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IMMUNOLOGICAL IMPACT OF TOLL-LIKE RECEPTOR (TLR 2 AND TLR4) IN PATIENTS INFECTED WITH *E. HISTOLYTICA*

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Abstract

Amoebiasis is the third cause of death worldwide, due to a parasitic infection, has a universal distribution affecting 10 to 20 percent of the world population and in some regions, up to 55 percent, Amoebiasis caused by the protozoan parasite *E. histolytica* was a first recognized as a deadly disease by Hippocrates who described patient with fever and dysentery, in this case- study design include 90 stool specimens were collected from patient with diarrhea attended to hospitals in Hilla city. The resultsshow high levels of TLR-2 in age group (1-10) compared to healthy controls, the increasing significantly ($p<0.05$). also levels of TLR-2 in age group (>40) was increase compared to controls but these results was non-significantly and show high levels of TLR-4 in age group (1-10) compared to healthy controls, the results significantly ($p<0.05$). While the level of TLR-4 was Decreased in age group (>40) compared to controls and the results was not significantly.

Key words: *E.histolytica*, Amoebiasis,TLR-2 , TLR-4

Introduction

Amebiasis remains a worldwide health problem accounting for up to 100,000 deaths yearly. (Lozano *et al*., 2012). Amoebiasis is a gastrointestinal disease, it may appear a symptomaticc or mild, severe symptoms including: abdominal pain, diarrhea, or bloody diarrhea, generally occurs by the ingestion of infected water or food due to fecal excretion of cysts and even fecal -oral transmission within house - hold and during male homosexual activity (Cheepsattayakorn and Cheepsattayakorn, 2014).

Entamoeba histolytica is an unicellular, intestinal protozoan parasite that infects humans, which is the etiological agent of amebiasis, In developing countries, *Entamoeba histolytica* is an important reason of morbidity and mortality in babies (WHO, 2005). Our host immune system sets up a series of defensive responses against the parasite. However, continued morbidity and mortality point out that this parasite is capable of escaping host defense responses to maintain its own survival (Moonah *et al*., 2013).

Toll-like receptors (TLRs) are a class of receptors involved in non-specific immunity, acting as a bridge to link the non-specific and specific immune responses (Husseinzadeh and Davenport, 2014). TLRs are single transmembrane domain-containing non-catalytic proteins that identify and bind conserved molecules from microorganisms (Lim and Staudt, 2013).

TLR-2 and TLR-4 receptors in intestinal cells bind to molecules, on the surface of pathogens denominated PAMPs, triggering signal transduction cascades that up-regulate expression of the TLR and expression of pro-inflammatory cytokines, both elements participating in the innate immune response (Cario *et al.,* 2007).). It has been reported that the LPPG molecule found on the surface of *E. histolytica* trophozoites is recognized by TLR-2 andTLR-4 and that the amoebic Gal/GalNAclectin, also found on the surface of trophozoites, can activate TLR-2 expression and signaling in murine monocytes (Kammanadiminti *et al.,* 2004; Maldonado- Bernal *et al.,* 2005).

Materials and Methods

Ethical approval

The necessary ethical approval was obtained by verbal consent from patients. This study was approved by the committee of publication ethics at college of medicine, Babylon University, Iraq.

Stool samples

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Stool specimens were collected from patients with blood and/or mucus diarrhea during period of October 2019 to march 2020 collected from patient to attended four (hospitals in Hilla city Imam Al_Sadiq Hospital, Babylon Hospital for Maternity and Children , Hospital Al_noor for Children and Teaching Merjan Hospital).

Ninety stool specimens were collected from patients between (1-50) ages. All collected samples were separately labeled with stickers having date, name of sample, name of collecting area. Stool samples were collected using a wide opened stool container and transported in cooler boxes to the lab-oratory, all specimens were properly labelled with patient's code and date of collection. The specimens were transported to the laboratory within 1h of passing of the stool, since amoebic trophozoites die and become unrecognizable after longer periods of time. Precautions were taken to prevent the samples from being contaminated with urine or dirt particles (Samie *et al*., 2020).

Examination of stool samples

The examination were done with direct swab method by taking a drop of Lugol'siod in solution and put on one end of the glass slide and a drop of normal saline put on the other end, took a small amount of stool trace the head of stick and mixed well The smears were covered with cover slides and examined under the microscope using 40× objective lens to detecting protozoan trophozoites and cysts as well as red and white blood cells (Lee *et al.,* 2009).

Blood Sample Collection

Blood samples were collected about (5ml) by disposable syringe from each patient diagnosed with *Entamoeba histolytica.* The blood was put in sterilize tube free from coagulant (Gel tubes), the blood was left in room temperature allow serum to clot for 10-20 minutes. Centrifuge at 2000-3000 rpm for 20 minutes to obtain serum for immunological tests (ELISA). The serum was put in sterile tube (Eppendorf tube), then served in -20C until required.

Statistical Analysis

The statistical analysis to the association study was performed by using a commercially available software program Statistical package for the social science (SPSS), Version 23.0, SPSS, Inc., Chicago, Illinois, USA. The statistical analysis of data had done by : P value less than 0.05 was considered statistically significant.

Results

Total number of *E.histolytica* parasites

The results of this study showed that the total percentage of infection with positive *E.histolytica* was 32/90(35.5%) as show in table 1. The results of the present study was agree with another studies who Showed positive and negative Percentage of parasitic infection. The presence of *Entamoeba histolytica* in Al-Nahda with (33.33%) done by (AL-Khalidy and Jabbar, 2020). Also Al- Masoudi *et al*., (2018) and Haque (2003) confirm that 20% of diarrhea cases associated with *E.histolytica*.

Table 1: The total number of E.histolytica parasites.

Stool samples	E. histolytica	
	Number	percentage
PositiveResults	32	35.5%
Negative Results	58	64.4%
Total	90	100%

Distribution of *E.histolytica* according to the type of diarrhea

In the current study, the highest rate of infections show in diarrhea that caused by *E.histolytica* 18/ 32(56.25%) who were suffering from bloody diarrhea and only 14/32(43.75%) cases were with watery diarrhea as show in table 2.

Table 2: Distribution of cases according to the type of Diarrhea.

Type of Diarrhea	*E.histolytica*	
	Number	percentage
Watery	18	56.25%
Bloody	14	43.75%
Total	32	100%

Other studies mentioned that the bloody diarrhea in *E. histolytica* isolates more than watery diarrhea because this parasite usually invades the intestinal lining and cause bleeding (Farrar *et al.*, 2013) this agree with the present study. Also another studies mentioned bloody diarrhea was not found in the age group 0–2 years old (Samie *et al.*, 2006). Other study showed a higher frequency of the patients with bloody diarrhea; while in patients with watery diarrhea, lower the frequency, Severe dehydration was observed in only (15.9%) of cases and the majority of them were seen in patients with bloody diarrhea done by (Ahmed *et al.*, 2011).

Distribution of *E.histolytica* according to the sex

The results of this study showed that the percentage of infection with *E. histolytica* was higher in male (65.7%) than female (34.3%), as in table 3.

This result is in agreement with other study by Younis (2007), also in agreement with results of Al-banea (2006) which founded that male is most frequent infection with *E. histolytica* than female, This is a clear reference of public healthrelated cause that males are more sensitive

Table 3: Distribution of *E. histolytica* according to the sex.

Sex	No. of Examination		Positive No. of *E.histolytica*	
	Numbers	percentage	Numbers	percentage
Male	56	62.22%	21	65.7%
Female	34	7.7%	11	34.3%
Total	90	100%	32	100%

to be feeding on an insecure food than female, while Al-Masoudi (2009) and Al-Ebrahimi (2013) which showed significant differences between percentages of infections in male and females. In this present study, prevalence of *E. histolytica* was higher in males than in females which was similar with the study from Nepal (Yogi *et al.,* 2018).

Distribution of *E. histolytica* according to the age groups

The result of current study showed that the infections with *E. histolytica* was the highest percentage 22(68.75%) for age group (1-10) years As show in below table 4. Our study agree with another studies done by (Haque *et al*., 2003) mentioned the Overall diarrheal morbidity was more in small age compare with old age, Although not statistically significant. The result of another study disagree with our results mentioned that infectious samples were increased in adults, These results is due to that the adults have more social activities and greater opportunity for exposure to *E. histolytica* (Al-Tufaili, 2020).

Table 4: Distribution of *E.histolytica* according to the age groups.

Sex	No. of Examination		Positive No. of *E.histolytica*	
	Numbers	percentage	Numbers	percentage
	71	78.8%	22	68.75%
11-20	8	8.8%	4	12.5%
21-30		55.5%	4	12.5%
31-40		44.4%	2	6.25%
>40	2	2.22%	0	0%
Total	90	100%	32	100%

The reasons of this difference may be due to the different socioeconomic levels and culture conditions in addition, the high frequency of amebic infection in males in comparison with females may be attributed to the anatomical structure and physiological factors "that affect individual 's resistance" in addition to the action of social and economic habits (Grodner *et al.,* 2000). In this present study, prevalence of *E. histolytica* was higher in males than in females which was similar with the study from Nepal (Yogi *et al.,* 2018).

Distribution of *E. histolytica* according to the months

The distribution of *E. histolytica* according to months of years was shown as in the table 5. The most frequent infection with *E. histolytica* was found in October, November and December with 31.25%, 21.9% and 18.7% respectively.

Table 5: Distribution of *E.histolytica* according the months.

Sex	No. of Examination		Positive No. of *E.histolytica*	
	Numbers	percentage	Numbers	percentage
October	25	27.7%	10	31.25%
November	16	17.7%	7	21.9%
December	20	22.2%	6	18.7%
January	14	15.5%	4	12.5%
February	9	10%	3	9.37%
March	6	6.6%	2	6.25%
Total	90	100%	32	100%

The table above showed the high percentage of *E. histolytica* infection in October and December. The reasons may be due to provide appropriate environmental condition for growth and spread of the parasite as intestinal parasite are more prevalent in this months of years (zeibig, 2014). The chances of human infection by *E. histolytica* increases significantly during the hot summer months as a result of the proliferation of disease carrying insects and spreading, including domestic flies, which is a mechanical vector foe protozoan parasites. The Monthly Infectious Diseases Surveillance Report produced by the Public Health Ontario (Kantor *et al.,* 2018).

Also reported that the percentage of *E. histolytica* decrease in winter due to rapidly killing of cyst in temperature below 5°C and above 40°C (Robert *et al.,* 2013).

Distribution of *E. histolytica* according to the residence

The results of this study showed that the percentage of infection with *E. histolytica* was higher in rural area 20/32(62.5%) than urban area 12/32(37.5%), as seen in table 6.

People from rural households were 1.9 times more likely to have diarrhea as compared to their counterparts (Alebel *et al.,* 2018). Our study agree with studies done

Table 6: Distribution of *E.histolytica* according to the residence.

Residence	*E.histolytica*	
	Number	percentage
Urban	12	37.5%
Rural	20	62.5%
Total	32	100%

by Fetensa *et al.*, (2020), who showed that people residing in urban settings had less infection when compared with those people living in rural residences.

Our study agree with studies done by Fetensa *et al.*, (2020), who showed that people residing in urban settings had less infection when compared with those people living in rural residences. Also another studies agree with our results done by Al-Masoudi (2009) and Al-Ebrahimi (2013), who showed the same results of that the people in rural areas are more susceptible to infection than urban areas the combined infections were more common in rural areas.

Toll Like Receptor 2 (TLR-2) Levels

The result in the table 7 showed high levels of TLR-2 in age group (1-10) compared to healthy controls, the increasing significantly ($p<0.05$). Also levels of TLR-2 in age group (>40) was increase compared to controls but these results was non-significantly.

Table 7: concentration of TLR-2 in patients and control.

Age (year)		Concentration pg\ml	P value
1-10 years	Case	2343.25± 50.78	0.01
	Control	1211.94 ± 80.66	
11-20years	Case	1943.17±21.71	0.02
	Control	697.86±15.22	
21-30 years	Case	1765.37± 26.22	0.4
	Control	682.10± 19.27	
31-40years	Case	1697.26± 71.11	0.2
	Control	597.42± 62.42	
>40years	Case	1483.84± 82.92	0.5
	Control	579.20± 29.72	

The current results are not relative with previous studies (Qian *et al.*, 2012) who mentioned the unchanged levels of TLR2 in monocytes from older individuals. Also not agree with other studies (Stewart *et al.*, 2005) who mentioned that no independent effect of age on TLR2 expression was observed. Other studies disagree with our study (Renshaw *et al.*, 2000) mentioned these data indicate that pro inflammatory cytokine responses decline with aging when TLR2, 3, 4, 5 and 9 on splenic or thioglycollate-elicited macrophages are stimulated (Renshaw *et al.*, 2000).

The results of other study revealed a significantly higher expression of TLR2 in patients than in controls (Karananou *et al.*, 2016). þAlso patients express significantly greater levels of TLR-2 compared with normal (Dispenza *et al.*, 2012). Another studies mentioned that patients express significantly greater levels of TLR-2 (Dispenza *et al.*, 2012).

TLR2 also interacts with a large number of non-TLR molecules, allowing for recognition of a great number and variety of PAMPs (Zähringer *et al.*, 2008) This diversity comprises different types of molecules from all microbial phyla including viruses, fungus, bacteria and parasites. TLR2 expression has been detected in immune cells, endothelial and epithelial cells (BrzeziDska-Blaszczyk and Wierzbicki, 2010).

The enhanced activation of TLR-2 may contribute to the amoebic pathogenesis through the exacerbation of inflammation. In the resistant individual the innate/ inflammatory and adaptive immune responses eliminate *E. histolytica* (Kammanadiminti *et al.*, 2004).

Innate immune receptors, such as Toll-like receptors (TLRs), is the first step in the inflammatory response to pathogens *Entamoeba histolytica*, the etiological agent of amebiasis, has a surface molecule with the characteristics of a PAMP. This molecule, which was termed lipo peptido phospho glycan (LPPG), is recognized through TLR2 and TLR4 and leads to the release of cytokines from human monocytes, macrophages and dendritic cells; LPPG activated dendritic cells have increased expression of costimulatory molecules. (Wong-Baeza *et al.*, 2010).

Toll Like Receptor 4 (TLR-4) Levels

The result in the table 8 showed high levels of TLR-4 in age group (1-10) compared to healthy controls, the results significantly ($p<0.05$). while the level of TLR-4 was Decreased in age group (>40) compared to controls and the results was not significantly.

Other studies mentioned that patients express significantly lower levels of TLR-4 compared with control (Dispenza *et al.*, 2012). While no significant association was found with TLR-4 in study of (Pandey *et al.*, 2009). Other studies could not confirm the age-associated decrease in TLR4 surface expression but found TLR4-induced cytokine production to be lower in older and associated with a decrease in basal expression done by Van Duin and Shaw (2007).

Table 8: Concentration of TLR-4 in patients and control.

Age (year)		Concentration pg\ml	P value
1-10 years	Case	1085.83± 118.93	0.5
	Control	727.31± 21.79	
11-20years	Case	597.12±31.01	0.09
	Control	209.85±51.92	
21-30 years	Case	570.57±28.59	0.1
	Control	198.98±9.31	
31-40years	Case	553.12± 12.84	0.00
	Control	623.62±11.99	
>40years	Case	499.06± 15.31	0.2
	Control	611.22 ± 0.34	

Surface expression of TLR 2 and 4 remained preserved in both aged and young (Tesar *et al.*, 2006). Another studies agrees with our study mentioned that older women who exercised regularly were found to have lower TLR4 expression (Flynn *et al*., 2003; McFarlin *et al*., 2004).

TLR 2 and TLR 4 constitute membrane-bound pattern recognition receptors resulting in the production of pro inflammatory cytokines, thereby causing inflammation rather than specific antiviral responses (Akira *et al*., 2006). Another studies of Devaraj (2008) mentioned the TLR4 expression was significantly up-regulated compared with controls. According to the results that obtained in this study we conclude The percentage of infection with *E.histolytica* was higher in male than female. The infections with *E. histolytica* was the highest percentage for age group (1-10) years, high levels of TLR-2 and TLR-4 in age group (1-10) compared to healthy controls, also levels of TLR-2 in age group (>40) was increase compared to controls.

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