Assessing some immune parameters during asymptomatic infection with Entamoeba histolytica.

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Abstract

Background and Aim: Entamoeba histolytica a protozoan parasite with high prevalence rates in tropical and subtropical regions of underdeveloped countries. Cellular and humoral immune system involves during invasive amebiasis but there is limited information about the exact immune response during asymptomatic infections which comprise the majority of the infected people.

Material and Methods: in a case-control study, serum from asymptomatic carriers of E. histolytica and healthy control individuals were collected, and then levels of IL-4, TNF-α, Ig G2 and Ig G4 were measured by ELISA. A structured questionnaire was filled out by each participant and statistically analysed using SPSS program.

Results: The results of present study showed that the levels of TNF-α and IL-4 were higher but not significant in the positive group compared to the negative control group. While, serum Ig G2 and Ig G4 levels were significantly higher in positive E. histolytica group compared to negative control individuals. Ig G2 levels were significantly higher in low-income people and individuals who always eating outside home compared to middle-income people and individuals who never or sometime eat outside, respectively, among the negative control group. There were also significant variations among the positive group, higher levels of IL-4 and Ig G2 were seen in people who washed their vegetables and fruits than who were not; while lower levels of Ig G4 were reported in the same category of participants.

Conclusions: The current study concludes that the parasite did not elicit cellular immune response but causes activation of humoral immune response against the asymptomatic E. histolytica isolates.

Keywords: Asymptomatic infections, IL-4, TNF-α, Ig G2, Ig G4, E. histolytica.

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Introduction

Entamoeba histolytica a protozoan parasite that inhabit human gastrointestinal tract, amoebiasis infects 50 million individuals globally, with an annual mortality rate ranging between 40,000 to 100,000 [1]. Amoebiasis can be asymptomatic in the majority of infected people or can lead to severe infection with amebic colitis and amebic liver abscess. Asymptomatic carriers representing an important group since they play role in spreading the parasite and prolong asymptomatic infection may turn to invasive amoebiasis with symptoms such as bloody diarrhea, abdominal pain, flatulence, nausea and vomiting [2, 3]. During the establishing of the infection, host cells elaborate various mechanisms for pathogen expulsion and removal, amebae have also established complex strategies to avoid host defense and enable their own survival [4]. Additionally, it has been proposed that host immune response tends to mediate pathogenesis rather than protection. Therefore, the path followed by CD4+ T cell immune response may be critical. For example, tissue destruction in amebic colitis can arise from both amoebic cytolytic factors and the subsequent gut proinflammatory response. Moreover, TNF-α induces neutrophils and macrophages to secrete reactive oxygen species (ROS) and nitric oxide (NO) to fight the parasite, but an extra amount of TNF-α can cause in direct damage to host tissue [5]. Therefore, the initiation of effective immunity against Entamoeba requires the activation of antigen-specific T cells, which cause either Th1 or Th2 phenotype depended on secretion of appropriate cytokines [6]. Although T cell cytokine responses during amoebiasis remain generally uncharacterized, in vivo evidence suggests that resistance to E. histolytica is associated to Thl responses. While in *vitro* data revealed that amebic components had favored the upregulation of Th2 cytokines, with suppression of the Th1 responses [7]. Limited information available on the pattern of immune response during asymptomatic infections with E histolytica, and there is a need to evaluate the host immune response during the presence of this parasite in the intestinal lumen. Invasive amoebiasis is also related with the development of high antiamebic immunoglobulin G (IgG) titers. Despite the production of elevated and long lasting specific antibody titers during symptomatic intestinal amebic infection, reinfection often occurs [8]. Thus, a more thorough analysis of the humoral immune response based on investigating the IgG subclasses might provide additional information associated with resistance or susceptibility to amoebic infection.

Material & Methods

Study design

The present case-control study involved collection of sera from the asymptomatic subjects (48 samples) and compared to sera

obtained from healthy individual without infections (40 samples) (negative control) for estimation of Ig G2, Ig G4, IL-4 and TNF-α using ELISA technique (ElabScience®, Wuhan, China). Each participant was submitted to a structured questionnaire and positive individuals with E. histolytica have been confirmed by PCR.

Measuring serum Ig G2: The ELISA kits that used in the present study perform upon the same principle, based on the Sandwich-ELISA principle. The micro plate that provided with the kit has been pre-coated with an antibody specific to Human Ig G2. Adding of samples or standards to the wells of the micro plates enabling combining with the specific antibody. Then a biotinylated detection antibody specific for Human Ig G2 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. The substrate is added to each well after washing away of the free components. The blue colored wells indicate the presence of Human Ig G2, biotinylated detection antibody and Avidin-HRP conjugate. Addition of stop solution terminated the enzyme-substrate reaction and turned the color to yellow. The spectrophotometer was used to measure the optical density (OD) at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Human Ig G2. The concentration of Human Ig G2 was calculated in the samples by comparing the OD of the samples to the standard curve.

Finally, the optical density (OD value) of each well was measured at once with a micro-plate reader set to 450 nm.

Measuring serum Ig G4, IL-4 and TNF-α: The brand and the manufactured company of the kits used for detection of serum Ig G4, IL-4 and TNF-α level are the same as that of I gG2, therefore, the principle of the reaction and the applied procedure are similar. Taking in consideration the differences in the type of the antibody pre-coated on the microplate for each respective immune marker. In addition to the differences in the serial dilutions and their standard curves.

Statistical analysis

Data were analyzed using SPSS software program version 23. Results were expressed as descriptive statistics as frequencies, exact Fisher test and Chi square; and mean± SD for numerical data using independent t-test and ANOVA. P value<0.05 was regarded as statically significant.

Ethical Consideration

The study was conducted in accordance with the Declaration of Helsinki – Ethical Principles for Medical Research, revised in 2008; and was approved by the Ethics Committee of Hawler Medical University. A verbal consent was obtained from each participants and information about the study research was provided prior to sample collection.

Results

Sociodemographic study

The statistical analysis of the data collected from 48 positive and 40 negative samples of E. histolytic were showed that there no significant differences among the age groups between the positive and negative samples (Table 1). There were significant lower female participant particularly as negative control compared with male participants. However, no significant differences were seen in the frequencies of urban and rural participants as well as applicants with various educational level and family sizes. Nevertheless, significantly more middle income-people were participated than low-income individuals. There were no significant differences between the samples that were collected from positive E. histolytica and negative samples regarding hygiene habits (washing fruits and vegetable or not), using different sources of drinking water and participants who always and sometime dining outside. There were almost significant higher rates of participant that took medications in more than two weeks prior sample collection as compared with participant that took medications in less than two weeks.

Table 1. *Frequency and statistical analysis of the studied variable from both E. histolytica positive and negative participants.*

Variants	E. histolytica Negative	E. histolytica Positive	Total	P value
Age Groups				
15-18	1	4	5	0.474
19-25	21	17	38	
26-35	12	17	29	
36-45	5	9	14	
>45	1	1	2	
Total	40	48	88	
Gender				
Male	40	16	56	>0.001
Female	0	32	32	
Total	40	48	88	
Residency				
Urban	23	30	53	0.667
Rural	17	18	35	
Total	40	48	88	
Educational Level				
Primary School	16	15	31	0.265
High school	15	26	41	
Bachelor	9	7	16	
Total	40	48	88	
Family size				

1-2	6	4	10	0.702
3-4	8	10	18	
5-6	13	14	27	
>6	13	20	33	
Total	40	48	88	
Income status				
Low-income	4	28	32	>0.001
Middle-income	36	20	56	
Total	40	48	88	
Hygiene habits				
Washing vegetable	39	46	85	0.668
Not washing vegetable	1	2	3	
Total	40	48	88	
History of drug intake				
In the last 2 weeks	6	4	10	0.502
More than 2 weeks	34	44	78	
Total	40	48	88	
Source of drinking Water				
Chlorinated water	24	24	48	0.394
Well Water	16	24	40	
Total	40	48	88	
Dinning Habits				
Never eat outside	9	14	23	0.062
sometimes	23	16	39	
Always	8	18	26	
Total	40	48	88	

Immunological Parameters

Serum levels of TNF-α, IL-4, Ig G2 and Ig G4 were measured using ELISA technique; the levels compared between the serums of asymptomatic participate (48) with E. histolytica and control negative group (40) without any parasitic infections. The results of TNF-α and IL-4 showed higher but not significant differences between the two studied groups with P-value 0.053 and 0.526, respectively (Table 2). While, serum Ig G2 and Ig G4 levels were recorded significant (p<0.001) higher levels in asymptomatic subjects with E. histolytica compared to negative control individuals.

Table 2. *Statistical analysis of serum TNF-α, IL-4, Ig G2 and Ig G4 levels, between control group and asymptomatic infection with E. histolytica, using independent t-test.*

Immune parameter	Asymptomatic Mean (48) ± Std Deviation	Control negative Mean (40) ± Std Deviation	Std. Error difference	95% confident Intervals Lower upper		P-value
TNF-α (pg/ml)	0.4837± 1.70748	0± 0	0.24645	-0.973	0.0056	0.053
IL-4 (pg/ml)	2.3544± 5.12702	1.7443± 4.20791	0.95735	-2.5109	1.2907	0.526
Ig G2 (µg/ml)	20.2097± 11.55886	0.7989± 1.29108	1.6787	-22.744	-16.0776	<0.001*
Ig G4 (ηg/ml)	270.57± 176.89097	20.3212± 23.28939	25.7523	-301.3806	199.1168	<0.001*

The statistical analysis showed no significant differences between the studied age groups with respect to the IL-4, TNF-α, Ig G2 and Ig G4 levels (Table 3). Similarly, the levels of IL-4, TNF-α, Ig G2 and Ig G4 were significantly not between different male and female; urban and rural participants as well as people with different educational levels. Families consist of variable members seems to not have significant influences on the levels of the studied immune parameters. However, people who live in poor life quality reported significant higher levels of Ig G2 in the negative control group. Significant higher levels of IL-4 and Ig G2 were seen in people who washed their vegetables and fruits than who were not, among the positive samples for E. histolytica; while Ig G4 levels were significantly higher in participants who were not washing their groceries. Participants who took medications prior of sample collection and who were not, reported no significant effects on the levels of the IL-4, TNF-, Ig G2 and Ig G4; likelihood participants who drink water from different source were not affected the immune parameters. The Ig G2 levels were significantly higher in participant who used to dining outside home than who sometime or never eat outside, among the negative groups.

Table 3. *statistical analysis for the studied variable for the negative and positive E. histolytica samples regarding each of IL-4 TNF-α, Ig G2 and Ig G4.*

Variants	Immune parameters							
	IL-4 Mean		TNF-α Mean	IgG2 Mean	Ig G4 Mean			
	E. histolytica		E. histolytica	E. histolytica	E. histolytica			
	Negative	positive	Negative	positive	Negative	positive	Negative	positive
Age Groups								
15-18	8.255	2.1128	0	0.1395	0	17.479	20.29	154.43
19-25	1.9561	2.6822	0	0.8079	1.13	17.9984	22.821	270.5229
26-35	2.0965	2.0926	0	0.5251	0.4377	21.2575	25.0933	257.6378

36-45	1.8474	2.5987	0	0	1.787	23.3378	34.9538	329.7854
>45	0	0	0	0	0.427	22.76	0	422.84
P value	0.739	0.988		0.825	0.342	0.806	0.715	0.483
Gender								
Male	2.0932	2.5796	0	0.5928	0.9586	20.0377	24.3855	233.5257
Female		2.2418		0.4292		20.2957		289.0922
P value		0.839		0.786		0.953		0.265
Residency								
Urban	2.9396	1.8475	0	0.4463	0.945	19.7335	21.0349	280.1683
Rural	0.9481	3.1993	0	0.546	0.9771	21.0033	28.9186	254.5728
P value	0.155	0.439		0.868	0.939	0.717	0.29	0.67
Educational levels								
Primary School	1.6952	2.5943	0	0.1674	1.1904	20.0168	26.8529	283.5588
High school	2.7254	2.6609	0	0.7964	0.4364	18.7582	20.8849	273.5072
Bachelor	1.7471	0.702	0	0	1.4169	26.0143	25.8333	231.8271
P value	0.605	0.388		0.287	0.089	0.151	0.563	0.511
Family Size								
1-2	3.6688	4.9628	0	0	1.1168	32.13	29.5833	218.0305
3-4	1.4741	1.5724	0	0.5858	0.5839	20.26	26.3586	281.789
5-6	2.4341	0.6317	0	0.1395	0.8563	15.2006	20.6454	270.1456
>6	1.4061	3.4296	0	0.7704	1.2185	21.3069	24.5123	275.7655
P value	0.355	0.116		0.413	0.387	0.288	0.484	0.518
Income Status								
Low-income	1.9655	3.4676	0	0.4407	2.483	18.6531	15.6318	262.546
Middle-income	2.1074	0.796	0	0.544	0.7893	22.3889	25.3581	281.036
P Value	0.954	0.06		0.865	0.016	0.257	0.439	0.723
Hygiene Habits								
Washing vegetable	2.1469	2.4568	0	0.5047	0.9304	20.6347	24.2723	262.5326
Not washing vegetable	0	0	0	0	2.059	10.435	28.8	455.43
P value	0.646	0.024		0.162	0.42	0.001	0.852	0.001
History of drug intake								
In the last 2 weeks	4.4878	1.2285	0	0	0.4738	31.4525	13.8583	166.77
More than 2 weeks	1.6706	2.4568	0	0.5277	1.0442	19.1876	26.2432	280.0064
P value	0.164	0.366		0.176	0.351	0.089	0.239	0.052
Source of drinking water								
Chlorinated water	2.8908	0.9909	0	0.3952	1.2683	18.8486	19.3882	302.4225
Well Water	0.8968	3.7179	0	0.5723	0.4941	21.5708	31.8814	238.7175
P value	0.177	0.065		0.748	0.078	0.432	0.1	0.206
Dining Habits								
Never eat outside	3.8436	3.3131	0	0.4184	0.654	22.8771	39.5389	272.9615
sometimes	1.4613	2.3339	0	0.1569	0.7011	17.7437	18.7675	263.572
Always	1.9409	1.6269	0	0.8249	2.0418	20.327	23.4896	274.9304
P value	0.261	0.663		0.31	0.037	0.248	0.076	0.869

Discussion

The exact protective mechanisms against amoebiasis are ambiguous, however it seems both innate and adaptive immune response participate to inhibit amebic infections [9-15].

Results of the present study reported that there were no significant differences in the levels of TNF-α and IL-4 between serums of asymptomatic carriers of E. histolytica and control negative group. Individuals that are asymptomatically infected with E. histolytica represent an important group enabling the study of immune responses that are critical to the outcome of an infection. The TNF-α is a pro-inflammatory cytokine, mainly produced by macrophages, it can cause tissue inflammation *via* activating macrophages, neutrophils recruitment, and inducing expression of other pro-inflammatory mediators [16, 17]. It may also result in increased cell permeability, causing impairment of barrier function and development of edema [18]. The TNF-α plays a central role in mucosal inflammation, and is elevated in the

gastrointestinal tract of some forms of inflammatory colitis [19]. It has been suggested that since inflammation causes alterations in the gastrointestinal microbiota, therefore there would be depletion in the nutrient sources of E. histolytica and this may trigger the parasite for searching for alternative sources, such as epithelial cells and erythrocytes consequently leading to amebic colitis [20]. A cohort study from Bangladeshi children, reported that an elevated levels of TNF-α can increase the risk of first and recurrent E. histolytica - related diarrheal infections, proposing that the production of TNF-α play role in future susceptibility to E. histolytica diarrhea and pathogenesis of amoebiasis. High levels of TNF-α protein expression was observed in children who had E. histolytica diarrhea compared with those who had asymptomatic infection or no infection [21]. The finding of this research is consistent with the result of the present work. Likewise, no significant differences in the TNF-α level were reported by another study, between asymptomatic carriers of E. histolytica compared with E. dispar infections [8]. Furthermore, data from animal model, showed that suppression or depletion of mice TNF-α levels result in protection and resistance to amebic colitis[22]. Since Th2 cells, produce immunosuppressive IL-4 and IL-10, therefore serum levels of IL-4 used as indicator to represent the Th2 response in the current study. The predominance of this immune response could facilitate the survival and dissemination of E. histolytica causing tissue damage and associated with the appearance of the disease [23]. The results of the current work showed no significant difference in serum levels of IL-4 between asymptomatic carriers and control negative group. Similarly, a study reported higher IL-4 levels in patients with acute amoebiasis and no significant differences documented in the IL-4 levels between asymptomatic carriers, E. dispar infections and control group [24]. The same study reported high levels of INF-γ in asymptomatic individuals, an indicator of Th1 response. Therefore, high levels of both IL- 4 and TNF-α are related with disease, while INF-γ have been documented to be associated with the clearance and protection from amoebiasis [25]. Moreover, animal model was used to investigate the immune status during amebic liver abscess, a Th1-like response was evident in the drug-cured gerbils, characterized by production of IL-2 but not IL-4, which were resistant to challenge infections and may help control the infection [7]. In *vivo* and In *vitro* studies showed that the Gal-lectin antigen of E. histolytica can initiate pro-inflammatory response characterized by elevated INF-γ and inhibition of IL-4, which provide protection from amoebiasis [26]. Data has showed that a systemic IgG response develops in subjects who are colonized asymptomatically or have invasive infection with E. histolytica, compared to those having E. dispar infection or no infection. However, the protective role of Ig G in amebic disease is difficult to establish [27]. Antibody-mediated immune response investigating the Ig G subclasses might provide further information related to resistance or susceptibility to invasive disease. The results of the present study showed significantly higher levels of Ig G2 and Ig G4 in asymptomatic carrier with E. histolytica compared to control negative group. There is limited information regarding Ig G subclasses of asymptomatic infection with E. histolytica, only single study investigating the immunoglobulin subclasses is available upto date. The study revealed that the Ig G2 and Ig G4 levels were higher compared to control group; which agreed with the result of the current work [8]. Principally, Ig G4 could be directly pathogenic, fulfill a protective role, and it has been reported to have exclusive structural and functional characteristics suggesting anti-inflammatory and tolerance-inducing effects [28]. This may explain the high level of this immunoglobulin subclass compared to that of the control group obtained from the present work. It has been documented that human Ig G 2s possess a greater resistance as compared with the other human IgG isotypes to microbial proteases [29]. This is may be the reason for the high level of Ig G2 in asymptomatic carriers which may prevent the development of invasive amoebiasis in these individuals. These studies reveal that the host can mount humoral immune responses against E. histolytica, and are somehow associated with protection. The Ig G2 levels were statistically higher in low-income people and in individuals who always dining outside home in the negative control group of the current study this is could be due to reason that the living in such an endemic region lead to acquiring the parasite at some time and developing of immunoglobulin as a result; additionally people live poor quality of life and frequent dining outside increases the possibilities to gain the parasite. People who washed their fruits and vegetables showed higher levels of IL-4 and Ig G2 this is could be due to their active immune system which developed against the parasite that might be acquired through contaminated water but Ig G4 levels were significantly high in people who not washing their vegetables and fruits this subclass of immunoglobulin could be raised during most asymptomatic infection with various parasites, therefore contaminated food with E. histolytica and other parasite lead to elevation of serum anti-inflammatory Ig G4 levels and cause asymptomatic infections. The current study concludes that there were no significant differences in serum IL-4 and TNF-α level between asymptomatic individuals and control negative group. These results suggest that the parasite did not elicit cellular immune response or may upregulate expressions of other cytokines. While, Ig G2 and Ig G4 levels in asymptomatic participants were significantly higher than control subject in the current study indicating an activation of humoral immune response against the asymptomatic E. histolytica isolates.

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