Immune response study of *salmonella mbandaka* isolated from human in (balb/c) mice

Afaf Abdul Rahman Yousif ; *Zinah Shakir Shallal

Department of Veterinary of Internal and Preventive Medicine, College of Veterinary Medicine /University of Baghdad, head of Zoonosis unit

* Department of biology/College of science/University of Wasit.

دراسة الاستجابة المناعية لبكتريا *Salmonella Mbandaka* المعزولة من الإنسان في الفئران نوع (BALB/c)

عفاف عبد الرحمن يوسف ; زينة شاكر شلال*

قسم الطب الباطني الوقائي / رئيس وحدة الامراض المشتركة/ كلية الطب البيطري /جامعة بغداد

*قسم علوم الحياة / كلية العلوم /جامعة واسط

المستخلص

أعد البحث لغرض تقييم الاستجابة المناعية نتيجة لإصابة الفئران نوع (BALB/c) تجريبياً ببكتريا *Salmonella Mbandaka*. أجريت الدراسة على مئة فأرة من نوع (BALB/c) من كلا الجنسين وتراوحت أعمار الفئران من ستة إلى ثمانية أسابيع. قسمت الفئران عشوائياً إلى مجموعتين (المجموعة (A) شملت 50 فأرة وجرعت فموياً بجرعة الإصابة بمقدار 1 ملي لتر يحتوي على (من البكتريا 1.3×10^7 C.F.U) والمجموعة (B) شملت 50 فأرة واعتبرت مجموعة السيطرة وجرعت فموياً بمقدار 1 ملي لتر من محلول دارئ الفوسفات الملحي).

أثبتت الدراسة الحالية إن الفئران المصابة تجريبياً عن طريق الفم أظهرت حصول استجابة مناعية خلطية وخلوية. قيست المناعة الخلطية باستخدام فحص الاليزا بعد أعطاء جرعة الإصابة للفئران وصل أعلى مستوى للأضداد (IgG) (211.13) بعد مرور أربعة أسابيع وانخفض قليلا ليصل معدله (29.47 ± 179.41) بعد ستة أسابيع من الإصابة بينما بعد الاسبوع الثامن انخفض معدل IgG ليصل إلى (34.56 ± 58.83) , بينما لم تظهر المجموعة B سوى المعدلات الطبيعية من ال IgG (1.11 ± 20.22 , 1.71 ± 20.79 , 19.81 ± 0.88 , 1.11± 20.22 , 0.57 , 20.97±) في وقت الصفر و2,4,6,8 أسبوع على التوالي. أما المناعة الخلوية فتم قياسها باستخدام الفحص الجلدي (فحص الحساسية المتأخر) فكانت درجة التثخن في راحة القدم اليمنى للفئران المجرعة بجرعة الإصابة هي (2.85±0.06) ملم بعد مرور 24 ساعة ثم ارتفعت لتصل إلى (3.69±0.07) ملم بعد مرور 48 ساعة ثم انخفضت لتصل إلى (2.71±0.09) ملم بعد مرور 72 ساعة ؛ استنتجت هذه الدراسة ان الفئران المصابة بالبكتريا قادرة على حث الاستجابة المناعية الخلطية والخلوية.

Abstract

The bases of this study is to evaluate the immune response to experimental infection of (BALB/c) mice by *Salmonella Mbandaka* . The experiment was carried out on one hundred mice of both genders with age range (6 – 8) weeks old, the mice were divided randomly into two groups (group A: includes 50 mice were given orally on infective dose (1.3×10^7C.F.U\ml) ,group B: includes 50 mice which inoculated orally with 1 ml of phosphate buffer saline (pH=7.2) and considers as control group).The study has showed that the experimentally infected mice (group A) are able to induce humoral and cellular immune response . The humoral immune response was detected by Enzyme-linked Immunosorbent Assay post induced infection in mice with infected dose. Maximum titers of (IgG) antibodies reached of (211.13) at four weeks , slight reduction at six weeks , reached a mean (179.41 ± 29.47), while in the eight weeks after infection, there was a reduction in the(IgG) titers to reach a mean (58.83 ± 34.56). While group B showed the normal range of IgG titer (20.22 ± 1.11 , 20.79 ±1.71 , 19.81 ± 0.88, 20.22 ± 1.11 , 20.97 ± 0.57) at zero time , 2 ,4 , 6 ,8 weeks respectively .

The cellular immune response was detected by using the skin test mean thickness of the right foot pad was (2.85±0.06) millimeter after 24 hours, then increased to reach (3.69 ±0.07) mm after 48 hours , then declined to reach (2.71±0.09)mm after 72 hours in mice infected by infective dose. This study concluded that *Salmonella Mbandaka* infection of the host was able to induce both humoral and cellular immune responses, and these responses are dose dependent.

Introduction

Salmonella bacteria causes a widevariety of diseases and disease syndromein human beeing, different animals and birds, it remains as serious problem with public health significance throughout the world (1).

In human enterocolitis caused by S*almonella* bacteria is characterized by sudden onset of diarrhea which may be bloody, nausea, vomiting, abdominal pain, and headache (2). If *Salmonella* organisms are ingested and pass successfully through the stomach, they will enter the intestine. From here *Salmonellae* may invade the bowel wall through specialized epithelial cells, which overlay intestinal lymphoid tissue. This encounter the first line of specific immune defense. As soon as the host is infected, the immune system produces a rapid humoral response. John and Bradford

(2004) (3) observed that when cows were vaccinated twice with killed *Salmonella* bacterins prior to parturition, They pass IgG antibodies to calves through colostrum, and this protection appears to be partial until the calf reaches 3 weeks of age, after which it wanes rapidly.A study done by Amany *et al.* (2009) (4) investigated the immune response elicited in mice after S/C immunization with flagellin from *S. enterica* serovar *enteritidis*. Grassl *et al*. (2008) (5) found during active clinical disease, the affected gut tissue often exhibits extensive damage to the epithelium and underlying tissues along with the expression of a characteristic cytokine profile dominated by interleukin IL-1, IL-17, tumor necrosis factor (TNF)-α, and interferon gamma (IFN)-γ. The concerted action of several cytokines, including tumor necrosis factor (TNF-α), interferon gamma (IFN-γ), interleukin IL-12, IL-15 and IL-18, is essential for the adaptive phase of the immune response. TNF-α is involved in the formation and persistence of granulomas, as well as, in the regulation of NADPH oxidase - mediated killing of *Salmonella* by macrophages.

Materials and methods

Laboratory animals

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

 Group (A): includes 50 mice orally administrated with infective dose of *Salmonella Mbandaka* .

 Group (B): includes 50 mice administrated orally with phosphate buffer saline.

Preparation of the bacteria:

 Salmonella Mbandaka was obtained from child with severe diarrhea, feverish and dehydrated. *Salmonellae* isolate were confirmed in the Central Public Health Laboratories (National Center of *Salmonellae* in Baghdad).

Preparation of infective dose (ID)

The ID dose of *Salmonella Mbandaka* was prepared according to procedure of Shallal (2011) (7). Each five colonies of *S. mbandaka* were 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 for 15 minutes. The sediment was washed three times with phosphate buffer saline (pH=7.2) and suspended by

using 1 ml of PBS (pH=7.2) and diluted at tenfold 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 Miles and Misra, (1938) (8) and diluention which contain (1.3×10^{7} C.F.U/ml) as infective dose.

Infection of the mice

The mice in group "A" were drenched orally with 1 ml of *S. mbandaka* suspension contain (1.3×10^{7} cells) as ID and group "B" mice also were 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 skin test was prepared according to Mitov *et al*. (9).

Briefly a bacterial suspension of *S. Mbandaka* 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 its use. The protein was estimated by using spectrophotometer (Ultra-violet absorption) according to Alan and Robin (1987) (10).

The ratio of absorbance 260:280 nm was calculated. This should be below 0.6; high ratio indicates that the protein was contaminated with interfering substances, notably nuclic acid.For mixtures of proteins or for any protein with unknown extinction coefficient:-

Protein Concentration =1.55 × absorbance at 280 nm - 0.77 × absorbance at 260 nm.

The immunological tests

(1) The Humeral Immunity Test: by using ELISA test after collection of serum as follows:-

Blood samples were collected from the both groups of mice at 2 weeks, 4 weeks ,6 weeks and 8 weeks post infection, then serum separated and kept at (-20 ˚C) .The test is carried by

Enzyme-linked immunosorbent assay (ELISA) method

A-This test was done according to manufacturer **(immunological consultents laboratory, Inc.)**

(2) - Cellular immunity:- Delayed type hypersensitivity test (DTH) (skin test):

Whichdone according to Hudson and Hay (1980) (11) as below:

(0.05) ml of soluble antigen of *Salmonella Mbandaka* which contains   protein as (250 μg/ml) was injected intradermally in right hind footpad of **A** and **B** group   while left hind footpad was injected by (0.05) ml of sterile phosphate buffer saline (pH=7.2) for two groups and thickness of skin was measured by Vernier caliper before and 24, 48 and 72 hours post injection.

Statically analysis

Chi square 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 discussion

(1) The results of Enzyme-linked Immunosorbent Assay (ELISA) method:

Table (1) showed the results of standard. The mice of group (A) showed different results of (IgG) titer at 2nd, 4th, 6th &8th weeks after induced infection as showed in table 2 , Fig 1.

The mice which infected by infective dose, after two weeks showed   (IgG) titers with a mean (29.27 ± 3.25),but after four weeks, the (IgG) titers raised to reach a peak with a mean (211.13 ± 50.11) .Infection after   six weeks, there was slight reduction in the (IgG) titers to reach a mean (179.41 ± 29.47) While in the eight weeks after infection, there was a large reduction in the(IgG) titers to reach a mean (58.83 ± 34.56).   Moreover the level of   (IgG) in group (B) remain with normal range in all times at zero time was (20.22 ± 1.11) , two weeks (20.79 ± 1.71) , four week (19.81 ± 0.88), six weeks (20.22 ± 1.11) and eight weeks (20.97 ± 0.57).

Table (1): The results of optical density compared with standards in ng/ml.

Standard	O.D* (nm/ml)	Concentration (ng/ml)
A	1.632	25500
7	1.579	600
6	1.380	300
5	1.181	150
4	0.965	75
3	0.701	37.5
2	0.410	18.75
1	0.318	9.375
0		

O.D*:Optical density

Table (2): Means of the antibody (IgG) titers in the two groups of mice

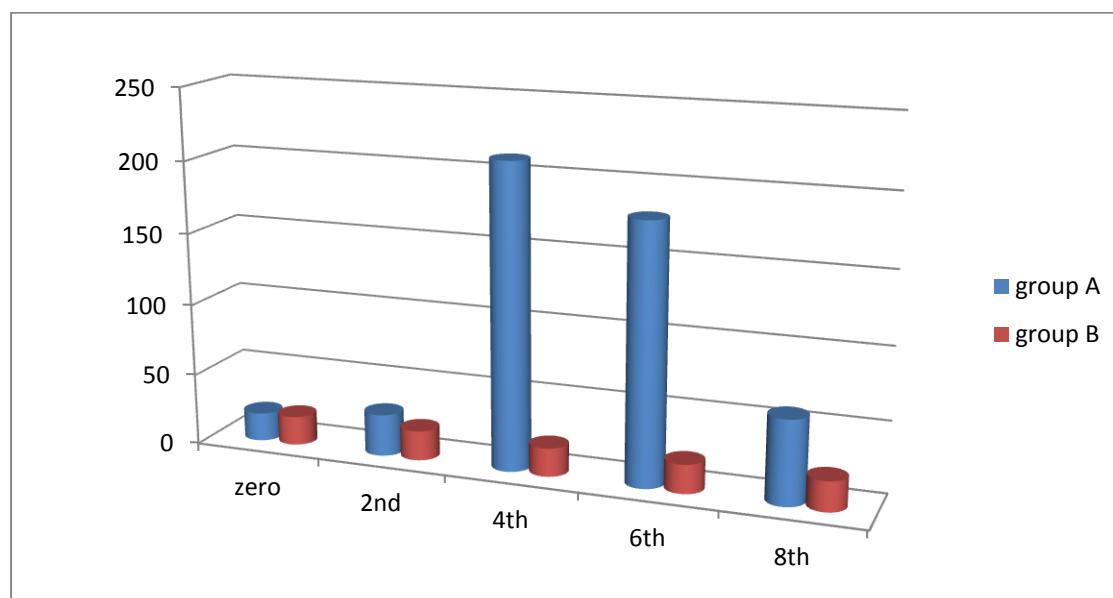| Weeks	Group (A) Infectious dose. Mean IgG ± SE**	Group (B) PBS. Mean IgG ± SE**
zero	19.81 ± 0.88	20.22 ± 1.11
2nd	29.27 ± 3.25	20.79 ± 1.71
4th	211.13 ± 50.11	19.81 ± 0.88
6th	179.41 ± 29.47	20.22 ± 1.11
8th	58.83 ± 34.56	20.97 ± 0.57

SE: Standard error.**

Figure (1): The antibody (IgG) titers of groups (A and B)

The increase of the (IgG) titers in group (A) indicated that the humoral immune response to the infection of *S. Mbandaka* (12). Immunoglobulin G (IgG) responses was due to both the heterologous antigen (recombinant pneumococcal antigen) rPspA ,*Salmonella* lipopolysaccharide (LPS) and outer membrane proteins (OMPs), after a single oral immunization in BALB/c mice .

This study showed that the experimentally infected mice were able to induce humoral immune response which represented by producing antibody against *S. Mbandaka*. The IgG level raised after two weeks, from infection and reached the peak after four weeks post infection, then decline slightly after passage of six and eight weeks post infection. This is compatible with that mentioned by Sara sombath *et al* . (1987) (13) which showed immunoglobulin G (IgG) rise at (4 weeks to 2 years)   from 14 patients suffered   from typhoid fever   by indirect enzyme-linked immunosorbent assay (ELISA). The mean of IgG in the controls was 30.7 an increment of antibodies was observed, and the peak was seen at week 4,and the IgG persisted (2 years) . Matsiota –Bernard *et al.*(1993) (14) reported IgG in mice during *S. typhimurium* at (7 to 35) days raised on day 15th and continued to increase slightly until day 35, Also Kusumawati *et al* .(2006) (15) measured IgG titers from serum samples of mice at 2 weeks after infection with *Salmonella typhimurium* which showed similar results .

(2) Delayed type hypersensitivity test -skin test response:

The results of delayed type hypersensitivity showed increases in the thickness of the right footpads of the mice of group (A) and the highest of the thickness was after 48 hours post immunization then declined after 72 hours.In group (A),the mean of footpad thickness (measured by mm unit) after 24 hours was (2.85 ± 0.06)mm and after 48 hours was (3.69 ± 0.07) mm then declined after 72 hours to reach (2.71 ± 0.09) mm .But the control group (B) did not show any reaction after injection the soluble antigen of *S. Mbandaka* , as shown in (table 3 , Fig. 2 and 3).

Table (3): Means and standard error of skin test in right foodpads of mice infected with *S. Mbandaka* in different times

Periods after injection of soluble antigen	Group (A)	Group (B)
	Skin thickness	Skin thickness
	Mean* ± SE**	Mean ± SE
24 hours	2.85 ± 0.06 mm	1.84 ±0.009
48 hours	3.69 ± 0.07 mm	1.84 ±0.009
72 hours	2.71± 0.09 mm	1.84 ±0.009

* The thickness of footpads was measured by millimeter unit (mm).

* SE: Standard error.

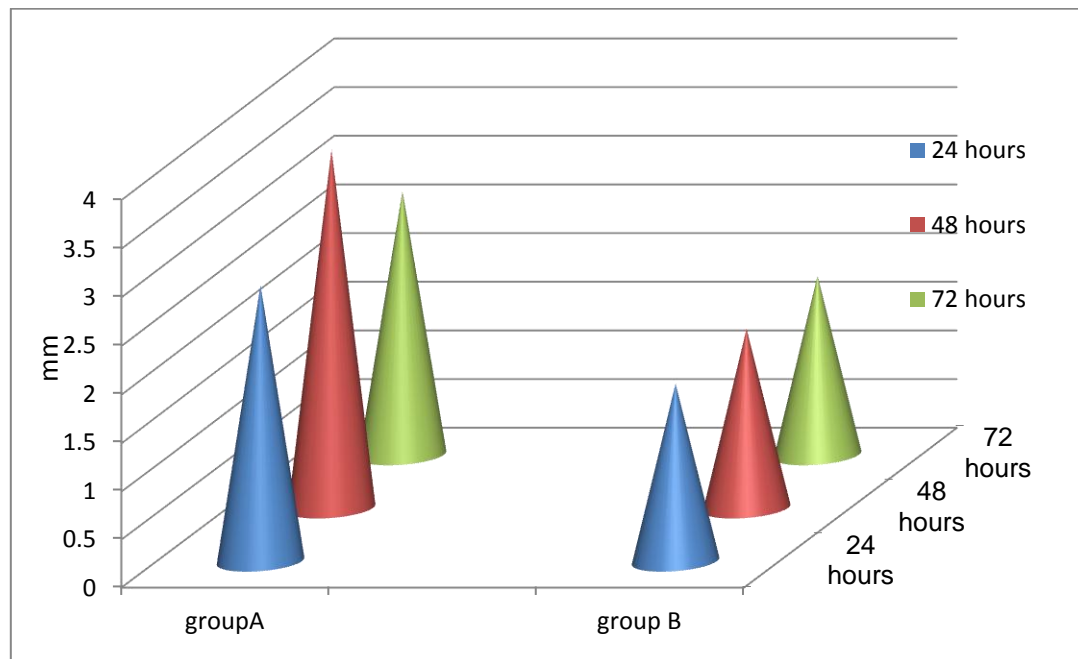**Figure (2): Thickness of the right footpads of the groups after injection with soluble antigen of *S. Mbandaka***

Group (A) after 24 hours post injection soluble antigen.

Group (A) after 48 hours post injection soluble antigen.
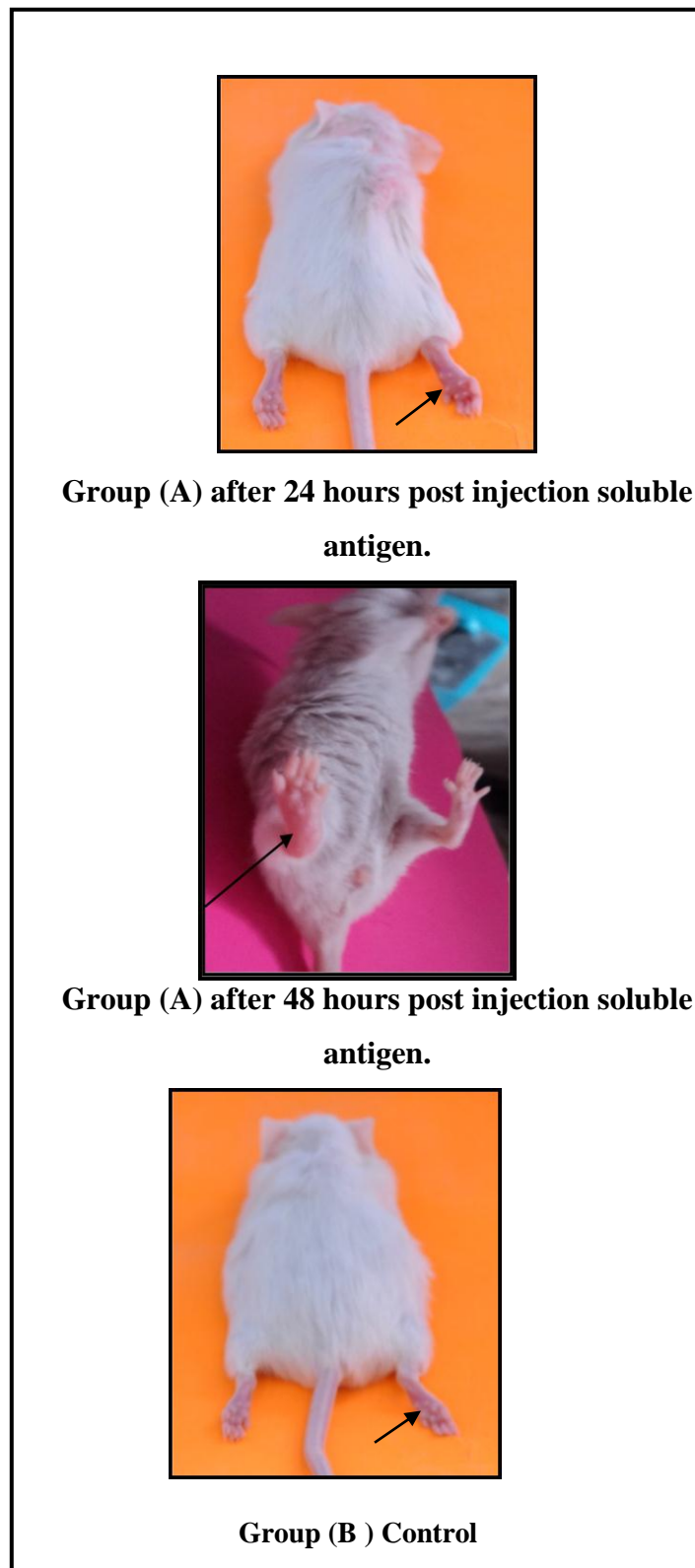
Group (B) Control

Figure (3): Thickness of the right footpads of the groups after injection   with soluble antigen of *S. Mbandak*

It is concluded that *S. Mbandaka* is able to induce cellular immune response during experimental infection with infective dose. So this result   is in agreement with results of Strindelius *et al*. (2002) (16) who used delayed-type hypersensitivity – skin test as a measure of cellular immunity in mice immunized with different types of *salmonella enteritidis* antigens, the mice showed a significant increase in all immunized groups. This cellular immune response induced by *Salmonella Mbandaka* appeared compatible with to that recorded by many researchers who found the same result of cellular immune response induced by some serovars belong to genus *Salmonella* such as *Salmonella hadar* (17) and *Salmonella typhimurium* (18) or by other different genus of bacteria such as *Listeria monocytogenes* (19) *Shigella dysenteriae* (20) and *Brucella melitenses* (21).

The skin test is widely used and considered as a diagnostic method for detecting the cellular immune response. The main cause of skin thickness is the aggregation of huge numbers of monocytic infiltration and macrophage which may reach to hundred times more than in normal condition; specifically previously sensitized T-h1 released the chemokines (IL-1 and IFN-γ) that attracted the phagocytic cells (especially the macrophages) and the inflammatory cells into injection site of the antigen (22).

Conclusion and recommendation

Conclusion

 (1) *Salmonella Mbandaka* stimulated humoral immune response, appeared after   4 weeks of infection showing higher IgG level.

(2) Good cellular immune response after 21 days of infection by *Salmonella mbandaka* .

Recommendations

(1) An immunological study of *Salmonella Mbandaka* in cattle, sheep, goat and chicken.

 (2)Making further studies aims to discover the role of antibodies in disminishing the infection by *Salmonellae.*

(3)Study the immunological characteristics of antigens   *Salmonella Mbandaka.*

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