EUROPEAN ACADEMIC RESEARCH
Vol. IX, Issue 3/ June 2021

Impact Factor: 3.4546 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

Molecular and Immunological Detection of Toxoplasma Gondii among Sudanese Pregnant Women

AMMAR A. ABDELMOLA[1]
ELAMEEN ABD ALKAREEM[2]
AYMEN M. MADKHALI[3]
MOHAMED ELFATIH ABDELWADOUD[4]

Abstract:

Background: *Toxoplasma gondii protozoon is widely distributed around the world and can infect all mammals and birds. While acquired toxoplasmosis is usually asymptomatic in healthy subjects, acute infection during pregnancy may lead to abortion, stillbirth, fetal neurological and ocular damages. This study aimed to determine the molecular and serological prevalence of Toxoplasma gondii and associated risk factors in women attending antenatal care in Khartoum during period 2015 to 2016.*

Method: *This was a cross-sectional descriptive study carried out between April 2015 and October 2016 involving 200 pregnant women attending antenatal care units at selected health centers in*

[1] Principal investigator and a PhD student, publishing these papers based on his researches in toxoplasmosis. He is a medical laboratory sciences graduate with M.Sc degree. I have been extensively trained in molecular parasitology and immunoparasitology and worked as lecturer in MLS, Trainer and researcher.

[2] He is a lecturer and associate professor in Parasitology and Medical Entomology, a graduate of MLS with M.Sc and PhD in Parasitology. Published several papers in parasitology and especially related to malaria. He is an educator, supervisor and trainer in the field of parasitology in Sudan. He also ranked as a college supervisor, dean and the main supervisor to this original investigator of this paper.

[3] Aymen M Madkhali has special expertise in Communicable Diseases working in Jazan University, UK tropical disease. He is published considerable contributions to cutting edge reviews, original articles in many well reputed journals.

[4] B.Sc and M.Sc & Ph.D degree Histopathology and Cytology- 22-2-2016 (OMDURMAN ISLAMIC UNIVERSITY- Sudan faculty of post graduate studies (Medical Laboratory Sciences). Currently serving as a dean faculty of MLS at UMST, Sudan. Associate Professor and researcher in pathology, cervical cancer and infectious disease pathology.

Khartoum state Sudan to determine prevalence of toxoplasmosis among pregnant women using serological and PCR techniques.

Results: *The positive cases of T. Gondii recorded 26 (13%) by Molecular Technique PCR with cut off level of IgG 9.35 with significant P. value 0.0000001 using independent T test which shows Negative PCR tests were 174 (6.02471±1.35242). In contradiction with IgG antibody, IgM antibody and PCR test showed insignificant association with p value of (0.549).*

Conclusion: *PCR is considered to be the golden standard test for Toxoplasmosis gondii. Advocating for the implementation of control and preventive measurements and routine screening services for T. gondii infection to be integrated with other antenatal care services to identify potential effects of the infection and its management should be considered as the main strategy to minimize congenital toxoplasmosis and abortion.*

Keywords: Toxoplasma gondii, Toxoplasmosis, Molecular diagnosis, IgG, IgM, PCR (Polymerase chain reaction)

INTRODUCTION

The protozoan parasite Toxoplasma gondii (T. gondii) is one of the most common parasites worldwide due to its ability to infect all warm blooded animals including humans. Serological Detection of T. gondii technique involves the detection of T. gondii antibodies in the serum of infected patients. Key among the serological methods employed in diagnosing toxoplasmosis include the Sabin-Feldman Dye test, the Indirect Hemagglutination assay, the Indirect Fluorescent antibody assay (IFA), the direct agglutination test (DAT), the Latex agglutination test (LAT), the Enzyme-Linked Immunosorbent assay (ELISA) and the Immunosorbent agglutination assay test (IAAT).

According to Hill and Dubey (2002), the most effective and preferred method and hence the gold standard for diagnosing toxoplasmosis is the Sabin-Feldman Dye test. This method is extensively used due to its high sensitivity and specificity for toxoplasmosis (1). However, it is costly and highly hazardous when used on live humans compared to ELISA (1,2). The initial evaluation is

to analyse toxoplasma IgG, IgM antibodies and IgG avidity. If toxoplasma infection is suspected in pregnancy, a new sample, ideally after three weeks, should be analysed for immunological changes and confirmation of the result. IgG seroconversion and at least a two-fold rise in IgG amount indicates primary infection. IgG and IgM positivity and low IgG avidity indicate the possibility of a primary infection (3,4). The strength of the antibody binding to the antigen (-avidity) increases with time. Different tests measure the antigen binding avidity of IgG antibodies. The method is most commonly used in order to decide whether the pregnant woman acquired the infection prior to conception or during pregnancy. Avidity cannot give precise information on the duration of the infection, but high avidity clearly indicates the occurrence of infection more than four months earlier. Low avidity indicates a possible but unconfirmed primary infection since low avidity in some patients may last for several months (5,6,7) The presence of T. gondii in a specimen can be confirmed by detection of the parasite DNAwith the PCR method. In 1990, Grover et al. described the efficacy of thePCR method for rapid diagnosis of congenital infection. PCR has been performed onamniotic fluid after amniocentesis with sensitivity 59 - 97% and specificity 94 -100% depending on gestation, method and laboratory. In the study by Jenum et al., the sensitivity was 59% and the specificity 94 % (8). Conventionally, the B1 gene of theparasite has been the target of PCR, but recently the 529-bp fragment has been shown to bemore informative. The sensitivity has been found to be highest in the secondtrimester and lowest in the first trimester (9.10,11,12).

The aim of this study to detect prevalence of Toxoplasmosis infections among Sudanese pregnant women by Serological and Molecular Techniques PCR.

MATERIALS AND METHODS

This was a cross-sectional descriptive study which was carried out between April 2015 and October 2016 among 200 pregnant women who attended health care centers located in Khartoum state. With those who agreed to participate by signing the consent form were included in the study while subjects with autoimmune disorders or those in immunosuppressive therapy were excluded from this study.

Furthermore, regarding collection of Data, the study participants were interviewed by administration of a standard questionnaire to obtain the socio-demographic and economic status information as well as epidemiological risk factors. As for detection of T. gondii antibodies (IgM and IgG) using ELISA assays, 5mL of venous blood were collected aseptically from each of the included pregnant women and divided into two EDTA anticoagulated containers. Plasma was separated from the whole blood in one container by centrifugation at 3,000 rpm for 5 minutes.

Detection of T. gondii infection by nested polymerase chain reaction

Real Time PCR was conducted using a pair of T. gondii-specific primers:
GAR6-F1: 5'-ATTTGTGTTTCCGAGCAGGT-3' and R1: 5'-GCACCTTCGCTTGTGGTT-3'.
Nested-PCR was performed with primers:
GAR6-F2: 5'-TTTCCGAGCAGGTGACCT-3' and R2: 5'-TCGCCGAAGAGTTGACATAG-3' (15).
Amplifications were conducted ina final volume of a 20 µL reaction mixturethat contained 10 µL of 2x Taq DNA polymerase Master Mix with 2 mM MgCl2 (Cat. no. A170301, Ampliqon, Denmark), 10 pmol of each primer, 5 µL of distilled water, and 3 µL of template DNA. For nested-PCR, one µL of the first round PCR product was used as the template. For each reaction, two positive controls (DNA extracted from T. gondii paraffin-embedded tissues and the RH strain of T. gondii) and a negative control (double distilled water) were included. Amplification was performed with initial denaturation for 5 minutes at 95°C, followed by 35 cycles at 95°C for 30 seconds (denaturation), annealing at 59°C in the first round, and 57°C in nested PCR for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 10 minutes. A total of 5 µl of nested-PCR products along with a 100-bp DNA ladder were electrophoresed in 1.5% safe stain (2) agarose gels and visualized under ultra-violet trans-illumination.

Genotyping of positive samples by restriction fragment length polymorphism

Positive samples were genotyped using GRA6 and SAG3 markers (13). First, we digested the nested-PCR products of GRA6 positive samples using Tru1I (MseI) restriction enzyme (Cat. No. ER0982, Thermo Fisher Scientific, USA) as previously described (2). Digestion was conducted in a final volume of 16 µL reaction mixtures that contained 5 µL of the nested-PCR products, 1µL of Tru1I enzyme, 1 µL of 10X Buffer R, and 9 µL of nuclease-free water. Then, the reaction mixtures were incubated at 65°C for 1 hour according to the manufacturer's instructions. A total of 10 µl of restriction fragments were electrophoresed by Tris-acetate-EDTA (TAE) buffer through 3% (w/v) agarose gel stained with safe stain and visualized under UV transillumination. We conducted genotyping of the positive samples by the SAG3 marker (2,13). Nested-PCR was carried out for positive samples using the SAG3 marker as previously described (14). Next, the products were digested using BcnI (NciI) restriction enzyme (Cat. No. ER0061, Thermo Fisher Scientific, USA) at 37°C for 6 hours according to the manufacturer's protocols. The restriction fragments were electrophoresed and visualized under UV transillumination. The type of T. gondii was determined according to the restriction patterns after digestion with restriction enzymes (2.13.15). In order to determine better illustrationpatterns of the genotypes, the GRA6 and SAG3 sequences of three types of T. gondii (RH type I, ME49 type II, and NED type III) were obtained fromGenBank and digested by their restriction enzymes using NEBcutter V2.0 (39/).

- **Nucleotide sequence analysis of the GRA6 gene**

We extracted two positive nested-PCR products of the GRA6 gene from the gel (Vivantis Gel Purification kit, Selangor DarulEhsan, Malaysia) according to the manufacturer's instructions. The products were sequenced in the forward and reverse directions by the Sequetech Corporation (Mountain View, CA, USA), edited with BioEdit software, (33) and compared with GRA6 partial sequences of T. gondii available in GenBank.

For analysis of data, Statistical Package for Social Sciences software, version 23.0 (IBM SPSSInc., Chicago, IL) and STATA 11 were used. Initially, all information gathered via Data master sheet and Interview based questionnaire then coded into variables. Both

descriptive and inferential statistics involving Pearson Fishers exact test, Independent T-test, Mann-Whitney U-Test, binary logistic regression and ROC curve for specificity and sensitivity were used to present results. A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In both **tables [1]** and table **[2]**, PCR testing to detect IgG antibody shows great significance with p value of 0.0000001 (p value less than 0.05) by using different types of statistical tests such as independent T test that illustrated that Negative PCR tests were 174 (6.02471±1.35242) and the positive were 26 (86.28846±35.780769) and the Mann-Whitney statistical U-Test that showed negative samples were 174 with mean rank of (87.5) and positive samples with a mean rank of (187.5)In contradiction with IgG antibody, The measurement of IgM antibody by PCR test showed insignificant association as shown in both **tables [3**] and **table[4]** which also use different statitstical tests. By using independent T test it shows that Negative PCR tests were 174 (0.02588±0.0557) and the positive were 26 (0.02258±0.017909) p value of (0.549) which is more than p value (0.05) and the Mann-Whitney statistical U-Test that showed negative samples were 174 with mean rank of (99.22) and positive samples with a mean rank of (109.04) and gave insignificant association of p value (0.419) which is more than p value 0.05

In **Figure [1]** theROC Curve represents sensitivity and specifity of IgG and IgM antibodies by using PCR molecular diagnosis test The smallest cutoff value is the minimum observed test value minus 1, and the largest cutoff value is the maximum observed test value plus 1. All the other cutoff values are the averages of two consecutive ordered observed test values.

It clearly shows that:

- IgG was considered as Positive test for toxoplasmosis if IgG ≥ 9.35000 (Sensitivity=100% and Specificity=100%) under area 100 %(CI 95%; 100%-100%) statistically significant with P value 0.0000001<0.5.
- IgM was considered Positive test for toxoplasmosis if IgM ≥ 0.1950 (Sensitivity=53.8% and Specificity=60.9%) under area

54.9 %(CI 95%; 42.2%-67.6%) statistically insignificant with P value 0.065>0.5.

The coordinate of the curve states that the test result variable(s): IgM has at least one tie between the positive actual state group and the negative actual state group.

Table [1] Shows molecular detection of IgG antibody by PCR testing using independent T-test

Independent T-Test					
Variables		Number	IgG		
			Mean	Std. Deviation	Std. Error Mean
PCR	Negative	174	6.02471	1.35242	0.102527
	Positive	26	86.28846	35.780769	7.017186
P value = 0.0000001**					

Table [2] Shows molecular detection of IgG antibody by PCR testing using Mann-Whitney U-Test

Mann-Whitney U-Test				
Variables		Number	IgG	
			Mean Rank	Sum of Ranks
PCR	Negative	174	87.5	15225
	Positive	26	187.5	4875
P value = 0.00000001**				

Tables [3] Shows molecular detection of IgM antibody by PCR testing using independent T-test

Independent T-Test					
Variables		Number	IgM		
			Mean	Std. Deviation	Std. Error Mean
PCR	Negative	174	0.02588	0.0557	0.004223
	Positive	26	0.02258	0.017909	0.003512
P value = 0.549*					

Table [4] Shows molecular detection of IgG antibody by PCR testing using Mann-Whitney U-Test

Mann-Whitney U-Test				
Variables		Number	IgM	
			Mean Rank	Sum of Ranks
PCR	Negative	174	99.22	17265
	Positive	26	109.04	2835
P value = 0.419*				

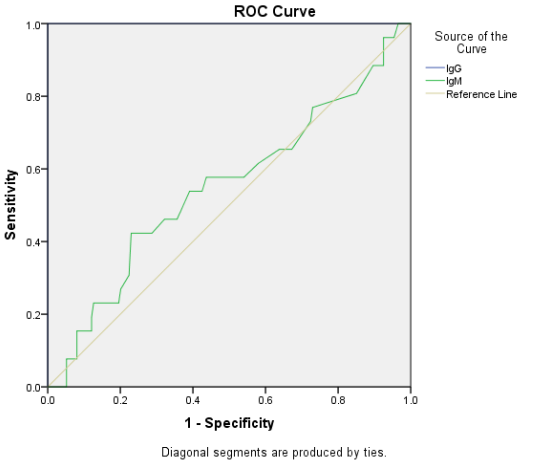

Figure [1] ROC Curve that represents sensetivity and specifity of IgG and IgM antibodies by using PCR molecular diagnosis test.

DISCUSSION

In current study out of 200 samples, the overall seroprevalence of anti-T. gondiiantibodies in women was 13%. A significant relation between non pregnant and pregnant women for IgG ($P<0.05$), the majority of IgG positive cases were observed in non-pregnant, as well as there were significant differences ($P<0.05$) between types of Toxoplasma antibodies for each non-pregnant and pregnant groups whereas the healthy women were 0% for all antibodies by ELISA technique. Although, in present study the highest seroprevalence was in anti- T. gondii IgG, this is due to that in T. gondii infection the maturation of antibody (T. gondii IgG) in response to infection generally are slow.

However, seroprevalence of toxoplasmosis in the present study was similar to many studies around the world and some Arabic countries in women with Bad Obstetric History (BOH), such as 44.8% 17, 35.1% 18, 41.9% 19 and 49.52% 20 (1.12.14,22, 23).

Although, the result of present study is agreement with other studies in Iraq, that deal with a seropositivity by using ELISA were 37.5% for IgG in Erbil. While, in Diyala province found high infection were 37 (46.26%) women out of eighty were infected with toxoplasmosis. A recent study in Iraq showed a different result than present study in which the ratio of infected female with toxoplasmosis was 330 (98.51%).

The present study revealed low anti- IgM in pregnant women, this finding is similar to the results of a study in Salah- Adden among pregnant women, the acute infection was 7(3.1%) of cases24 and in Zanjan, Northwest of Iran 38.6% (IgM and IgG were positive in 1.4% and 37.2% respectively).

The variation in the prevalence rates of T. gondii among studies can be attributed to the differences in the study populations and study areas, as well as the different diagnostic methods used for blood examination. Also, the differences in toxoplasmosis rates can be interpreted on the basis of similarity or differences of climatic conditions of location and that the various ages of patient's study, nutritional and immune status, hygienic habits, sanitary supplies and socioeconomic conditions in study's region. In general, the prevalence of protozoans was strongly associated with a variety of risky factors including host, sociodemographic environmental and zoonotic transmission 24,25.26.27.28.29).

CONCLUSION:

The findings of this study demonstrated a relatively lower prevalence of molecular-seropositivity than studies reported from other countries. Existence of disease in rural areas with contact with of cats and animal at homes and farms, consumption of undercooked meat and unpasteurized milk were identified as risk factors for T. gondii infection (31,32,33). Therefore, a health education program to increase the mother's knowledge about toxoplasmosis towards the source of infection, modes of transmission and prevention methods by avoiding

eating raw or undercooked meat, contact with cats and consumption of unpasteurized milk during pregnancy is recommended. Encourage the laboratories to implement Real time PCR as golden standard test for Toxoplasma Gondii (34.35.36,37). Advocating for the implementation of control and preventive measures and routine screening services for T. gondii infection to be integrated with other ANC services to identify potential effects of the infection and its management should be considered as the main strategy to minimize congenital toxoplasmosis. Furthermore, our results suggested that the implementation of newborn screening and follow-up testing can lead to reducing of toxoplasmosis associated complications (38).

REFERENCES

1. Hill D, Dubey JP. Toxoplasma gondii: Transmission, diagnosis and prevention. Clin Microbiol Infect 2002; 8: 634–640.
2. Abdoli A, Dalimi A, Soltanghoraee H and Ghaffarifar F. Molecular detection and genotypic characterization of Toxoplasma gondii in paraffin-embedded fetoplacental tissues of women with recurrent spontaneous abortion. Int J Fertil Steril. 2017 Jan-Mar; 10(4): 327–336.
3. Liesenfeld O, Montoya JG, Kinney S, Press C, Remington JS. Effect of testing for IgG avidity in the diagnosis of Toxoplasma gondii infection in pregnant women: experience in a US reference laboratory. J Infect Dis. 2001;183(8):1248-53.
4. Al-Hadraawy SK and Hadi FA. Immunological and Molecular Study of Toxoplasma gondii in Al- Najaf Governorate – Iraq. International Journal of Pharmacognosy and Phytochemical Research 2017; 9(4); 482-492 ISSN: 0975-4873
5. Lappalainen M, Hedman K. Serodiagnosis of toxoplasmosis. The impact of measurement of IgG avidity. Ann Ist Super Sanita. 2004;40(1):81-8.
6. Alsaide FM, Elagib AA, El-Rayah IA and Hamad MNM. Immunological and Molecular Diagnosis of T. gondii Infection among Aborted Women in Sana'a Capital and Capital Trusteeship, Yemen. International Journal of Medical Research & Health Sciences, 2019, 8(7): 122-133
7. Baron S. Medical Microbiology. 4th edition. University of Texas Medical, 1996, Galveston, Texas, USA.
8. Jenum PA, Stray-Pedersen B, Melby KK, Kapperud G, Whitelaw A, Eskild A, Eng J. Incidence of Toxoplasma gondii infection in 35,940 pregnant women in Norway and pregnancy outcome for infected women. J Clin Microbiol. 1998 Oct;36(10):2900-6.
9. Wallon M, Franck J, Thulliez P et al. Accuracy of real time polymerase chain reaction for Toxoplasma gondii in amniotic fluid. Obstet Gynecol 2010;115(4):727-33.

10. Caroline P and Mark HY. Toxoplasmosis in pregnanacy: Prevention, screening and treatment. J. Obstet Gynaecol Can 2013; 35(1 esuppl A); S1-S7.
11. Dannemann BR, Vaughan WC, Thulliez P, Remington JS. Differential agglutination test for diagnosis of recently acquired infection with Toxoplasma gondii. J Clin Microbiol. 1990;28(9):1928-33.
12. Darde ML. Genetic analysis of the diversity in Toxoplasma gondii. Ann Ist Super Sanita 2004; 40, 57-63.
13. Darweesh NH , Hussein RA , Salman ST and Shaker MJ. Immunological and Molecular study of Toxoplasma gondii from aborted women in Diyala / Iraq. SJMR, Vol. 2, Issue 6, pp 75-82, Spring 2018
14. Dubey JP, Lindsay DS, Speer CA. Structures of Toxoplasma gondii tachyzoites, bradyzoites and sporozoites and Biology and development of tissue cysts. Clin Microbiol Rev 1998; 11(2):267-299Gajria B, Bahl A, Brestelli J et al. ToxoDB: an integrated Toxoplasma gondii database resource. Nucleic Acids Res 2008; 36, 553-556.
15. El Gamal RL, Selim MA, Mohamed SM, Fathyand GM and Abdel Rahman S. Comparison of PCR with ELISA in Diagnosis of recent Toxoplasmosis in Pregnant women. Journal of American Science 2013;9(12): 824-832.
16. Fabiana Maria Ruiz Lopes et al. Toxoplasma gondii Infection in Pregnancy. BJID 2007; 11 (October) 496-506
17. Falah hasan hadi et al. Comparative study for recurrent aborted women infected with Toxoplasma gondii. J. Pharm. Sci. & Res. Vol. 10(12), 2018, 3268-3272
18. Fatemeh Sadat Ghasemi. Molecular Diagnosis of Toxoplasma gondii in Aborted Women. Jundishapur J Microbiol. 2015 January; 8(1): e15925.
19. Ghoneim NH, Shalaby S, Hassanain NA, Zeedan GS, Soliman YA, Abdalhamed AM. Comparative study between serological and molecular methods for diagnosis of toxoplasmosis in women and small ruminants in Egypt. Foodborne Pathog Dis. 2010;7(1):17-22.
20. Gomez CA, et al. Dual-target, real-time PCR for the diagnosis of intraocular Toxoplasma gondii infections. Br J Ophthalmol 2019; 103:569–572.
21. Grigg ME, Dubey JP, Nussenblatt RB. Ocular toxoplasmosis: lessons from Brazil. Am J Ophthalmol. 2015;159(6):999-1001.
22. Hadi HS, Kadhim RA, Al-Mammori RT. Seroepidemiological aspects for Toxoplasma gondii infection in women of Qadisiyah province, Iraq. International Journal of PharmTech Research, 2016,9(11): 252-259.
23. Hashoosh DA and Majeed A. Comparison of two assays in the diagnosis of toxoplasmosis: immunological and molecular. Eastern Mediterranean Health Journal 2014;20;1:46-50.
24. Hassanain ME, El Bolaky HA , Younis AI , Abu- Sarea EY , Abd El-Razik KA, El Fadaly HA, Abd El Wahab WM. Serological and molecular diagnosis of T. gondii in complicated pregnant Egyptian women. Basic Research Journal of Medicine and Clinical Sciences 2015; 4(9):231-236.
25. Herrmann DC, Pantchev N, Vrhovec MG et al. Atypical Toxoplasma gondii genotypes identified in oocysts shed by cats in Germany. International Journal for Parasitology 2010; 40, 285-292.

26. Howe DK and Sibley LD. Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease. J Infect Dis 1995; 172,1561-1566.
27. Ibrahim HM, Mohamed AH2, El-Sharaawy AA, El-Shqanqery HE. Molecular and serological prevalence of Toxoplasma gondii in pregnant women and sheep in Egypt. Asian Pac J Trop Med. 2017;10(10):996-1001.
28. Khan A, Taylor S, Su C et al. Composite genome map and recombination parameters derived from three archetypal lineages of Toxoplasma gondii. Nucleic Acids Res 2005; 33, 2980-2992.
29. Kheirandish F. Toxoplasma Serology Status and Risk of Miscarriage, A Case-Control Study among Women with A History of Spontaneous Abortion. Royan Institute, International Journal of Fertility and Sterility Vol 13, No 3, October-December 2019, Pages: 184-189
30. Kwofie KD (2012). Risk of mother-to-child transmission of Toxoplasma gondii infection among pregnant women in the Greater Accra region. M.Phil dissertation, Department of Clinical Microbiology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. pp. 34-39.
31. Lindsay DS and Dubey JP. Long-term survival of Toxoplasma gondii sporulated oocysts in seawater. J Parasitol2009;95,1019-1020.
32. LuftBJ and Remington JS. Toxoplasmic encephalitis in AIDS. Clin Infect Dis 1992; 15, 211-222.
33. Mercier A, Devillard S, Ngoubangoye B et al. Additional haplogroups of Toxoplasma gondii out of Africa: population structure and mouse-virulence of strains from Gabon. PLoS Negl Trop Dis 2010; 4, e876.
34. Montoya JG, Liesenfeld O. Toxoplasmosis. Lancet. 2004;363(9425):1965-76.
35. Paola DI CARLO et al. Investigation and management of Toxoplasma gondii infection in pregnancy and infancy: a prospective study. Acta Pharmacologica Sinica (2011) 32: 1063–1070
36. Saki J, Mohammadpour N, Moramezi F, and Khademvatan S. Seroprevalence of Toxoplasma gondii in Women Who Have Aborted in Comparison with the Women with Normal Delivery in Ahvaz, Southwest of Iran. Hindawi Publishing Corporation, The Scientific World Journal Volume 2015, Article ID 764369, 4 pages
37. Villard et al. Serological diagnosis of Toxoplasma gondii infection; Recommendations from the French National Reference Center for Toxoplasmosis. Diagnostic Microbiology and Infectious Disease 84 (2016) 22–33
38. Boyer KM,Holfels E, Roizen N, Swisher C, Mack D, Remington J, et al. Risk factors for Toxoplasma gondii infection in mothers of infants with congenital toxoplasmosis: Implications for prenatal management and screening. Am J Obstet Gynecol. 2005;192(2):564-71.
39. www.neb.com accessed 2020 Dec, 13