Evaluation of Oxidative Stress in Molecular Diagnostic Patients with *Toxoplasma gondii* in A Najaf province

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ABSTRACT

Toxoplasmosis is caused by infection with Toxoplasma gondii. This coccidian protozoan is normally transmitted to humans by ingestion of either oocysts from cat feces, or by tissue cysts in raw or undercooked meat It is one of the most important problems in immunocompromised individuals. The frequency of primary maternal toxoplasma infection depends on the proportion of seronegative pregnant women who are susceptible to infection and on the prevailing infection risk. In the study you mentioned, the researchers likely used molecular diagnostic techniques to evaluate the presence of T. gondii in patients in Najaf province. They may have also assessed oxidative stress in these patients, which is a type of cellular damage caused by the production of reactive oxygen species (ROS). Elevated levels of ROS can cause damage to DNA, proteins, and lipids, and may be associated with the development of various diseases. The researchers may have measured markers of oxidative stress, such as certain enzymes or biomolecules, in order to understand the potential relationship between T. gondii infection and oxidative stress.

Keywords: Toxoplasmosis, Toxoplasma gondii, Coccidian protozoan, Oxidative stress and Molecular diagnostic techniques

Introduction: -

Toxoplasma gondii was first discovered in an African desert rodent, Ctenodactylids gundi, in 1908 by Nicolle and manteaux (Dubey and Beattie, 1988) and described the same year by Samuel T. Darling (De la Cruz, 1989). The name was derived from the Greek toxon, bow or arc, alluding to lunate shape (Frenkel, 1973). It was then found in many species of mammals and birds worldwide and also in humans. In 1923, Janku described T. gondii in the retina of a hydrocephalic child, but the role of the parasite as a human pathogen was not widely known until Wolf and Cowen reported congenital T. gondii infection in man. Their report stimulated considerable interest in human toxoplasmosis. Pinkerton and Weinman reported the first known cases of fatal

toxoplasmosis in adult human patients. The development of the dye test by Sabin and Feldman was and still is the key to much of our present knowledge of toxoplasmosis (Dubey and Beattie, 1988). In 1970, the life cycle of T. gondii was first described. Until then Isospora species were considered parasites of carnivores (dogs, cats) and birds and were thought not to be host specific. T. gondii, was first known to parasitize extraintestinal tissues of virtually all warm-blooded hosts, but then was found to be an intestinal coccidia of cats and to have in its life cycle an isosporan-like oocyst. Further life cycle studies indicated that some of the Isospora species that had been considered to have only an intestinal cycle also had stages in extraintestinal tissues (Dubey, 1993). The knowledge of T. gondii life cycle was completed by the finding of the sexual phase of the parasite in the small intestine of the cat. Toxoplasma gondii oocysts, the product of the merogony and gametogonia, were found in cat feces and characterized morphologically and biologically (Dubey et al., 1970a) In 1977, an outbreak of toxoplasmosis involved patrons of a riding stable in Atlanta (Teutsch et al., 1979).

The worldwide prevalence of toxoplasma IgG antibody among the pregnant population or among females of reproductive age ranges from 1 to 84%. The highest prevalence has been reported in Africa, South East Asia and Central America with the lowest in Japan, Korea and the northern parts of Scandinavia (Pappas et al., 2009). Toxoplasma infection can be diagnosed by serologic tests, amplification of specific nucleic acid sequences, histologic demonstration of the parasite and/or its antigens, or by isolation of the organism (Remington et al., 2001). Toxoplasma infection can be diagnosed by serologic tests, amplification of specific nucleic acid sequences, histologic demonstration of the parasite and/or its antigens, or by isolation of the organism (Remington et al., 2001).

Oxidative Stress Oxidative stress may occur if the production of ROS exceeds the antioxidant capacity of the cell (Jacot et al.,2013). It is assumed that the malondialdehyde (MDA) arising from the lipid peroxidation is an indicator of the oxidative stress in tissue and cells. Lipid peroxidase is a derivative enzyme of feeble unsaturated fatty acid which is produced as a result of decomposition of a set of complex components (Koltas,2005). Malondialdehyde has been shown to be produced by reaction of hydroxyl radicals with deoxyribose moieties (Patton et al.,2006) . High reactivity of this molecule mainly based on its electrophilicity production it strongly reactive toward nucleophiles, such as basic amino acid residues (i.e., lysine and histidine). This reactivity is not only based on MDA's aldehydic nature but is also influenced by its 1,3-dialdehydic structure making it possible to form mesomerically stabilized Schiff base(Kijlstra and Jongert,2008). Also it is react with protein such as collagen. In addition to that the MDAproduct can cause the cross-linkage of membrane elements by affecting the ion exchange from cell membranes(Granseth and Norsk,2012) , which gives way to aftermaths including a change in ion permeability and enzyme activity (Norway,2009) It is assumed that the malondialdehyde (MDA) arising from the lipid peroxidation is an indicator of the oxidative stress in tissue and cells. Lipid peroxidase is a derivative enzyme of feeble unsaturated fatty acid which is produced as a result of decomposition of a set of complex components (Koltas,2005).

Materials and Methods

This study was carried out, during the period from June 2018 to February 2019., in the laboratories of Pediatric and Maternal Hospitals of Al-Najaf, provinces Al-Zahraa Hospital in Najaf , and ((PCR tests)) Molecular study) in the Department of Laboratory Investigation in the Faculty of Science – Kufa University

Study Groups

Five ml was the total blood collected from each clinical suspected woman with *T.gondii* infection and non-suspected women (as control group) by disposable syringe, 2.5 ml of blood kept at room temperature for 30 minutes. The collection of samples was approved by the Institutional Ethics Committee of the Faculty of Science at the University of Kufa and all participants signed informed consent forms.

1. Patients Group: including 62 women aged 15-50 years, admitted to pediatric ward of

Pediatric and Maternal Hospitals of Al- Najaf, provinces. Most cases had clinical manifestation pregnancy complication is fetal loss and can be defined as two or more failed pregnancies.

2. Control group: consists from 5 healthy women all were with no history of parasitic infection, and without clinical manifestation of any disease.

A. Molecular Study

DNA Extraction Kit:

It is a product of Favogene corporation blood DNA Extraction Kit Cat. FABGK 001

The primers

Identification of *E. histolytica* and exclusion of *E. dispar* were verified by PCR using the following two sets of specific primers from a noncoding short tandem repeat (STR) in locus DA, also known as locus 1-2:

Forward Primer: TOXO-F (5-TCCCCTCTGCTGGCGAAAAGT-3)

Reverse Primer:TOXO-R (5-AGCGTTCGTGGTCAACTATCGATTG-3)

Reaction Mixture:

It is a product of AccuPower® TLA PCR PreMix, Bioneer Corporation,USA. Cat. No. K-3201. It contains 0.2ml thin-wall 8-strip tubes with attached cap / 96 tubes.

Agarose:

Norgen Biotek Corp. Canada. Agarose-Biotechnology Grade. Cat. No. 28034. Kit Size 100 g.

TE Buffer:

Promega Corporation USA. TE Buffer, 1X, Molecular Biology Grade (pH 8.0). Cat. No. V6231. Kit Size 100ml.

TBE Buffer:

Promega Corporation USA. TBE Buffer, 10X, Molecular Biology Grade (pH 8.3). Cat. No. V4251. Kit Size 1,000ml.

Ethidium Bromide:

Promega Corporation USA. Ethidium Bromide Solution, Molecular Grade. Cat. No. H5041. Kit Size 10ml. Conc. 10mg/ml.

DNA Markers

1- DNA ladder: Fermentas International Inc. Canada. GeneRuler™ 100 bp DNA Ladder, 100-1000 bp. Cat. No. SM0243. Kit Size 50 µl (0.1 µg/µl).
2- DNA Loading Dye: Fermentas International Inc.Canada. 6X DNA Loading Dye. Cat. No. SM0241. Kit Size 1.00 ml.

Stages in PCR Procedure

I. Extraction of DNA Template

Transfer up to 200 µl sample (whole blood, buffy coat) to a micropcentrifuge tube (not provided). ·If the sample volume is less than 200 µl, add the appropriate volume of PBS. If RNA-free genomic DNA is required, add 4 of 100 mg/ml RNase A to the sample and incubate for 2 minutes at room temperature. Add 20 Proteinase K and 200 µl FABG Buffer to the sample. Mix thoroughly by pulse-vortexing . Do not add Proteinase K directly to FABG Buffer. Incubate at 60 for 15 minutes to lyse the sample. During incubation, vortex the sample every 3 - 5 minutes 0^{c}. Briefly spin the tube to remove drops from the inside of the lid. Add 200 µl ethanol (96~100%) to the sample. Mix thoroughly by pulse-vortexing for 30 seconds. Briefly spin the tube to remove drops from the inside of the lid. Place a FABG Column to a collection tube. Transfer the sample mixture (including any precipitate) carefully to FABG Column. Centrifuge for 1 minute and discard the flow-through then place FABG Column to a new Collection tube. Immediately, Wash FABG Column with 500 µl W1 Buffer by centrifugefor 1 minute then discard the flow-through. Make sure that ethanol has been added into W1 Buffer when first open. Wash FABG Column with 750 µl Wash Buffer by centrifuge for 1 minute then discard the flow-through. Make sure that ethanol has been added into Wash Buffer when first open. Centrifuge for an additional 3 min to dry the column. This step will avoid the residual liquid to inhibit subsequent enzymatic reactions. ·Important Step!. Place FABG Column to Elution Tube. Add 100~200 µl of Elution Buffer or ddH O (pH 7.5~9.0) to the membrane center of FABG Column. Stand FAGB Column for 3 min. For effective elution, make sure that the elution solution is dispensed onto the membrane center and is absorbed completely. Standard volume for elution is 200 µl, If sample has low number of cells, reduce the elution volume (50µl - 150µl) to increase DNA concert ration. Centrifuge for 2 min to elute

the DNA . Store the DNA fragment at 4°C or -20°C.

Amplification

Amplify 115 bp fragments for T.gondii. Amplification consisted of 35 cycles of 1 min at 93 °C, 30 seconds at 58 °C, and 60 seconds at 72 °C, with a final extension of 5 min at 72 °C. The PCR products were isolated by electrophoresis.

Electrophoresis and Visualization

The gel electrophoresis method was done according to Sambrook and Russell (2001) as the following:

1. Preparing the Gel

A 1% agarose gel was made by mixing 1g agarose with 90 ml distilled water and 10 ml 10X TBE buffer. The mixture was heated in a microwave oven until all the agarose powder was melted and the solution started to boil. The gel solution was then left to cool (to approximately 65 °C). 5µl of ethidium bromide solution (10mg/ml) was added after the mixture was cooled, and gently mixed. The gel was poured slowly into a gel rack, the comb was set at one side of the gel, and any bubbles in the solution removed. The gel was allowed to set (20 to 30 minutes). After 20 minutes, when the gel had solidified, the comb was removed, and the gel, together with the rack, was soaked into a chamber with 1X TBE gel running buffer. The gel was placed with the wells facing the electrode that provide the negative current.

2. Loading and Running the Gel

A DNA ladder was loaded into the first well. This was used to determine the absolute size of the separated DNA strand by comparing their migration with that of the ladder. The samples were loaded into the wells (5 µl for each) without adding loading dye. The lid of the electrophoresis chamber was closed and the current was applied. The gel was run at 70 volts for 1hour. The ethidium bromide stained gel was visualized under UV light and photographed.

B. Malondialdehyde (MDA) Assay

It is a product of Elabscience corporation blood Malondialdehyde Kit Cat.No.E-BC-K025. Pretreatment of sample Collect the cells into a centrifuge tube, add 0.5 mL Reagent 5 (extraction solution), mix fully for 2 min, then treat the cell with sonication or homogenization. Meanwhile, determine the protein concentration of supernatant (E-BC-K318, E-BC-K168, E-BC-K165)

Result and Discussion

1. Distribution of Cases According to Age:

The study population was included 71 women confirmed or clinically suspected Toxoplasma gondii in an Naiaf provinces. Assessment of age presentation of patients revealed that 7 (9.85%) patients were seen in age group (15-20)years , 26 (36.61%) in age group (20-25) years, 12 (16.90%) in age group (25-30) years, 8 (11.26%) in age group (30-35) years, 10 (14.08%) in age group (35-40) years , 7 (9.85%) patients in the age group (40-45) years, and 1 (1.4%) (Figure 4.15). The estimated incidence of Toxoplasmosis increases significantly ($P < 0.05$) in the second age group (20-25) years which contains 36.61% of Toxoplasmosis patients (Figure 3.1).

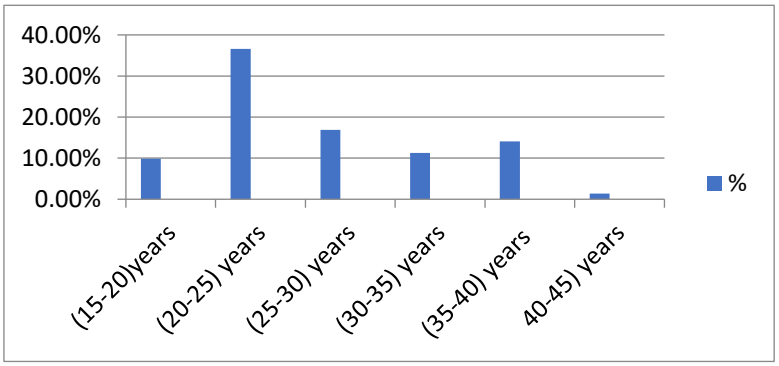

Figure 3.1: The Distribution of Toxoplasmosis Cases According to age.

The most common age group infected with Toxoplasmosis was between (20-25) years, they represented 36.61%, which was higher as compared to other age groups. This result is in agreement many studies. Nijem and Al-Amleh (2018) mentioned that the increase of infection among the urban women married at age < 20 years, and This result is in agreement with Shawky and Milaa(2000) which had recorded that the high incidence rate was between 20 year and 30 year (69.64%). He suggested that pregnant women are at higher risk probably because of low immunity.

2. Molecular Study

Toxoplasma gondii DNA was detected in 30 (42.25%) patients suspected of having the disease but was not detected in any healthy control (Figure 3.2), while 41 (57.74%) cases were negative (Table 3.1).

Table 3.1: The Results of the PCR Data:

Subjects	**PCR results**		**Total**
	Positive	Negative	
Healthy controls	0	5 100%*	5 6.58%
Disease suspects	30 42.25%	41 57.74	71 93.42%
Total	**30** **39.48%**	**46** **60.52 %***	**76** **100%**

***P < 0.05 significant**

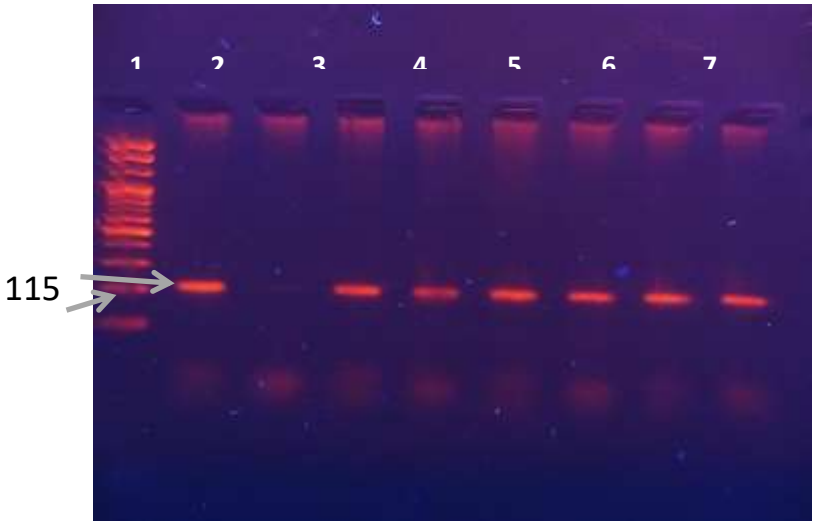

Figure 3.2: PCR Amplified 155 bp of *Toxoplasma gondii* DNA Gene, 1: (50 bp) DNA Ladder, 3: Control, 2,4-9: *Toxoplasma gondii* DNA.

In this study, we evaluated the use of PCR in detecting Toxoplasma parasite in blood his finding correlated with the natural course of primary infection, where the parasite

disseminates through bloodstream, causing transient parasitemia before they localize in various tissues. However, the appearance of blood parasitemia in primary infection differs among parasite strains and routes of infection. (Weiss et al.,2018). These findings illustrate that blood PCR is more helpful in the early diagnosis of toxoplasmosis, as has been indicated by other investigators(Dupouy-Camet et al.,2013; Filice et al.,2016). This result is in agreement with Silveira et al. (2011). which had recorded PCR sensitivity was poor, 8% positive blood samples and negative blood samples 25%.

3. Oxidative stress Study

This study showed that T. gondii infection exert significant effects on erythrocyte MDA concentrations.

Table 3. 2: Descriptive statistics of malondialdehyde seropositive patients and healthy controls.

Parameters	Control	Infected	P value
MDA (nmol/g Hb)	119.76±17.09	198.89±20.30*	P<0.05

Lipid peroxidation, which generates free radical species, plays a role in the pathogenesis of many parasitic and protozoan infections. Lipid peroxidation, which generates free radical species, plays a role in the pathogenesis of many parasitic and protozoan infections (Asri-Rezaei and alir-Naghadeh,2006 ; Matanovic et al.2007). Lipid peroxidation, as determined by erythrocyte MDA concentrations in the present study, was significantly increased due to toxoplasma infection. This may have resulted from the excess production of free radicals and oxidants following infection. Free radicals can react with DNA, resulting in genetic mutations or cytotoxicity. They also can bind to erythrocyte membranes, which are rich in polyunsaturated fatty acids (Fisher, 2003), thereby causing membrane destruction and cellular damage (Gilbert et al., 2018). Karaman et al. 2008 reported that serum MDA concentrations were significantly increased in T. gondii-infected humans, and Yang et al. (50) showed that serum concentrations of free oxygen radicals (NO, -OH, O2-) increased in T. gondii-infected mice. Our results are in accordance with Karaman et al. 2008, which suggested that erythrocyte MDA concentrations were significantly increased by T. gondii infection. In contrast, Engin et al. ,2012 found no change in serum MDA concentrations of mice infected with T.gondii. Preventing lipid peroxidation is essential in biological systems. To counteract the harmful effects of ROS, all oxygen-metabolizing cells are equipped with cellular antioxidant defense systems (Akkus , 1995). Superoxide dismutase is an important physiological antioxidant defense mechanism in aerobic organism. This enzyme prevents the formation of the hydroxyl radical by detoxifiying hydrogen peroxide (Halliwell and Chirico ,2014). The decrease in SOD activity observed in our study may be related to the increasing the severity of parasitemia and oxidative stress (Asri-Rezaei and alir-Naghadeh,2006).

Conclusion:

In conclusion, the parasitic infections are associated with significant degree of free radicals formation as indicated by significantly higher MDA. Toxoplasma gondii had high prevalence and unusual presentation by affecting high proportion 20-25 severe clinical manifestations, and laboratory findings that were known to be usually encountered in invasive Toxoplasma gondii

Recommendations:

Use real time PCR for the diagnosis of Toxoplasma gondii. The oxidative stress can be used to study the pathogenesis of Toxoplasma gondii.

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