EVALUATION OF THE IMMUNE RESPONSES INDUCED BY EXPERIMENTAL INFECTION OF (BALB/c) MICE WITH SALMONELLA HADAR

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ABSTRACT

The present study bases on evaluate the immune responses due to experimental infection of (BALB/c) mice by *Salmonella hadar* .

The experiment was carried out on eighty mice of both genders with age range (6 – 8) weeks old, the mice were divided randomly into three groups (group A:- contain 20 mice were administrated orally with infectious dose (1.5×10^7C.F.U\ml) ,group B:- contain 40 mice were administrated orally with LD_{50}dose (1.5×10^9 C.F.U\ml) and groupC:- contain 20 mice which inoculated orally with 1 ml of PBS (pH=7.2) and consider as control group).

The study has noticed that the experimentally infected mice are able to induce humoral immune response which represented by producing antibody against *Salmonella hadar* and this production was elevated after two weeks of administration and reach the peak after four weeks post infection then decline sharply after passage of six weeks post infection in both groups (A and B) but the titration of the antibodies in group B was higher on that recorded in group A.

It is obvious that *S. hadar* is able to induce cellular immune response during experimental infection with infectious dose and LD_{50} dose and the results of delayed type hypersensitivity have showed increases in the thickness of the right footpads of the mice of both groups (A and B) and the highest mean of the thickness was after 24 hours post immunization.

Finally, we concluded that *Salmonella hadar* in infected the host was able to induce both humoral and cellular immune responses and these responses are dose dependent.

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INTRODUCTION

The bacterium *Salmonellae* consider as an important epidemiological zoonotic agent that cause diarrhea, enteritis and systemic disorders in both human and animals (1).Immune responses to *Salmonellae* depend on the host species and the *Salmonella* serotype infecting the host. Three lines of defenses are present in intestine (2):

(i)Natural defenses: stomach acidity, bile salts, mucus, motility and permeability.

(ii)Innate immune responses: Ag capture, cytokine secretion and Toll like receptors (TLRs).

(iii)Acquired immune responses namely oral tolerance (OT) and secretory IgA (sIgA) response. All of them interact together.

It is widely accepted that cell-mediated immunity is more important than humoral responses in protection against *Salmonella* infections (3) ; although most of these studies come from *S. typhimurium* infection in mice where a typical typhoid like infection is produced and how far this is true for disease-free gut colonization is unclear (4).

Both cellular and humoral immune responses are stimulated by intra-peritoneally administered heat-killed and live *Salmonella* vaccines in mice, the difference being the stimulation of Th1 or Th2 responses, which either direct B-cells to switch to IgG2α via live organism stimulation of Th2/IL-4 or switch to IgG1 following stimulation of IFN-γ, producing Th1 cells by killed *Salmonella* (5 , 6).

The humoral immune response is not completely protective, but the free or cell bound antibodies play an important role as opsonizing agents in the initial stages of infection (7). Secretory IgA is considered to be particularly important, as these antibodies may bind to the bacteria and prevent their uptake by macrophages and epithelial cells of the intestine (8).

In mice, Th1 cytokines, which enhance cell-mediated responses, are crucial for protective immunity against a primary *Salmonella* infection (9 , 10). Evidence for the importance of Th1 responses comes from experiments using interferon gamma (IFN-γ) receptor knockout mice and mice with neutralizing antibodies to interleukin-12 (IL-12), which are unable to resolve infection by an attenuated *Salmonella* strain (11, 12 , 13).

The aim of this study was to evaluate the humoral and cellular immune response after infection of mice with two doses of *Salmonella hadar*

MATERIALS AND METHODS

❖ **Laboratory animals :**

A total of 80 mice (BALB/c) of both gender with age range (6 – 8) weeks old, which obtained from the (National Center of Researches and Drugs Monitor in Baghdad) that adapted for two weeks before started experiment then divided into 3 groups:

- Group (A): contain 20 mice orally administrated with infective dose (ID).
- Group (B): contain 40 mice administrated orally with LD_{50} .
- Group (C): contain 20 mice administrated orally with phosphate buffer saline.

❖ **Preparation of the bacteria**:

In this experiment, *Salmonella hadar* strain which was isolated from goats and identified in the Central Public Health Laboratories/Ministry of Health, and it was preserved in brain heart infusion broth and brain heart infusion agar.

❖ **Preparation of Infectious dose (ID) and LD_{50} :**

The ID and the LD_{50} dose of *Salmonella hadar* were prepared according to(14).

Each five colonies of *S. hadar* was removed from brain heart infusion agar and inoculated in 10 ml of Brain heart infusion broth at 37 ˚C for 18 hours then centrifuged in cooling centrifuge (8000) rpm (round per minute) for 15 minutes then the sediment was washed three times with PBS (pH=7.2) and suspended by using 1 ml of PBS (pH=7.2) and diluted at ten fold dilution (10^{-1},10^{-2}.10^{-3},10^{-4},10^{-5},10^{-6},10^{-7},10^{-8},10^{-9} and 10^{-10}).

The viable count of the bacteria in each diluent was made according to method of (15) and diluention which contain (1.5×10^7 C.F.U/ml) as infectious dose and (1.5×10^9 C.F.U/ml) as LD_{50} were selected.

❖ **Infection of the mice:**

The mice in group "A" were drenched orally with 1 ml of *S. hadar* suspension contain (1.5×10^7 cells) as ID and group "B" mice also were drenched orally with 1 ml of bacterial suspension contain (1.5×10^9 cells) as LD_{50} while group "C" mice was drenched orally with 1 ml of phosphate buffer saline (PBS, pH=7.2) and considered as control group.

❖ **Preparation of soluble antigen (salmonellin):**

Soluble antigen which used for the passive haemagglutination test and skin test was prepared according to (16).

Briefly a bacterial suspension of *S. hadar* obtained from overnight brain heart infusion agar culture was sonicated for 50 minute at intervals in a water-cooled sonicator oscillator at 40 MHZ/second and the homogenate was centrifuged twice by using cooling centrifuge at 8000 rpm for 30 minutes each time to remove cellular debris. The supernatants passed through a (0.22 µm) Millipore filter and stored at (-20 ˚C) until used. The protein was estimated by using Biuret kit (Randox Lab.) according to (17).

❖ **The immunological tests:**

(1) The passive haemagglutination test:

This test was used to estimate the humoral immune response according to method of (18) and estimating the titrations of the antibodies in response to *S. hadar* infection in intervals of 2^{nd} , 4^{th} & 6^{th} weeks post infection.

*** Materials of this test:**

- Sheep RBCs (which preserved in AL-Sever's solution for 72 hours pre-using.
- Tanned RBCs "tRBCs" **(**the sheep RBCs are mixed with equal volume of tannic acid solution (1:20000).
- Sensitized washed tanned RBCs **(**Serial dilutions of soluble antigen were prepared by using PBS (pH=6.4) to sensitize washed tRBCs and to detect the optimal antigen concentration which give a positive reaction with higher antibody dilution**).**
- Serum samples **(**The serum samples were incubated 56 ˚C for 30 minutes in a water bath to impair the complement activity).

(2) The delayed type hypersensitivity test (skin test):

This test was used to estimate the cell mediated immune response according to method of (19). briefly about (0.05) ml of soluble antigen of *Salmonella hadar* which contain protein at a concentration of (800 µg/ml) was injected intradermally in right hind footpad of (A, B and C groups) while left hind footpad was injected by (0.05) ml of sterile PBS (pH=7.2) for all groups and thickness of skin was measured by Vernier caliber before and 24, 48 and 72 hours post injection.

❖ **The statistical analysis:**

Statistical analysis was conducted to determine the statistical differences among the tested groups by using ready–made statistical design: statistical package for social science (SPSS).

RESULTS AND DISCUSSIONS

(1) The result of The passive haemagglutination test:

All groups have showed negative results for passive heam-agglutination test before starting the experiment, but after inducing infection by *S. hadar* , the group (A) which infected

by infectious dose and group (B) which infected by LD_{50} showed different results of antibodies titer at 2nd, 4th and 6th weeks after induced infection as showed in the (tables No. 1 , 2).

The mice which was infected by infective dose, after two weeks showed antibody titers with a mean ± SE(28.00 ± 4.00) but after four weeks, the antibody titers raised to reach a peak with a mean± SE (64.00 ± 22.62) while in the six weeks after infection, there was drastic reduction in the antibody titers to reach a mean± SE (9.00 ± 2.51). In contrast, the mice were infected by LD_{50} showed antibody titers after two weeks after induced infection with a mean± SE (33.00 ± 12.26) then after four weeks from adiminstration, the antibody titers raised to reach a peak with a mean ± SE (320.00 ± 235.15) but after six weeks post infection, a drastic reduction in the antibody titers to reaches a mean± SE (18.00 ± 5.03). No antibody titers have detected in the mice of the control group (C) during the same intervals.

Statistically, there was a significant inside and between groups at a level (5%) only. In the group (A) there was a significant variation between the value of antibody titers in the forth weeks and sixth weeks post infection at a level (5%), while in the level (1%) there was no significant variation in the antibody titers among the 2nd , 4th and 6th weeks post infection while in group (B) there was only significant variations between the antibody titers in the 4th weeks and 6th weeks post infection at a level (5%) only, while in the level (1%) there was no significant variations in the antibody titers among the 2nd, 4th and 6th weeks post infection.

Also there was significant variations between the antibody titers in the mice of group (A) and that found in group (B) at the two levels (5% and 1%) with highest titer of the antibody titers of the mice of the group (B) which infected by LD_{50} dose.

Table (1): Means± SE of the antibody titers in mice infected with *S. hadar*

Weeks after infection	Group (A)	Group (B)	Group (C)
	Infectious dose	**LD_{50}**	**PBS**
	Mean ± SE*	Mean ± SE	Mean ± SE
0	0	0	0
2nd	28.00±4.00	33.00±12.26	0
4th	64.00±22.62	320.00±235.15	0
6th	9.00±2.51	18±5.03	0

* SE: Standard error.

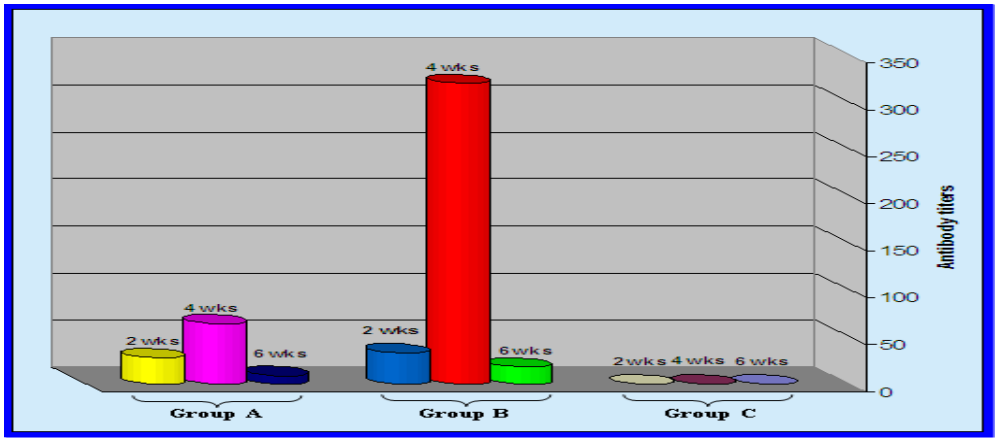

Fig. (1): Antibody titers of the three groups (A, B and C)

The present study has noticed that the experimentally infected mice are able to produce humoral immune response which represented by production of antibody against *Salmonella hadar*, this is compatible with that mentioned by (20and 21).

It is noticed that the titers of the antibody were highly in survival mice of group (B) which was administrated by large cells numbers of *S. hadar* (1.5×10^9 cells) when compared with the mice of group (A) which was administrated less numbers of *S. hadar* (1.5×10^7 cells), this gives an impression that the antibody levels increase with increasing the given dose, so the study agrees with (21) in that the antibody response is dose dependent.

(2) The result of delayed type hypersensitivity test(skin test):

The results of delayed type hypersensitivity have showed increases in the thickness of the right footpads of the mice of both groups (A and B) and the highest mean of the thickness was after 24 hours post immunization then declined after 48 hours and returned to normal thickness after 72 hours post injection of soluble antigen as showed in the (Table 2).

In group (A), the mean of footpad thickness (measured by mm unit) after 24 hours was (1.80 ± 0.01) and after 48 hours was (0.50 ± 0.04) then declined after 72 hours to reach (0.07 ± 0.04). While in group (B) the mean of footpad thickness after 24 hours was (2.20 ± 0.12) and after 48 hours was (0.85 ± 0.15) then declined after 72 hours to reach (0.02 ± 0.02). But, the control group (C) does not show any reaction after injection of the soluble antigen of *S. hadar*.

Table(2):The thickness of the right footpads of mice infected with *S.hadar*

Periods after injection of soluble antigen	Group (**A**)	Group (**B**)	Group (**C**)
	Skin thickness	**Skin thickness**	**Skin thickness**
	Mean* ± SE**	Mean ± SE	Mean ± SE
0 hours	0	0	0
24 hours	1.80 ± 0.01	2.20 ± 0.12	0
48 hours	0.50 ± 0.04	0.85 ± 0.15	0
72 hours	0.07 ± 0.04	0.02 ± 0.02	0

* The thickness of footpads was measured by millimeter unit (mm).
** SE: Standard error. Numbers of mice in each group = 4

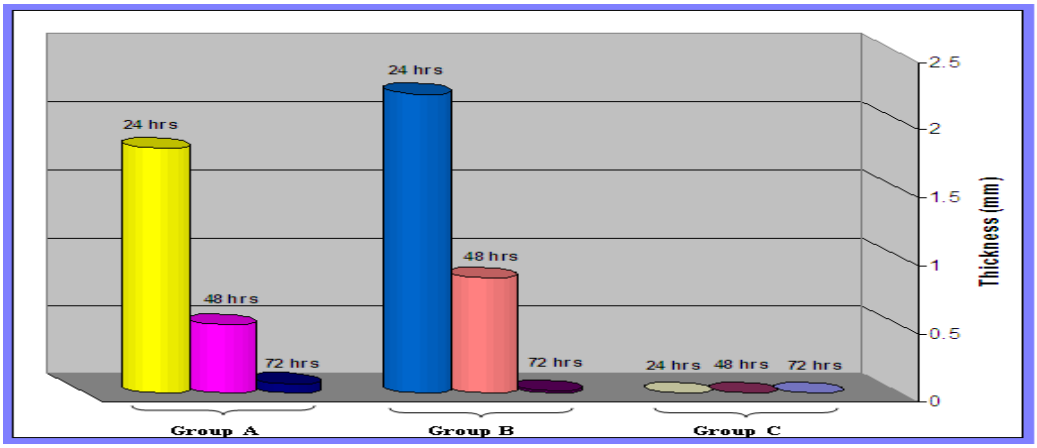

Fig. (2): Thickness of the right footpads of the three groups after injection of soluble antigen of *S.hadar*

The delayed type hypersensitivity test used to detect the cell mediated immune response in the infected mice with live virulent strain *S. hadar*.

this cellular immune response induced by *Salmonella hadar* was appeared resemble that recorded by many researchers who have found the same result of cellular immune response induced by the some serovars belong to genus *Salmonella* such as *Salmonella dublin* (22) and *Salmonella typhimurium* (23) or by other different genus of bacteria such as *Listeria monocytogenes* (24), *Shigella dysenteriae* (25) and *Brucella melitenses* (26).

The skin test is widely used and considered as a diagnostic method for detecting the cellular immune response. The main cause of the skin thickness is the aggregation of huge numbers of lymphocytes which may reach to hundred times more than in normal condition; specifically previously sensitized T-lymphocytes released the chemokines that attracted the phagocytic cells (especially the macrophages) and the inflammatory cells into injection site of the antigen.also concluded that the increases in the antibodies level at 4^{th} weeks post infection was certainly due to systemic dissemination of the bacterium in the host body and causes bacterimia that initiate the B cells to excessive production of antibodies specific for *Salmonella hadar*,this support the consider of *Salmonella hadar* is invasive strain among *Salmonellae* strains.

تقييم الاستجابة المناعية نتيجةً لإصابة الفئران نوع (BALB/c) تجريبياً بجرثومة *Salmonella hadar*

الخلاصـــــة

صمم االبحث لغرض تقييم الاستجابة المناعية نتيجة لإصابة الفئران نوع (BALB/c) تجريبياً بجرثومة *Salmonella hadar* .

أجريت الدراسة على ثمانين فأرة من نوع (BALB/c) من كلا الجنسين وتراوحت أعمار الفئران من ستة إلى ثمانية أسابيع . قسمت الفئران عشوائياً إلى ثلاثة مجاميع (المجموعة(A) احتوت على 20 فأرة وجرعت فموياً بجرعة الإصابة بمقدار 1 ملي لتر يحتوي على(1.5×10^{7}C.F.U)والمجموعة (B) احتوت على 40 فأرة وجرعت فموياًً بللجرعة القاتلة النصفية بمقدار 1 ملي لتر يحتوي على (1.5×10^{9} C.F.U) والمجموعة الأخيرة (C) احتوت على 20 فأرة واعتبرت مجموعة السيطرة وجرعت فموياًً بمقدار 1 ملي لتر من محلول دارئ الفوسفات الملحي).

أثبتت الدراسة الحالية إن الفئران المصابة تجريبياً أظهرت استجابة مناعية خلطية والتي قيست باستخدام اختبار التلازن الدموي المنفعل والمتمثلة بإنتاج الأجسام المضادة لجراثيم *S. hadar* وان إنتاج الأجسام المضادة ارتفع بعد مرور أسبوعين من إحداث الإصابة وبلغ اعلى معيار خلال الأسبوع الرابع ثم انخفض بحدة بعد مرور ستة أسابيع من الإصابة في المجموعتين(A و B) ولكن مستوى الاضداد في المجموعة ب تفوق على مستوى الاضداد الذي سجل في فئران المجموعة A.

ان جرثومة *S. hadar* لها القابلية على إحداث استجابة مناعية خلوية عند إصابة الفئران بالجرعة الاصابة أو الجرعة القاتلة النصفية حيث أظهرت نتائج فحص الحساسية المتأخر (الفحص الجلدي) زيادة في سمك جلد راحة القدم اليمنى في فئران المجموعة (A) وفئران المجموعة (B) وكان أعلى معدل لفرق السمك لراحة القدم اليمنى بعد مرور 24 ساعة من حقن المستضد الذائب لجرثومة *S. hadar* .

أخيرا نستنتج إن جرثومة *S. hadar* بعد إصابتها للمضيف لها القدرة على إحداث استجابة مناعية خلطية وخلوية وهذه الاستجابة تعتمد على جرعة البكتريا المحدثة للإصابة .

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