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Estimation of Immune Response in Rabbits Infected with Attenuated *Entamoeba histolytica* by Gamma Radiation

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Abstract

Out of 1279 stool sample only 245 were found to be infected with *E. histolytica* with total percentage 19%. Diagnostic study for *E. histolytica* by using techlab Eliza test showed that the non-pathogen *E. dispar* was significantly higher (78.9%) than *E. histolytica* (22%). We test the effect of the attenuated *E. histolytica* by gamma Radiation on the mortality rate in laboratory animals infected with *E. histolytica* by using increasing doses of radiation (5, 10, 15, 20 and 25) Rad., the results showed that the percentage of mortality decrease when increase the dose of attenuated *E. histolytica* cyst in comparison with positive control group (non – attenuated group) it reached (100%, 66.7%, 33.3% 0% and 0%) respectively. Also we found that gamma radiation elevate some immunological parameters in laboratory animal. So the level of gamma interferon (IFN- γ) reached to (3000pg/ml) and level of (IgE) reached to (550 mg/L) in comparison to control group (130.5 and 78.20 mg/L) respectively. In conclusion, the high level (IFN- γ) and (IgE) may be used as indicator for induction of immune responses which may have protective effect against amoebiasis infection.

Keywords: *Entamoeba histolytica*, radiation, immune response.

تقييم الاستجابة المناعية في الارانب المصابة بالاميبا الحالة للنسيج المضعفة بواسطة اشعة كاما

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الخلاصة

من ما مجموعه 1279 عينة براز فقط 245 كانت موجبة للاميبا الحالة للنسيج بنسبة اصابة 19%. اظهرت الدراسة التشخيصية باستخدام اختبار Techlab Elisa ان طفيلي *E. dispar* الغير مرضي كان اعلى نسبة اصابة (78.9%) من طفيلي الاميبا الحالة للنسيج (22%). تم اختبار تأثير اشعة كاما في نسبة هلاكات الحيوانات المختبرية باستخدام جرع تصاعدي من اشعة كاما (5, 10, 15, 20, 25)% حيث اظهرت النتائج بان نسبة الهلاكات تقل عند زيادة جرعة الاشعاع بالمقارنة مع مجموعة السيطرة الموجبة (المجموعة غير المضعفة) حيث وصلت الى (100%, 66.7%, 33.3%, 0% and 0%) على التوالي. كذلك وجد ان اشعة كاما رفعت بعض المعايير المناعية في الحيوانات المختبرية، حيث ارتفع مستوى كاما انترفيرون الى (3000pg/ml) ومستوى IgE الى (550mg/L) بالمقارنة مع مجموعة السيطرة (130.5 و78.20) على التوالي. نستنتج من هذا ان المستوى العالي لمستوى (IFN- γ و IgE) دليل على حث الجهاز المناعي ضد الاصابة بالاميبا الحالة للنسيج.

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Introduction

Amoebiasis caused by the intestinal parasite *Entamoeba histolytica*, has an estimated worldwide prevalence of 500 million infected people and is responsible for 40,000 - 100,000 deaths each year. It is an important health problems, especially in developing countries [1]. The strains found in asymptomatic individuals (nonpathogenic) were so distant from pathogenic strains that they have now been accorded a separate species status, namely *E. dispar* [2]; and the name *E. histolytica* has been retained for the pathogenic strains. The amoebapore and cysteine proteinases from *E. dispar* have been analyzed in some detail. Both proteins do exist in *E. dispar*, although differing considerably from the homologous proteins in *E. histolytica*. The specific activity of *E. dispar* amoebapore is less than half that of *E. histolytica* [3].

The immune response of host stimulated by parasite infection, the lysozyme and mucous substance of digestive system that secreted by acidity of intestine according important factor in defense of host against parasite, the members of the lymphatic secondary in the large intestine mesenteric lymph and patches Bayer and white cells between epithelial cells and tensile arbitrator to epithelial cells by protein complexes are all working together as a barrier immunophysiology is also an important source of the outputs of the inflammatory immune to contribute effectively to protect the body against invading parasite [4].

The invasive stage of the parasite to host intestinal tissue stimulates the formation cellular immune response as to attempt parasite adhesion epithelial cells lining the large intestine in order to form colonies induce the inflammation process and thus stimulate the cells to produce and edit the kinetics of cellular cytokines such as interleukin -8 (IL- 8) and interleukin -1 (IL-1) as well as tumor necrosis factor (TNF) Tumor Necrosis Factor and interferon gamma (IFN-gamma) and this dynamics act as attracting chemical for cells of systemic immune to reach the area of inflammation as macrophages, neutrophils and mononuclear cells where these cells attack and lyses the parasite [4].

There are several methods in the preparation of vaccines parasitic, such as the use of doses a few eccentric sexual parasite or antigens specific parasite and used directly or following the methods of genetic engineering for the preparation of these antigens, as well as attenuation which means weakening susceptibility Pathogens to stimulate the immune system methods of chemical, physical or use radiation, which is one of the most important methods used in the multiplication of pathogens and production of vaccines for being easy and inexpensive, has been proven effective in many applications [5] that the use of X-ray and gamma ray after numerous studies and knowledge of its impact on living materials and organic parasite that causing a series of physical changes and structural, which leads to weakening the ability of the parasite to induce the infection with maintaining its ability to stimulate immune response in the host [6].

The aim of this study to estimate immune response in animals infected with attenuated *E. histolytica* By gamma radation

Material and Methods

Laboratory Animals

In this study 21 New Zealand white rabbit from local market were brought and put in breeding cages in the animal house in the College of Medicine in Babylon University during experimental study. Rabbit infected with *E. histolytica* through intra esophageal administration of cysts suspension by using syringe with fine small tube entre through mouth to esophagus and push the cysts suspension to stomach directly and examined daily by direct method. The rabbits grouped into seven groups each one consist of three rabbits. One group administrated with normal *E. histolytica* cyst suspension .Five groups administrated with one of the following doses of attenuated *E. histolytica* cyst of (5,10,15,20 and 25) unite of gamma ray, in addition to control group which administrated with normal saline .

Sample Collection

Stool Sample

During the period, from March to September/2013, (1279) stool samples were collected from people suffering from severe diarrhea admitted to Babylon Maternity and Children Hospital/province of Babylon. Each stool sample was placed in glass sterile tube with a wide opening which tightly to keep moisture sample and prevent dry, then (1gm) of stool sample was taken and diluted by 10 ml of normal saline and mix well and then filtered through a layer of sterile gauze to remove the large particles. [7]. Cysts were washed with phosphate-buffered saline (PBS) pH 7.2 several times and the number of cysts per ml was determined by Neubauer hemocytometer.

Examination of Stool Samples:

Microscopical Examination

The examination were done with direct swab method by taking a drop of normal saline and put on one end of the glass slide, and a drop of iodine Lagos solution on the other end, and took a small amount of stool as much as the head of a match stick and mixed with both solutions separately, then put the lid slide and examined under the light microscope [8].

TechLab *E. histolytica*II Test.

The ELISA kits were used on the frozen stool specimens. For Triage parasite panel (Triage), this test was done to differentiate between pathogenic (*E. histolytica*) and non-pathogenic (*E. dispar*). The assay procedure was completed according to the manufacturer's directions.

Blood Sample

At the end of vaccination period, blood samples were taken from vaccinated rabbits and put in sterile tubes free from coagulant for serum collection, the blood was left in room temperature for 30 minute then centrifuge at 3000 rpm for 10 minute, then the aspirated serum put in sterile eppendorf tube and preserved at -20 C° until using [9] . Serum was used to determination of gamma interferon and IgE level by ELISA assay according to the manufacturer's directions.

Statistical Analysis

The results were analyzed by using Fisher-exact test , and the significance level was designated at ($P < 0.05$) All data were checked for normality and homogeneity of variances using SPSS Inc. (2007) program.

Results and Discussion

During The period from March to September (2013), 1279 stool sample were obtained from patients suffering from diarrhea who admitting to (Babylon Maternity and Children Hospital) out of 1279 stool sample only(245) were found to be positive for *E. histolytica* . The related results of this study showed that the total percentage of infection with *E histolytica* was 19 %. Results of this study in agreement with those results of [10] who showed the percentage of infection with *E.histolytica* was 22%, while it was disagreed with results of [11] who confirmed that the total infection of children in Babylon was 34.3 % .This difference in total percentages of infection in Babylon province may be return to the different in sample size and inadequate and contamination of water supplies and poor sanitation favor.

Diagnosis of *E.histolytica*/*E.dispar* by Techlab ELISA Test.

During the study period, 245 samples with amebic *E. histolytica*/*E. dispar* trophozoites or cysts in feces were detected by microscope. We determine the ratio between the pathogenic species *Entamoeba histolytica* and non-pathogenic species *E. dispar* using an enzyme linked immunosorbent assay (Techlab ELISA test) to detect the lectin (1 galactose N-acetyl D-galactosamine) of *E. histolytica* in feces. The result showed that non-pathogen *dispar* significantly ($p \leq 0.05$) higher (78.9%) than *E. histolytica* (22%) figure-1.

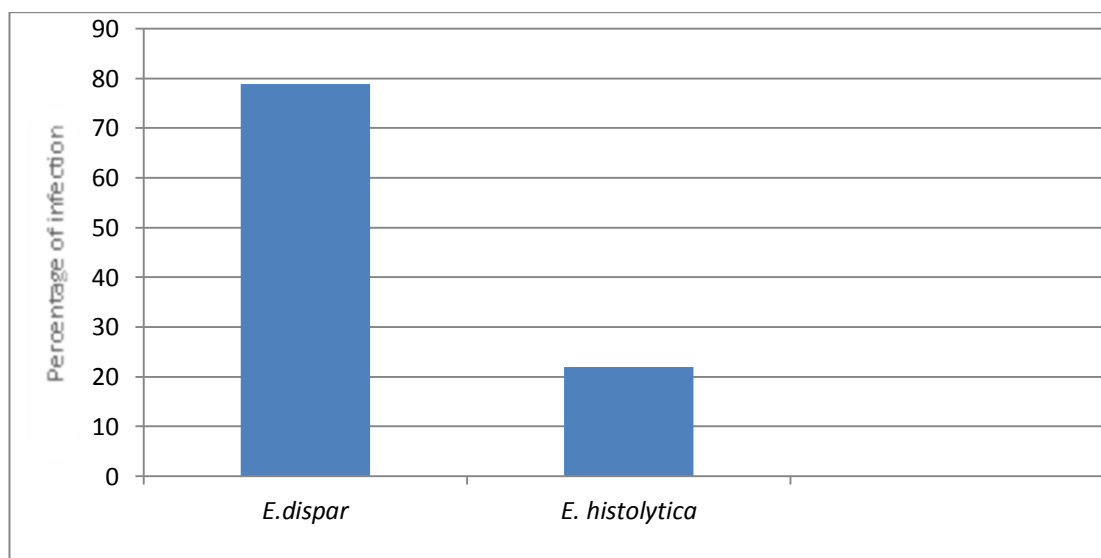


Figure 1- Diagnosis of *E.histolytica* / *E.dispar* by Techlab ELISA test .

The results of antigen detected by (Techlab ELISA test) showed that the percentage of *E.histolytica* (22%) less than *E.dispar* (78%) .This reason may be due to the galactos adhesions of *E. histolytica* can be differentiated from those of *E. dispar* by those monoclonal antibodies, resulting in a diagnostic ELISA test [12]. The frequencies of *E.histolytica* using the Techlab antigen detection kit was agreed with [13] they proved that the direct microscopic diagnosis of amoebiasis is not an efficient method for the diagnosis of *E.histolytica* infection. Comparative study carried out in different parts of the world showed the high sensitivities of TechLab antigen kit between 95 and 100% [14]. The result also closes to [15] in Bangladesh who recorded infection rate 4.29 % for *E. histolytica* using TechLab *E. histolytica* II ELISA kit, [16] in Northeastern Brazil recorded higher rate 10.2 % (41 of 401), and 58.9 % (110 of 187) for *E. histolytica* compared to 9.2 % (37 of 401), and 41.1 % (77 of 187) for *E. dispar* respectively, using TechLab *E. histolytica* II ELISA kit.

Effect of Gamma Radiation on the Percentage of Mortality in Laboratory Animals.

The result in table-1 showed that the percentage of mortality decreased positively with the increase of radiation doses in comparison to positive control group (non – attenuated group) at the second week of infection and it reached (100% , 66.7%, 33.3%) respectively when give the rabbits attenuated *E.histolytica* cyst (5,10 and 15) rad respectively . Also we found no mortality was happened in rabbits given attenuated *E.histolytica* cyst with dose (20 and 25) rad.

From this results we found and proved that gamma radiation reduce the pathogenicity of *E. histolytica in vivo* , This results is in agreement with [17] who confirmed that gamma radiation kill the *E. histolytica* cyst *in vivo* . However [18] confirmed that radiation with (20, 21) kilorad caused the reduce of development of *Eimeria tenella* sporozoite in chicken, also radiation gave protective against sporozoite of *plasmodium* [19]. Other study found that attenuated oocyst of *cryptosporidium* by using gamma radiation lead to reduce ability to disease in Buffalo [20].

Table 1-Effect of attenuated *E. histolytica* cyst on mortality rate of rabbits .

Dose (1000 cyst /ml)	No. of dead rabbit in first week	Mortality % in First week	No. of dead rabbit in second week	Mortality% in second week
Control group	0	0	0	0
Non-attenuated group	1	33.3%	2	100%
Attenuated with 5 Rad	0	0	3	100%
Attenuated with 10 Rad	1	33.3%	2	66.7%
Attenuated with 15 Rad	0	0	1	33.3%
Attenuated with 20 Rad	0	0	0	0
Attenuated with 25 Rad	0	0	0	0
P value Fisher-exact test	1.000		0.006	

Relationship between Attenuated *E. histolytica* Cyst with Clinical Sign Appears in Rabbits

The clinical signs and virtual changes in the behavior of animals during the testing with doses of attenuated and non-attenuated *E .histolytica* cyst by gamma radiation were studied. The results in the table-2 showed the clinical signs appear in animals after vaccinated with attenuated and non-

attenuated cyst orally .The clinical signs showed in all groups but appear lowest in groups that vaccinated with high dose of gamma radiation with 20 and 25 rad.

Table 2-Effect of gamma radiation on clinical sign appears in rabbits which administrated with attenuated *E. histolytica* cyst.

Dose (1000 cyst /ml)	Hypoactivity		Diarrhea		Anorexia	
	Yes	No	Yes	No	Yes	No
Control group	0 (0%)	3	0(0%)	3	0(0%)	3
Non-attenuated group	3(100%)	0	3(100%)	0	3(100%)	0
Attenuated with 5 Rad	3(100%)	0	2(66.7%)	1	3(100%)	0
Attenuated with 10 Rad	2(66.7%)	1	2(66.7%)	1	2(66.7%)	1
Attenuated with 15 Rad	1(33.3%)	2	1(33.3%)	2	1(33.3%)	2
Attenuated with 20 Rad	0(0%)	3	0(0%)	3	0(0%)	3
Attenuated with 25 Rad	0(0%)	3	0(0%)	3	0(0%)	3
P value Fisher-exact test	0.013		0.067		0.013	

These results of our study confirm the probability of using attenuated *E. histolytica* cysts to decrease the effectiveness ability of the parasite on animals in comparison with non- attenuated parasite. So, the hypoactivity of animals reach to 33.3%when gave attenuated cyst with 15 rad, while it reach to 100 % in animal which administrated with non- attenuated cyst .Also other clinical signs such as anorexia and diarrhea which reduced when administrated with attenuated cyst specially in dose 20 and 25 rad . Our study was in agreement with result of [17]who found that administrated of mice with attenuated *E.histolytica* cyst lead to decrease the clinical signs which appear in mice such as hypoactivity (10%) and Anorexia(20%) when used (20 and 25) Rad respectively . several factors are involved in the successful infection with *E. histolytica* in animals [21]. These include strain of *E. histolytica* (clinical isolate) used; age, weight, and the susceptibility of the host to *E. histolytica*. The infection persisted among control group and the cure rate was 0%.

Effect of Gamma Radiation on IFN-gamma Level.

The result of Interferon gamma by ELISA test after vaccinated of rabbits with different doses of attenuated *E. histolytica* cyst by gamma radiation showed in the figure-2 . The group that exposure to high dose of radiation gives high level of (IFN-gamma) in the serum, it was 130,5% of control group and increase significantly (p <0.05) when increase dose of attenuated cyst it reach to (187.5,373,750,1500 and 3000) pg/ml at (5,10 ,15 ,20 and 25) rad respectively . Gamma interferon is producing from peripheral mononuclear cell, it is important to activate macrophage to kill *E. histolytica* [22] or stimulate cell mediated immunity against amoeba [23]. *E. histolytica* trophozoites lyse resting macrophages and neutrophils but macrophages or neutrophils activated by treatment with gamma interferon (IFN- γ) [24]. The killing of *E. histolytica* trophozoites by activated macrophages is mediated by nitric oxide (NO) [25].

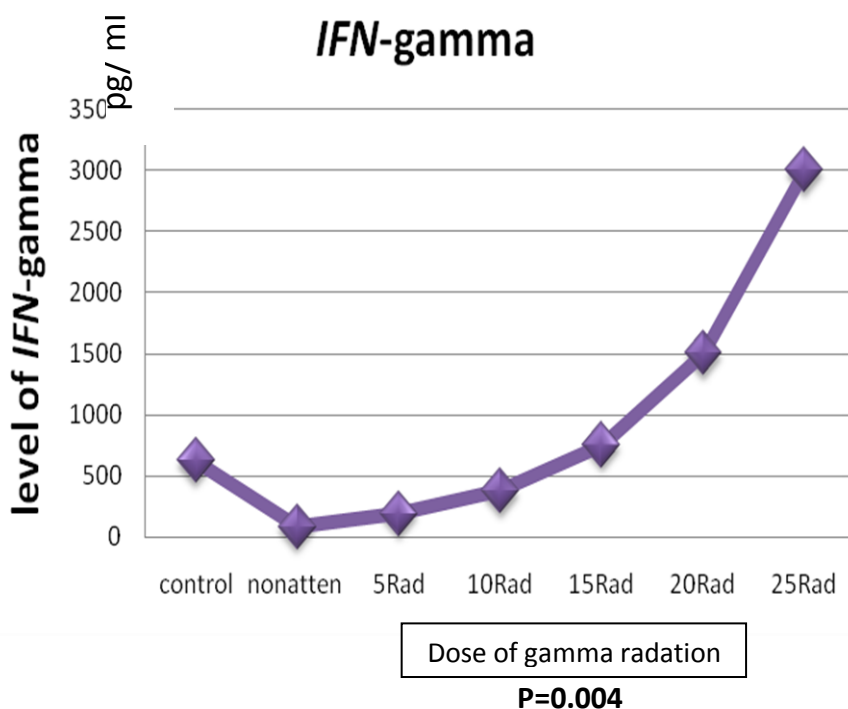


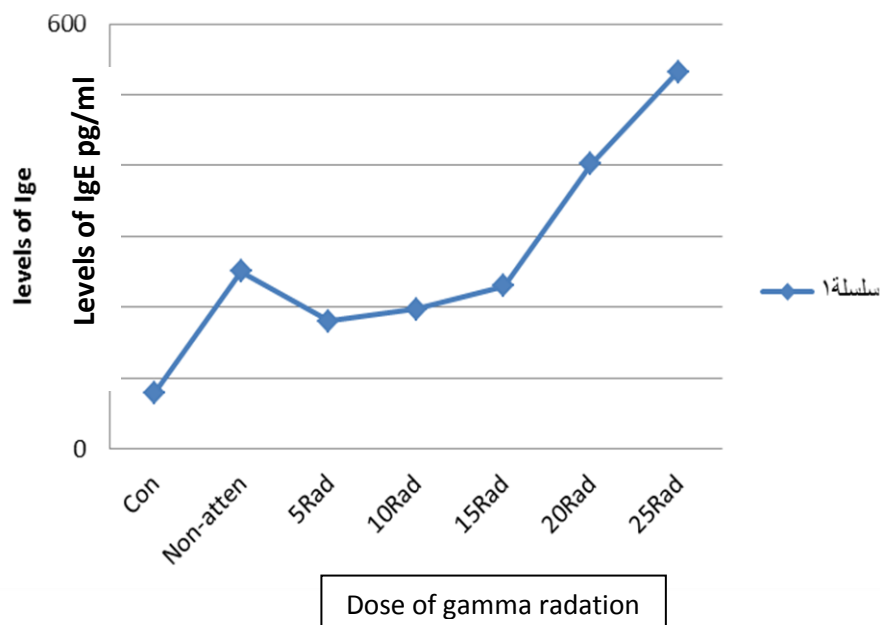
Figure 2-level of *IFN-gamma* in serum of vaccinated rabbits.

The results showed increasing of $IFN-\gamma$ level accompanied with reduce pathogenicity of *E. histolytica* which exposure to gamma radiation. The elevation in the levels of interferon-gamma ($IFN-\gamma$) from stimulated peripheral blood mononuclear cells (PBMCs) and a mucosal IgA antilectin antibody response have been previously associated with protection from *E. histolytica* infection [16]. In result of this study showed that the levels of ($IFN-\gamma$) is higher in animals group that exposure to 25 Rad of gamma radiation than other groups of study from this result we suggested that 25Rad a good dose which recommended in prepared vaccine against *E. histolytica*. this result is compatible with [26] who proved that the immune response of good vaccine type evaluated by($IFN-\gamma$) levels, the good vaccine give higher levels of ($IFN-\gamma$) in the serum. $IFN-\gamma$ expression levels were significantly higher in asymptomatic *E. histolytica* infections than in asymptomatic *E. dispar* infections, which suggested a role of $IFN-\gamma$ in limiting *E. histolytica* invasion[4].

Effect of Gamma Radiation on Immunoglobulin E (IgE) Level.

Levels of IgE in rabbits serum was increase when administrate the rabbits with different doses of attenuated *E.histolytica* cyst with (5,10,15,20,25)Rad of gamma radiation(180.7, 197.6, 230.11, 402.33, 532.44) mg/ L in comparsion to non-attenuated *E.histolytica* cyst and control groups figure-3. It reach to (78.20) mg/L.

The immunological response to parasite infection remain unknown, the parasite antigens stimulated Th2 response with production of interleukin -4 and interleukin- 5 which induce to the synthesis of Immunoglobulin E (IgE) and eosinophils activation [27]. IgE is considered to play a central role in protective immunity against parasites, not only helminths but also some protozoa such as *E.histolytica* [26].From our study we found that the level of IgE elevated in animal vaccinated with attenuated *E.histolytica* cyst (25Rad) more than in control group or group with non- attenuated cyst (alive cyst), meaning that we can used gamma radiation in order to elevate the host immune response against *E. histolytica* infection.



P=0.012

Figure 3- level of (IgE) in the serum of vaccinated rabbits.

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