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Seroepidemiological and Molecular Study of Toxoplasmosis in the Blood Donors and Applicants for Marriage peoples in the Babylon Province, Iraq.

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ABSTRACT

Identification and seroepidemiological and molecular study were conducted for blood donors and applicants for marriage to investingate the infected person with Toxoplasmosis in the Babylon province for two sexes during the period from November 2014 till February 2015, ELISA technique for 140 serume samples for blood donors and 166 serume samples for Applicants for marriage and by polymerase chain reaction (PCR) technique for 66 blood samples (where positive with ELISA technique), according to epidemiological criterions (area region , Sex ,age group). The results showed that the total toxoplasmosis samples positive using the ELISA antibody IgG (34%) and antibody IgM (% 3) for blood donors and antibody IgG (24%) and antibody IgM (% 5).polymerase chain reaction technique was 8(21.6%) for blood donors while was 12(41.37%) for Applicants for marriage the results showed that Prevalence of toxoplasmosis was slightly higher in rural area than compared to cities when using the three detecting methods, infection in male highly frequency when using ELISA and PCR technique compared with females . all age groups and ages generally more than 35 years more vulnerable to infection .

Keywords: Toxoplasmosis, ELISA technique, PCR technique.

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INTRODUCTION

Toxoplasma gondii is a coccidian parasite which an obligate intracellular protozoan it is an opportunistic singlecelled parasite (Jones ,2001; Switaj et al., 2005) . with the capacity to infect humans any warm blooded animal It considered one of the most widespread parasites in the world (Innes 2009;Pereira et al., 2010).

The present study has been high lights the blood donors infection and applicants for marriage peoples that infected with toxoplasmosis .the laboratory diagnosis of toxoplasmosis can be performed in several ways: in one kind, the observer a certain color of tachyzoites under a microscope, as it is with the dye test (DT) Indirect fluorescent antibody test (IFAT), another based on the principle of agglutination of tachyzoites Toxoplasma, and red blood cells or latex particles like with direct agglutination test (DAT), indirect haemagglutination test (IHAT) and latex agglutination test (LAT), respectively.

With enzyme-linked immunosorbent assay (ELISA) for the quantity of IgM and IgG class antibodies against Toxoplasma direct detection of parasite-specific DNA in biological samples using polymerase chain reaction based molecular methods, The molecular diagnosis is more sensitive and cost-effective than the conventional methods (Remington et al., 2004).

In previous studies indicated of toxoplasmosis infection in blood donor as Saleh (2011), Mahmood *et al*.,(2013), Mahmood *et al*.,(2014) in Iraq and Meymand *et al.,*(2015) in Iran and in applicant for marriage Hasan(2011) in Iraq .

MATERIAL AND METHODS

Enzyme linked immunosorbent assay (ELISA)

This assay was performed by using two kits .One for detection of IgG antibodies, and the other for detection of IgM specific antibodies against *Toxoplasma* antigen in the patient's serum, (Young, 1995).

Detection of anti- *Toxoplasma gondii* antibody (IgG) by Enzyme linked Immunosorbent Assay(ELISA) technique.

The bioCheck *Toxoplasma* IgG ELISA (BC-1085) kit was used .The *Toxoplasma* IgG ELISA is intended to evaluate a patient's serolgic status to *T.gondii* infection.

Principle

Purified *T.gondii* antigen (Ag) is coated on the surface of micro wells. Diluted patient serum was added to the wells, and the *T.gondii* IgG- specific Ab, if present, will bind to the Ag.All unbound materials were washed away. Horse radish peroxidase (HRP) conjugate is added, which binds to the Ab-Ag complex. Excess HRP-conjugate is washed off and solution of tetra methyl benzidine (TMP) reagent was added. The enzyme conjugated catalytic reaction is stopped at a specific time. The color intensity generated is proportional to the amount of IgG -specific Ab in the sample.The results were read by ELISA reader (Roller *et al*., 1987).

Reagents

- Micro titer wells: purified *T. gondii* Ag-coated wells (8x12 wells).
- Enzyme conjugated reagent 1 vial (12 ml).
- Sample diluents: 1 bottle (22ml).
- Negative control: range stated on label. (150µL/vial).
- Cut-off calibrator: 32 IU/ml, (150µL/vial).
- Positive calibrator: 100 IU/ml (150µL/vial).
- Positive control: range stated on label, (150µL/vial).
- Wash buffer concentrate (20x): 1 bottle (50 ml).
- TMB reagent (one-step): 1 vial (11 ml).
- Stop solution: (1N HCL), 1 vial (11 ml).

Assay procedure

1. Before start the work, the plates were opened and left for (5 min) at RT to equilibrate there and select the required number of strips for the run.
2. Prepared (1:40 dilution) of test samples, negative control, positive control, and calibrators by adding (5 µl) of sera samples, negative control, positive control, and calibrators to (200 µl) of sample diluents and mixed well.
3. Dispensed (100 µl) of diluted sera, calibrators and controls into the appropriate wells. For the reagent blank, (100 µl) sample diluents was dispensed in 1A well position. Tap the holder to remove air bubbles from the liquid and mix well.
4. Incubated at (37°C) for (30) minutes.
5. At the end of incubation period the liquid from all wells was removed , the microtiter plate was rinsed and flicked five times with diluted wash buffer.
6. Dispensed (100 µl) of enzyme conjugated to each well, and mixed gently for (10 sec) and covered with adhesive seal.
7. Wells were incubated at (37°C) for (30 min.).
8. Enzyme conjugated was removed from all wells after adhesive seal was removed.
9. The microtiter plate was rinsed and flicked five times with diluted wash buffer.
10. About (100 µl) of TMB reagent was dispensed into each well, and mixed gently for (10 sec).
11. Wells were covered with adhesive seal and incubated at (37°C) for (15 min).
12. About (100 µl) of stop solution (1N HCL) was added to each well to stop reaction, this well change the color in wells from blue to yellow.
13. At (450 nm) the O.D. was read within (15 min) with micro well, reader

Detection of anti-*Toxoplasma gondii* antibody (IgM) by Enzyme linked Immunosorbent Assay (ELISA) technique

The bioCheck *Toxoplasma* IgM ELISA (BC-1087) kit was used .The *Toxoplasma* IgM ELISA is intended for using in detection of IgM status to *T.gondii* in human serum.

Principle

The same principle, reagents, assay procedure of ELISA-IgG technique was adopted in ELISA-IgM detection with few exception, the specific antibody IgM was used instead of IgG specific antibody (Turune *et al*.,1983).

Calculation of results

Calculation of results for both IgG and IgM antibodies was done in the same mannar.

1- The mean of duplicate cut-off calibrate was calculated which represents the cut-off value.
2- The mean of duplicate positive control, negative control and patient sample were calculated.
3- The mean value of each sample (absorbance) was divided by the cut-off value ,and then the results were compared with the following ratios to differentiate between positive ,negative and equivocal samples.

Positive: ratio absorbance / cut-off ≥ 1.0
Negative: ratio absorbance / cut-off < 0.9
Equivocal: ratio absorbance /cut-off ≥ 0.9 < 1.0 (sample was retested).

Primer

Use a primer and manufactured from a company (Bioneer –Korea) for the purpose of investigating the gene 's DS29,DS30(914bp) and B5 B6(191bp) genetic patterns parasite *T.gondii* (Savva *et al* ,.1989).

Primer used in present study with sequence nucleotides (Savva *et al* ,.1989).

Primer sequence	Direction	Primers Gene name
TTG CCG CGC CCA CAC TGA TG	Forword	1-DS29,DS30(914bp)
CGC GAC ACA AGC TGC GAT AG	Revers	

Mixture chain reaction polymerization volume 20 microliter

Volume (microliter)	Ingredients
5	Master mix
5	DNA template
1.5	Forward primer (F)
1.5	Reverse primer(R)
7	Nuclease free water
20	Total

Thermocycler for to amplify of DNA

Interactions conditions used in thermocycler:

Number of Cycles	Time (minutes)	Temperature c°	steps
30	5	95	**Initial Denaturation**
30	1	95	**Denaturation**
30	1	58	**Annealing**
30	3	74	**Extension**
30	7	72	**Final Extension**

Agarose electrophoresis

Make electrophoresis to gel Agaros 1.5% the Polymerase chain reaction product analysis as Balader *et al*. (2009) as fallowing:

1. Melted 1.5gm from gel Agaros in 100 ml from (TBE Buffer) a concentration of 1X
2. Leave the gel cooled at room temperature and after the dye was added Ethidium bromid nuclear and radioactive and mix well with gel
3. Agaros gel was poured in deportation Tray containing the comb template to locate samples PCR and then he left the gel solidifies at room temperature for 15 minutes and then removed from the comb gel removed carefully.
4. Used DNA ladder sequence from 25 /100bp To measure the result of the interaction in the first pit.
5. Immersion Agarose gel by used (TBE buffer) a concentration of 1X and operation of the device electrophoresis current 100 volt and 80 AMP for one hours.
6. After complete the electrophoresis is checked gels containing the product PCR by use ultraviolet rays at a wavelength 260 Nm To determine the results are positive and matched him with measurement by with DNA ladder.and then photography of bands that revealed by digital camera (Sony).

RESULTS

306 blood samples were collected from applicants for marriage and blood donors, that attending to health care center and blood banking center in the AL-Hilla Teaching Hospital Babylon province, during the period from November 2014 till February 2015, their age groups ranged between, 17-42 and more year.

Table(1): Percentage of infection toxoplasmosis in the blood donors and applicants for marriage peoples by using the ELISA technique in the Babylon province according to the residence area.

Applicants for marriage			Blood donors			Residence area
Positive for antibody IgM(%	Positive for antibody IgG(%)	Examined No.	Positive for antibody IgM(	Positive for antibody IgG(%)	Examined No.	
(%1.5)1	(%14.9)10	67	(%2)1	(%24)12	50	Town(city center)
*(%4)4	(%14.1)14	99	(2.22)2	(%24.4)22	90	Rural area
(%3)5	(%14.5)24	166	(2.1)3	(%24.3)34	140	Total
2.3 sign rural area (IgM) and 0.4 sign for IgG			non sign2.3 rural area (IgM), non sign 0.8 (IgG)			Statistical testing)**Z**-test(

*Significant differences (P< 0.05).

Table(2): Percentage of infection toxoplasmosis in the blood donors and applicants for marriage peoples according to the sex by using ELISA technique.

Applicants for marriage			Blood donors			The sex
Positive for antibody IgM(%	Positive for antibody IgG(%)	Examined No.	Positive for antibody IgM(%	Positive for antibody IgG(%)	Examined No.	
*(%5.4)4	(%12.2)9	74	3(2.14%)	(%24.3)34	140	Males
(%1.1)1	*(%16.3)15	92	0	0	0	Females
(%3)5	(%14.5)24	166	3(2.14%)	(%24.3)34	140	Total
2.4 sign IgG, Female and 2.1 sign IgM, Male						Statistical testing)**Z**-test(

*Significant differences (P< 0.05).

Table(3): Relationship of percentage infection of toxoplasmosis in the blood donors and applicants for marriage peoples according to the age group of patients by using ELISA technique.

Applicants for marriage			Blood donors			Age groups (years)
Positive for antibody IgM(%	Positive for antibody IgG(%	Examined No.	Positive for antibody IgM(%	Positive for antibody IgG(%)	Examined No	
(%2.2)1	(%13)6	46	(%0)0	(%20)2	10	21 -17
*(%7.1)2	(%17.9)5	28	*(% 11)2	(%21)4	19	26 -22
(%5.4)2	(%8.1)3	37	(%4.5)1	(%27.3)6	22	31 -27
(%0)0	(%18.5)5	27	(%0)0	*(%45)9	20	36 -32
(%0)0	(%10)2	20	(%0)0	(%13.3)4	30	41 -37
(%0)0	*(%37.5)3	8	(%0)0	(%23.1)9	39	42- and more
(%3)5	(%14.5)24	166	(%2.1)3	(%24.3)34	140	Total
3.6 sign (22-26) IgM, 7.5 sign 42)and more (IgG			4.6 sign (22-26) (IgM), 6.5 sign (32-36) (IgG)			Statistical testing)LSD -test(

*Significant differences (P< 0.05).

Table(4): Percentage infection of Toxoplasmosis in the blood donors and the applicants for marriage peoples by using the PCR technique in Babylon province

Residence area	Examined No.	Positive PCR for IgG	Positive PCR for IgM	(%)PCR for IgG	(%)PCR for IgM	Total infected No.	Total percentage of infected%
Town(city center(	24	9	2	37.5*	*8.3	11	45.8
Rural area	42	6	3	14.3	7.1	9	21.4
Total	66	15	5	22.7	7.6	20	30.3
Statistical testing **Z-test((**	3.4 sign town IgG, 1.3 non sign IgM						

* Significant differences (P.(0.05 >

Table(5): Percentage infection of Toxoplasmosis in the blood donors and applicants for marriage peoples according to the sex of patients by using the PCR technique .

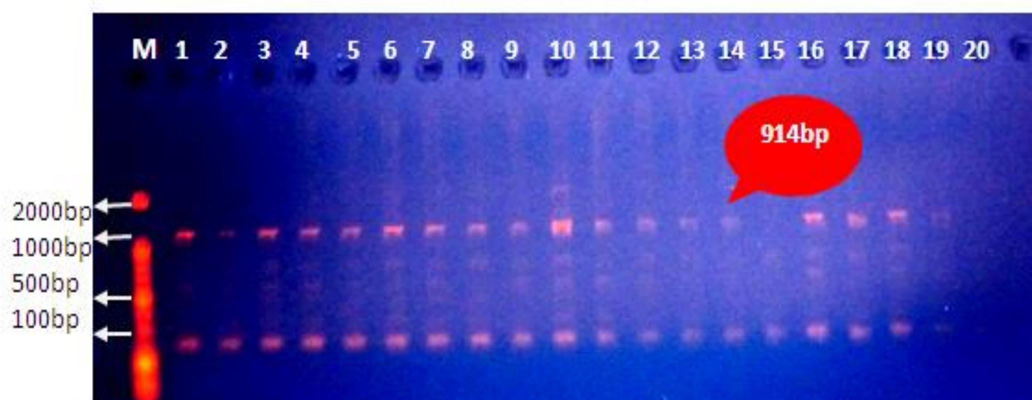
The sex	Examined No.	Positive PCR for IgG	Positive PCR for IgM	(%)PCR for IgG	(%)PCR for IgM	Total infected No.	Total percentage of infected %
Males	50	12	3	24.5	6.1	15	30.0
Females	16	4	1	*26.7	*6.7	5	31.2
Total	66	16	4	24.2	6.1	20	30.3
Statistical testing Z-test((	2.4 sign IgG female IgM 0.3 sign ,						

*Significant differences (P.(0.05 >

Table(6): Percentage infection of Toxoplasmosis in the blood donors and applicants for marriage peoples according to the age groups of patients by using the PCR technique.

Age groups	Examined No.	Positive for IgG	Positive for IgM	(%)PCR for IgG	(%)PCR for IgM	Total infected No.	Total percentage of infected %
21 -17	9	4	2	44.4	*22.2	6	66.6
26 -22	13	1	0	7.7	0	1	7.7
31 -27	12	4	1	33.3	8.3	5	41.6
36 -32	14	2	0	14.3	0	2	14.3
41 -37	6	3	1	*50	16.7	4	66.6
42- and more	12	1	1	8.3	8.3	2	16.6
Total	66	15	5	22.7	7.6	20	30.3
Statistical testing **LSD-test((**	8.5 sign (37-41) IgG and 5.6 sign(17-21) IgM						

*Significant differences (P< 0.05).



Figure(1): Electrophoresis to gel agaros with dye Ethidium bromid to amplify the results DS29,DS30(914bp) of parasite *T.gondii* by PCR technique into the circumstances(70V ,100V, Time 1-1.5 hr).that as M=DNA Ladder for 20 samples DNA containing the parasite.

DISCUSSION

Relationship with toxoplasmosis in the blood donors and applicants for marriage peoples with residence area.

There is many Iraqi provinces , previous similar studies were done but the present study it's the first on the blood donors and applicants for marriage peoples in the Babylon province by use ELISA test , (140 in blood donor and 166 in applicants for marriage). in blood donor that they indicated no significant difference

of infection by *T.gondii* according to habitation. by ELISA (IgG and IgM) in rural area where (24.4%,2.22% , respectively), compared to town(city center) residents (24%,2%, respectively) . In applicants for marriage indicated for IgG no significant difference urban area where 14.9%, compared to rural area 14.1% .

The present study agree with the study of the Al-Jubori (2005) regarding the residency of the patients and its relation with seropositive *Toxoplasma* antibodies showed no significant difference between *Toxoplasma* Abs distribution and both urban and rural areas, through which the rate were (33.98%) and (32.08%) respectively.study of Hamza (2006) revealed a non significant association between the residency and prevalence of total *Toxoplasma* antibody by ELISA IgM test urban has (32.8%), and rural has (40.3%). And study of Jassam (2010) showed that among the positively rate of anti-*Toxoplasma* IgG antibodies selected explanatory variable among schizophrenic group was non significant among rural (49.4%) compared to urban residence (47.1%).

While IgM in applicant for marriage in the rural area was (4%) higher than Town(city center) was(1.5%) agreed with the study of Salibay *et al. ,* (2008) in Philippines where they showed that was higher in suburban patients than urban residents and study of Al-Saadii (2013) in males and females highest in rural area which where 111(91.73%) compared to 10 (8.26%) urban residents.

By used PCR technique in Town(city center) was (45.8%) higher than rural was 21.4%, agreed with study of AL-Maamuri (2014) who found (80.92%) urban area and (66.67%) rural area. The prevalence of schizophrenia is higher in urban areas than in rural areas(Takei *et al*., 1995) and disagree with study Zoe *et al.,*(2009) in Romania who revaled that higher seropositivity of toxoplasmsis was among rural enviroment (63.68%) compared to the urban one (55.12%)and with study of Sroka *et al.*(2010) in Poland also showed that human living infarms had significantly greater percentage of anti-*Toxoplasma* antibodies with (59%) compared to urban dwellers (41.0%).

Difference in the readings toxoplasmosis according to a place living may be because lack of health education in some rural areas, sufficient appropriate of treatment and direct contact with cats and other animals, as they generate animals in the home parks. Other possible explanation increased exposure to infection during pregnancy and children due to the more crowded living conditions or perinatal period of living conditions in urban areas (Mortensen *et al*., 1999).

May be the different studies in city(urban area) and country side rural may be due to the hygiene and the difference in sample size , frequent ready-made foods , eat more in restaurants in the city from the countryside, and may be , also the fact that specimens in this study, most of them were from city residents compared to rural areas by PCR test . Perhaps people in Iraq do not have the habit of consuming raw meat, it is very likely that casual ingestion of oocysts is the primary route of transmission in Iraq..

Relationship with toxoplasmosis in the blood donors and applicants for marriage peoples with the Sex

Percentage of infection by use ELISA test the present study results according to gender , in applicants for marriage by ELISA for IgG The results showed seropositive percentages for both men and women(12.12 %,16.3%, respectively) agreed with Al-Maamuri(2014) were (82.43%)in female and (76.8%) in male,as well as study of ,(Mahmood *et al*.,2014) was 13.3% in male and 43.3% in female Saleh (2011) was 17.83%in female and 9.1%in male and disagree with study of Salman and Mustafa (2014) in Kirkuk city show higher in males was 31.26% than in female was26.19%.

While, ELISA-IgM was recorded variable results in male 5.4% higher than female was 1.1%. and the present study agreed with pervious study with Walle *et al*.(2013)was in male 4.0% and in female was 2.0% and study of the Al_Ghargholi (2015) showed dissimilar findings with *Toxoplasma* seroprevalence 52.3% in male and 50.3% in female (IgG) and (IgM) in male 5.6% and 18.7% in female respectively.while in blood donor were submitted male only chronic infected in blood donor higher 24.3% for IgG than 2.14% for IgM acute infected.

The present study in the blood donors and applicants for marriage according to gender by PCR technique was they indicated no significant difference in male and female 30%,31.2% ,respectively, the present study agree with study of Khademvatan *et al*., (2013)in Iran, who found no significant difference in prevalence of IgG positivity between men and women this differences may be due to the samples taken from

students of Medical Science University in Iran. and disagreed with pervious study Xiao *et al*.(2010), who found a higher prevalence in males than in females,and in study of Al_Ghargholi (2015) who found high signification in female 61.7% higher than male 30.18%.

This differences may be due to variable of size specimen were taken was males only from blood donors with males in applicants for marriage compared with females therefore was percentage by PCR non significances, or may be due to of probably both males or females exposed to infected with toxoplasmosis according to mode of live or residence area or not to follow Hygiene habits .

Relationship with toxoplasmosis in the blood donors and applicants for marriage peoples with age group.

While the highest percentage of infection with toxoplasmosis by ELISA for IgG in blood donor where focused in (32-36) years as 75%,agreed with Abdulla *et al*.(2015)who found in age group(31-40)years with IgG was 35%. while in applicants for marriage by ELISA(IgG) focusedin the 42- and more years was 37.5% were agreed with Saleh (2011) was (40-49) years was 30% % and Al- Maamuri(2014) was 41-50 years was 85.39%. and disagreed with Al_ Mosawi(2012) showed dissimilar findings with *Toxoplasma* seroprevalence 25-29 years for IgG was 66.6% and 45and more years for IgM was 26.4% and agree with both IgG and IgM in age group 20-24 years as 49.2%.

While, ELISA-IgM was recorded variable results characterized by the presence of highest in blood donor and applicants for marriage in age group(22-26)years were (11.76%, 7.14%, respectively) , were agreed with Mahmood *et.al*.(2013*)* showed similar findings with *Toxoplasma* was (18-25)years was 30% with IgM and Al-Saadii (2013) in which had noticed(18-25) was 30%.and Saleh (2011) age group 19-29 years was 10.8%.

By PCR technique blood donors and applicants for marriage according to age group the higher among age group 37-41 years was 66.7% The present study agreed with pervious study Al_Ghargholi (2015), who found high signification among age group 39-43 years was 52.1% . These differences between previous results and current result given the differences in the specificity and sensitivity of method used for diagnosis and response of every host to the strian of parasite, the deffernces in parasite strains may play an important role in the stimulation of host immune response against the parasite (Suzuki and John, 1994). The presennt result might be relevant to the different number of each infected person at each age group ,also the pepols may be contact with *Toxoplasma* in childhood ,through cats connected ,soil exposure has resulted to accumulate of anti *Toxoplasma* antibodies at different percentages within human being that lead to the chronic infection with toxoplasmosis(Spalding *et al*.,2005).

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