruence and compatibility between infection rates or its absence at the level of serological and genetic testing and genetic pattern of those aborted women may explain possibility of sexual transmission among couples making pregnant women at risk through semen subsequently fetal infection during pregnancy [8].

Prenatal diagnosis of Congenital Toxoplasmosis relies commonly on the molecular based detection of *T. gondii* DNA. Here it was reported a severe case of congenital Toxoplasmosis which resulted in termination of pregnancy. The isolate has the most similarity with type I of *Toxoplasma gondii*. In Europe, type II genotype infects humans and this type is related to acquired and also congenital Toxoplasmosis [9, 10]. In a study done by Ajzenberg *et al.,* genotype of 86 *T. gondii* isolates collected from patients with congenital Toxoplasmosis and their association with clinical findings were evaluated.

Results of the study demonstrated that type II isolates are the largely predominant type and type I and atypical isolates was not found in asymptomatic or benign congenital toxoplasmosis [10]. Genotype analysis of *Toxoplasma* strains in Spain revealed that *Toxoplasma gondii* type II were the most prevalent (52%) genotype in immunocompromised patients, whereas strains of type I were present in 75% of the congenital infection cases. This is rather different from previous study in the region and also the study in France which showed that type II strains were mostly associated with all kinds of human Toxoplasmosis; these results go well with the current study. Effects of selection in the process of culturing and isolation, prior to strain characterization, have been accounted for by these differences [12]. Type I genotype is considered to be the most virulent type, with a high level of parasitemia [13]. This may cause an increase in the risk of trans-placental transmission, producing severe symptoms in the fetus or newborn. Findings of the current case report were keeping with this concept.

In this study PCR technique has been used for detection of *Toxoplasma* DNA in amniotic fluid of aborted Iraqi women. This procedure has a relatively high sensitivity and almost 100 percent specificity [14]. A PCR negative test on amniotic fluid does not rule out the infection but a positive result guarantees the infection of the amniotic fluid. Taken together, findings of this study are important as they will provide a better understanding of congenital Toxoplasmosis and its outcome and also possible association between parasite genotypes and congenital infection. This study also highlighted the lack of efficient medication for treatment of toxoplasmosis during pregnancy [13].

The fact that toxoplasmosis linked to Type II strain as previously mentioned by [10, 11]. Aborted women and the fact that most of them are women infected with type II so, this is referred to by many researchers [14], concerning that the prevalence is increasing proportionally with age, as was noticed that the highest prevalence rate among women who ranged in age from 36 years and older[15,16], this compatible with the current result. The current result was not in harmony with or disagree with that for Al-Obaidy (2004), in Mosul who observed the highest infection rate was among older age group(26-33) years age group, and referred to in Al-Obeidi (2004)[17], that the highest infection rate found in Category age 26-30 years, as well as (Al-Qurashi 2009), [18] which stated that the highest infection rate is in the age group 21-29 years also bucked the study conducted by Al-Shikhly [19], in Baghdad, so as the study conducted by Al-Shikhly (2010) in Baghdad, who noted that highest infection rate among the age group 15 - 24 years. These vibrations may be attributed to the difference in the topography of the area studied, behavior and food habits and presence or absence of cats and method of examination, as well as the number of samples and quality within all of these involve differences between sero-prevalence in all countries of the world and within the same geographical area as well. This explanation is that the common type II to be is at the many older ages, that means that all species are the most presence at older ages in accordance with the above researchers, and it is compatible with[10,15], Also type III, which uncovered for the first time in Iraq.

AFP was found mainly to be originated from the fetal yolk sac endoderm and liver beside some from intestinal epithelium of the fetus, according to AFP gene coding for it which situated at 4q25 locus on

chromosome 4. Any variation in AFP related to difference in the gene length [20].

In view of these results the level of AFP ranges from 200 to 400 ng/ mL during the first trimester while it elevates at 14 weeks of gestation from 25.6 ng/mL up to 74.9 ng/mL at 21 weeks of gestation, [22]. It was reported that the normal concentration of AFP is less than 5.4 ng/mL (4.5 µg/L) which is in concordance with the results of the present study in which the optimum level was found 10 ng/mL [23].

Considering correlation between IgM anti-*Toxoplasma gondii* and AFP although; the specificity, sensitivity and accuracy of the latter are less than the first one. These data attributed to linkage between the antibodies and the parasite, while AFP is well known to be associated with carcinoma cases particularly liver carcinoma and malformation [21].

Because this is the first stud in Iraq revealed the existence of the type III of *T. gondii* in aborted women, there are significant differences between the positive infections and healthy control, there is a consensus among IgM and Alpha-fetoprotein level, the fact that this elevation during acute infections. Due to the third type as found in both cases of acute and chronic toxoplasmosis [20, 23].

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