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Investigation of the role of TLR-2 and TLR-4 immune cytokines and study of some blood and immune markers related to Toxoplasmosis disease infection

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Abstract:

In a study at the Women and Children's Hospital in Ramadi, Iraq, 68 cases of *Toxoplasma gondii* infection were identified from 88 female patients, representing 77.27% of the total sample, while 20 samples (22.73%) were non-infected controls. The 26-35 age group showed the highest infection rate (40.90%), whereas the 36-45 group had the lowest (13.63%). Infection prevalence was notably higher in rural areas (48.86%) compared to urban areas (28.41%). Physiologically, infected individuals exhibited elevated white blood cell counts (8.66 ± 0.26) compared to controls (8.17 ± 0.35). Furthermore, counts of red blood cells, packed blood cells, and platelets were significantly elevated in the infected group (12.86 ± 0.45), with a marked decrease in hemoglobin (HGB) levels ($P \leq 0.05$). Immune responses revealed significantly increased concentrations of Toll-like receptors (TLR-2 and TLR-4) in infected patients. TLR-2 levels averaged 0.2313 ± 0.01309 in infected individuals, compared to 0.1858 ± 0.00882 in controls, while TLR-4 levels were 0.2235 ± 0.007478 in infected patients versus 0.2012 ± 0.008517 in controls, highlighting the immune system's role in response to infection.

Keywords: *Toxoplasma gondii*, infection rate, immune response, Toll-like receptors, rural-urban differences

Introduction:

A dangerous zoonotic illness that affects both people and animals is toxoplasmosis. This condition is brought on by infection with Toxoplasma gondii, an intracellular parasite. It is prevalent in many homoothermic species and affects a significant portion of human populations worldwide. The only required definitive hosts for it are cats and other members of the feline family. The infection is asymptomatic in a host with good immune efficiency, as the host's immune system is able to stop the parasite from multiplying and forming tissue cysts in most body tissues, which are highly concentrated in the central nervous system, cardiac muscles, and skeletal muscles, without any symptoms appearing in most cases [1]–[4].Toxoplasmosis lacks well-defined symptoms; however, it can be diagnosed using a variety of techniques. The most popular method is serological immunological testing; other

techniques involve removing the parasite from bodily fluids such as blood, amniotic fluid, spinal fluid, and tissues like the brain and placenta after injecting it into lab mice and tissue cultures [5], [6].The parasite elicits a strong innate immune response in the infected host followed by strong adaptive immunity. In the past few years, the importance of innate immune mechanisms has been identified, which depend on the role of specific independent pathways and the role of inflammatory monocytes and innate lymphocytes, as innate immunity is considered the first line of defense against invading pathogens [7]. Part of this early response is mediated by pathogen recognition receptors such as Toll-like receptors (TLRs), C-type lectins, Nod-like receptors and other receptors important against the Toxoplasma parasite [8]. Once inside the human body, the parasite divides and hides from the immune system by creating a parasitic gap inside the cells. This allows the parasite to spread throughout the body and eventually reach the fetus, where it may cause congenital or post-natal deformities or even abort the child. Interleukins 1 and 2, natural killer cells, and dendritic cells—which aid in the development of CD4+ and CD8+ T cells—are produced by the phagocytic cells in response to infection.

These processes will stimulate other immune cells to produce interleukins 6 and 12, which stimulate other immune cells to manufacture and release interferon gamma (INF-gamma), which helps stimulate the production of some cytokines such as TLR2 and Toll Like Receptor 4, which determine the (glycosyl phosphatidylinositol (GPI) pathway and stimulate the (immunity related GTPases (IRGs) pathway caused by the release of IFN-γ and contribute to the removal of ***T. gondii*** in the body's cell types and contribute to the dissolution of the parasite's vacuole envelope, which helps the parasite reproduce. Therefore, removing the vacuole envelope will help eliminate the parasite and help stop its reproduction and stop its immune evasion from the body [9],[12].

The purpose of this study was to determine the level of the relationship between TLR-2 and 4 and some other parameters when infected with toxoplasmosis, in light of the increased incidence of toxoplasmosis in women, the risk it poses to the fetus's life, and the difficulties it causes the pregnant mother.

Material and Methods:

This research was carried out on eighty-eight women between the dates of 18\7\2023 and 8\2\2024, twenty of whom were in the control group and sixty of whom were infected with the ***T. gondii*** parasite. The other sixty-eight women were healthy. During a one-on-one interview, each woman provided personal information about herself, including her name, age, place of residence, date of marriage, educational attainment, number of children born, number of miscarriages, type of pregnancy treatment, and frequency of chronic conditions like high blood pressure, diabetes, and asthma.
Sterile medical syringe with a (10) ml capacity was used to extract blood samples from the vein. After dividing the blood into two parts, 8 ml of the serum was transferred into sterile test tubes devoid of any anticoagulant. The serum was then incubated at 37 C for 30 minutes until clotting occurred. The serum was then separated using a centrifuge running at 3000 rpm for 10 minutes. The resulting serum was then transferred into eight Eppendorf tubes and kept at -20 C until it was needed for the required tests.The other part of the blood, 2 ml, was

placed in tubes containing an anticoagulant Ethylene Diamine Tetra Acetic acid (EDTA) and used to calculate the total and differential white blood cell count. The parasite was diagnosed using the Torch cassette method at the Women's and Children's Hospital in Ramadi City / Anbar Governorate / Iraq. This cassette can detect four tests, including Toxoplasma, Cytomegalovirus, Rubella virus, and Herpes virus, to exclude samples infected with other than Toxoplasma, in addition to the Latex test with a single test. To further confirm, the samples were diagnosed using the ELISA method by taking the Serum containing the Antibody and the ELISA kit containing the antigen, according to [13].The concentration of Human Toll-like Receptor 2 and 4 was measured according to the measurement kit (kit) used by SUNLONG BIOTECH CO.,LTD for the year 2022_2023 and according to the sandwich-elisa method, and the work was done according to the recommendations of the kit manufacturer. The data were analyzed using the SPSS statistical program.

Results:

Serological tests of *T. Gondi:*

For all research groups, the latex test was used as a quick diagnostic technique; the findings are listed in Table (1). Seventy samples from the toxoplasmosis infection group tested positive for the disease, whereas the eighteen samples from the control group tested negative. This confirms the effectiveness of this test for diagnosis, as it reflects the actual positive diagnosis for people infected with toxoplasmosis at a rate of 79.55% compared to those not infected at a rate of 20.45%, while it confirms the effectiveness of the ELISA test for diagnosis, as it reflects the positive responses for people infected with toxoplasmosis, which numbered 68 people at a rate of 77.27% compared to 42 uninfected people at a rate of 22.73%.

Table (1) Latex and ELISA test results and their percentages for the study groups

Total NO	Analysis method	positive	%	negative	%
	cassette	70	79.55	18	20.45
	ELIZA	68	77.27	20	22.73

Infection according the age

Based on the results of our current study, the age group that was most affected and infected was 26–35 years old. The percentage of infection in this group was 40.90%, compared to 9.09% in the control group. The age group that had the lowest percentage of infection was 36–45 years old, with 13.63% compared to 5.68% in the control group. (Table II).

Table (2) shows the relationship between age group and Toxoplasma gondii diseas e.

Age	Examined case	%
(16-25)	Infected (20)	22.72

	Control (7)	**7.95**
(26-35)	**Infected (36)**	**40.90**
	Control (8)	**9.09**
(36-45)	**Infected (12)**	**13.63**
	Control (5)	**5.68**

Variations according to residential area:

According to the results of our current study, there are disparities in infection rates between rural and urban areas. The infection rate in the countryside was greater than the infection rate in the city, reaching 48.86% in the former and 28.41%) in the latter.

Table 3 shows the association between the residential area and *Toxoplasma gondii* infections and non-infections.

residential area	**Positive samples**		**negative samples**		**Total**	
Rural	**no**	**%**	**no**	**%**	**no**	**%**
	43	48.86	12	**13.63**	**55**	**62.5**
City	25	28.41	8	**9.10**	**33**	**37.5**
Total	**68**	**77.27**	**20**	**22.72**	**88**	**100**

Physiological blood tests:

Our study's findings demonstrated a rise in white blood cell values: those infected with the parasite had values of 8.66 ± 0.26 compared to 8.17 ± 0.35 for the control group, and there were no appreciable differences in the treatments given to the infected and control groups (Table 4).

Table (4) shows the changes in white blood cells of infected and control patients.

parameters	Mean ±SE		T-test	P-value
	Control	unfected		
WBC (x10^3)	8.17 ±0.35	8.66 ±0.26	1.089 NS	0.370

Lymphocyte (x10^3)	2.455 ±0.13	2.720S ±0.16	0.442 NS	0.231
Monocyte (x10^3)	0.575 ±0.04	0.500 ±0.03	0.140 NS	0.283
Granulosa (x10^3)	5.13 ±0.27	5.30 ±0.40	0.973 NS	0.723
Lymphocyte (%)	30.36 ±1.30	33.29 ±2.90	5.613 NS	0.294
Monocyte (%)	7.13 ±0.47	5.90 ±0.23	1.438 NS	0.0498
Granulosa (%)	62.50 ±1.39	60.80 ±2.67	5.562	0.535
* (P≤0.05), NS: Non-Significant.				

Table (5) shows the blood variables of the infected and the control.

Variables	Mean ±SE		T-test	P-value
	Control	infected		
RBC (x10^6)	4.14 ±0.07	4.36 ±0.03	0.221 *	0.0482
HGB (g/dl)	12.86 ±0.45	11.55 ±0.28	1.374 *	0.050
HCT	33.35 ±0.60	38.90 ±0.20	1.785 **	0.0001
PLT (x10^6)	315.75 ±10.58	381.00 ±43.00	58.54 *	0.050
* (P≤0.05), ** (P≤0.01).				

Our study's findings demonstrated a significant decrease in hemoglobin B (HGB) concentration at the probability level of P≤0.05. The results of the parasite-infected group were 11.55 ± 0.28, while the control group's results were 12.86 ± 0.45.

Cytokines test (TLR-2 and TLR-4)

88 samples were tested, including 68 samples infected with toxoplasmosis and 20 control samples, and the concentrations of some immune cytokines related to infection were detected, which included Toll Like Receptor - 2 and 4. After statistical analysis, it became clear to us that there was a clear significant increase in both factors between infected and uninfected women, as the results of TLR-2 for infected women were 0.2313±0.01309 and the results of

TLR-4 for infected women were 0.2235±0.007478 compared to the control 0.1858±0.00882 and 0.2012±0.008517, respectively. Table (6).

Table 6 the level of TLR-2 and TLR-4

p-value	Test satistic	Patients			Healthy control			Parameter
		SEM	SD	Mean	SEM	SD	Mean	
0.005	2.88	0.01309	0.10795	0.2313	0.00882	0.03946	0.1858	TLR2
0.04	1.97	0.007478	0.06167	0.2235	0.008517	0.03809	0.2012	TLR4

Discussions

The results of our current study as shown in tabl(1)This result was close to what was reached by [14]. in Egypt when using the Elisa test, and [15] in Baghdad and [16] in Erbil and less than the rates recorded in [17]) in Najaf Governorate with an infection rate of 76%, 84%, 80% 77% 90% respectively, and less than what was recorded by [18] in Sulaymaniyah with an infection rate of 47.7%. Many studies have indicated that the latex agglutination test is used to detect antibodies to the parasite in survey studies due to its ease of implementation, reasonable cost, and the little effort and time required to perform it [19].

The reason for the difference in infection rates between the LATex and ELISA tests could be attributed to a variety of factors, including biological factors like common antigenicity caused by the presence of non-specific antibodies or technical factors depending on the test type used. However, the ELISA test is more accurate than the cassette test due to the presence of a false positive in the cassette test [20] Perhaps the geographic location and environmental factors of each nation also play a significant role in the variation in infection rates between governorates and neighboring countries. As the infection rate rises in warm, humid regions and is higher than in dry regions, these factors may also account for the variation in infection rates between governorates and neighboring countries. Also, the increase in rainfall and the availability of moisture in some areas encourages the maturation and sprouting of egg sacs while they remain in the soil, maintaining their vitality for a long time, which leads to increased transmission and exposure to infection in those areas, in addition to the difference in the habits of eating undercooked meat in some areas, as well as the increase in dealing with cats, which is the main cause of infection with toxoplasmosis [14].

The results of our current study as shown in table(2) are consistent with [21], which showed that the highest rate of infection with toxoplasmosis was within the age group (26-35 years), in addition to the convergence of the results of our study with the study of [22], which indicated that the highest rate of infection was within the age group (20-31 years).

While our study's results were inconsistent with those of [23], who suggested that the age group of 36–45 years had a higher percentage of infected women than the age group of 26–35 years, our results also did not align with those of [24], who suggested that the age group of 30-41 years had the highest percentage of infected women. the reason for the difference in results or the consistency with researchers in toxoplasmosis infection within different age groups may be due to the difference in social and cultural conditions from one place to another [25]. Alternatively, hormonal changes during pregnancy may be the cause of the high rate in the age group of 25 to 35 years. These changes result in a decrease in immunity in pregnant women, which raises the risk of infection. Additionally, pregnant women may interact with the home environment more precisely and assiduously [26], as mentioned by [27] The reason for the spread of toxoplasmosis may be due to the fact that societies are mostly multicultural and are characterized by very different living conditions and eating habits. Additionally, it has become common practice to raise pets because this age group is more likely than others to be more susceptible to disease because they are eager to explore their surroundings and interact with them more in order to achieve their basic needs.

As shown in Table (3), our study's findings corroborated those of [23], [28], , who reported that toxoplasmosis infection rates were greater in rural areas than in urban areas.

Our study's findings, however, disagreed with those of [29], [30] in Mosul, [31] in Dohuk, who reported that women residing in urban areas had a higher rate of ***Toxoplasma gondii*** infection than women residing in rural regions.

The high incidence of Toxoplasmosis in rural areas may be due to lack of awareness, livestock herding and frequent contact with them, in addition to drinking water from running streams in those areas, which are originally a place for grazing, settlement, food and drink for domestic and farm animals, in addition to the difference in environmental conditions among people living in the countryside, as well as depending on the type of exposure (contact with animals, types of food consumed, contact with wild areas, sanitation conditions, etc.) [32]

As shown in table (4) the current study's findings corroborated those of studies on toxoplasmosis infections in aborted women by [33] and [34], since both studies showed a rise in the proportion of white blood cells in infected women.

The reason for the increase in the number of white blood cells in women infected with Toxoplasma gondii may be due to the stimulation of the immune response resulting from the presence of the parasite, through which the immune system can be stimulated, which in turn leads to an increase in the production of white blood cells [35] because white blood cells have the ability to perform a high activity in movement and phagocytosis [36]. Our findings, however, did not support the findings of [37] and [38] on the incidence of a reduction in white blood cell (WBC) count in toxoplasmosis infection.

The results of our study for hematological variables in Table (5) agree with the findings of [39] regarding an increase in the concentration of HCT in women infected with toxoplasmosis compared to the control group, as well as the findings of [34] and [38]), who reported an increase in the number of red blood cells (RBC) in women infected with Toxoplasma gondii. [33]) have reported that when infected with the ***T. gondii*** parasite, there is an increase in platelet count (PLT). The increase in platelets may be due to infection with the parasite, as the human body produces platelets at a higher rate and larger size in order to avoid damage and bleeding in the body resulting from the parasite's feeding and attack on cells [39].

While the results of our study do not agree with those of [33] and [40], who indicated in their results that there was a decrease in the number of red blood cells, it is likely that the occurrence of anemia and low Hb may have caused the decrease in the number of red blood cells [41].

Anemia brought on by an increase in interferon gamma levels may be the cause of the drop in red blood cell count [42]. This is because high interferon gamma concentrations disrupt the development and differentiation of blood-forming stem cells. Additionally, the introduction of the parasite Toxoplasma gondii into the body of a living organism triggers an immune response, which in turn raises interferon gamma concentrations [43]. The decrease in the proportion of HCT in toxoplasmosis-affected women was another area where it deviated from [44] findings. He provided an explanation for this, stating that the number of red blood cells in infected women decreases as a result of the smaller packed cells. This is because the percentage of HCT is based on the quantity of red blood cells, and the breakdown of the latter causes hemolysis, which in turn reduces the size of the compact cells. Moreover, low iron causes fewer red blood cells, which in turn causes the compact cells to shrink in size. Furthermore, our study's findings contradict those of [37], who reported a decline in the number of platelet, The reason was explained by the fact that the bleeding that occurs in women who have miscarried and are infected with ***Toxoplasma gondii*** can be the cause of the decrease in the number of platelets (PLT) and red blood cells, which is accompanied by a decrease in the concentration of hemoglobin.

Our findings aligned with those of [40], who also found a decrease in hemoglobin Hb concentration. However, our findings did not support those of [21], who conducted a study among women infected with ***Toxoplasma gondii*** in Tikrit city and reported a decrease in hemoglobin Hb concentration.

These changes in the Hb concentration of infected women are not a cause of anemia, but rather a condition of bleeding that occurs in women who have had miscarriages as a result of infection with Toxoplasma gondii and the occurrence of miscarriage, which leads to the occurrence of anemia, as the breakdown of red blood cells in the infected tissue causes a decrease in the Hb level [45].

The results of Our study's findings as shown in table (6) were in line with those of [46] who found that women infected with Toxoplasma parasite had higher levels of both TLR2 and

TLR4 than the control group [46], also explained that elevated levels of TLR2,4 are important during acute infection with Toxoplasma gondii, and our findings also supported the findings of [47]. who found that women infected with ***Toxoplasma gondii*** had higher levels of TLR2,4 than the control group. while our study did not agree with what was reached by [48], as he indicated in the results of his study that the levels of both TLR2,4 were lower in infected women compared to the control.

Upon infection, phagocytes will begin to produce interleukins IL-1 and IL-2, natural killer cells and dendritic cells that contribute to the production of CD4+ and CD8+ T cells. These processes will stimulate other immune cells to produce interleukin 6 and 12, which stimulates other immune cells to manufacture and release interferon gamma (INF-gamma), which helps stimulate the production of some antioxidants, including NO, and some cytokines represented by TLR2 and TLR4, which determine the glycosyl phosphatidylinositol (GPI) pathway and stimulate the immunity-related GTPases (IRGs) pathway, which is caused by the release of IFN-γ and contributes to the elimination of ***T. gondii*** in all types of body cells.

It contributes to the dissolution of the parasite's vacuole envelope, which helps the parasite reproduce. Therefore, removing the vacuole envelope will help eliminate the parasite, stop its reproduction, and stop its immune evasion from the body. In addition, the release of the above factors helps in the differentiation of the myeloid differentiation primary response gene 88 (MyD88), which encodes a protein essential in the process of modifying TLRs to match the surface receptors of the parasite and invalidating the process of parasite infection of cells and inhibiting it and controlling the infection by releasing another protein called profiling, which alerts the rest of the other cells to the presence of the parasite infection [49]–[52].

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