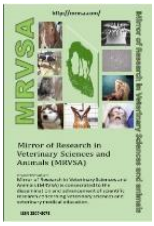
ORIGINAL ARTICLE **Mirror of Research in Veterinary Sciences and Animals MRVSA/ Open Access DOAJ**

Mirror of Research in Veterinary Sciences and Animals (MRVSA)
Journal homepage: http://mrvsa.com/
E-ISSN 2307-8073; 2520-324X (Print)



Cellular immune responses induced in mice by *Salmonella hadar* O antigen

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ARTICLE INFO
Received: 09.11.2012
Revised: 09.12.2012
Accepted: 20.12.2012
Publish online:20.12.2012

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Abstract

This study was designed to investigate the cellular immune response in mice after parenteral immunization with *Salmonella hadar* O antigen with and without Adjuvant. BALB/c mice were divided into three groups (each 25 mice). First group was injected subcutaneously with *S. hadar* O antigen (heat killed O antigen), second group similarly injected with (*S.hadar* O antigen suspended in alum as adjuvant) and control group injected with phosphate-buffered saline. All mice were immunized two times on days 0 and 14. Two weeks after the last immunization, cellular immune responses to *S.hadar* were assessed using E. rosette test at 2nd, 5th and 8th week, while delayed type hypersensitivity test (DTH) skin test used after 21 days of immunization. Then all mice were challenged intraperitoneally with 4LD50 of virulent *Salmonella hadar* eight weeks post immunization. The E.rosette test in mice injected with O antigen and O antigen suspended in adjuvant showed a significant increase in the activation of lymphocyte at 2nd ,5th & 8th week after immunization. While the control group gives normal range of active lymphocyte in all weeks of experiment. DTH skin showed significant increase in thickness of the footpad skin after 24 and 48 hours post inoculation with *S hadar* soluble antigen while the control group didn't show any reaction. A significant protection was observed in the immunized groups challenged with 4 LD50 {4(1X108)} compared with control group of mice which died within 1-2 days. In conclusion, this study indicate that the administration of *S.hadar* O antigen suspended in adjuvant can induce a significant cellular immunity more than non-suspended *S.hadar* O antigen only, but both antigens give a good protection against salmonellosis in mice.

To cite this article: Afaf Abdulrahman Yousif; Hawraa, G. Al-Saadi,2012 Cellular immune responses induced in mice by Salmonella hadar O antigen. Mirror of Research in Veterinary Sciences and Animals. MRVSA 1(1), 35-43.
DOI: 10.22428/mrvsa. 2307-8073.2012.00115.x

Keywords: cellular Immunity, O antigen, adjuvant, E.rosette, DTH skin test

Introduction

Salmonella enterica are Gram-negative bacterial pathogens capable of infecting humans and animals and causing significant global morbidity and mortality (Coburn *et al.,* 2007; Antoine *et al.,* 2008). *Salmonella hadar is now one of the five most frequently isolated serotypes in human and animals (*Valdezate *et al., 2000;* Cailhol *et al.,* 2006*).it* is a highly prevalent food borne pathogen and therefore a major cause of human gastroenteritis worldwide *(*Snoussi *et al.,* 2012*). It is isolated firstly in the early 1950s, from a stool sample of a subject with gastro-enteritis and fever (*Hirsch *et al.,1954).* In Iraq *S. hadar* was isolated from liver and bile of slaughtered goats by (Yousif *et al.,* 2011) also *S. hadar* was isolated from feces of affected children with diarrhea in Thi-Qar province (Harab and Yousif, 2011). Over the last century, the use of vaccines has profoundly reduced the morbidity and mortality caused by infectious diseases in both human and animal populations. In fact, vaccination has proven to be the most successful medical intervention ever developed (Schijns, 2003). It is widely accepted that cell-mediated immunity is more important than humoral responses in protection against *Salmonella* infections; but Mastroeni *et al.,* (1993) found that both cellular and humoral immune responses are stimulated by intraperitoneally administered heat-killed and live *Salmonella* vaccines in mice. Berndt *et al,* (2007) observed that the CD8-T-cell response were as high in infected birds with *S. hadar* also they found that *Salmonella* serovars *typhimurium* and *hadar* are moderately invasive and intermediate immune stimulators. Wilson-Welder *et al.,* (2009) report that the development of vaccine suspended adjuvant can enhance the effectiveness of vaccine, these adjuvants should have the ability to elicit a potent immune response. The efficacy of vaccines depends on the presence of an adjuvant in conjunction with the antigen. Of these adjuvants, the ones that contain aluminium, which were first discovered empirically in 1926, are currently the most widely used. Jazani *et al.,* (2011) conclude that the administration of the alum-naloxone mixture as an adjuvant, in combination with the Heat killed *S. typhimurium* vaccine, can enhance both humoral and cellular immunity and shift the immune responses to a Th1 pattern. The aim of this study is to investigate the efficacy of *S. hadar* O antigen of with and without adjuvant in inducing cellular immunity in mice against challenge with virulent strain of *S hadar*.

Materials and Methods

Bacterial strain

Salmonella hadar was isolated from feces of children suffered from diarrhea in Thi-Qar province in Iraq by (Harab and Yousif, 2011), using selective media, biochemical tests and API 20 (Quinn et al., 2004) . Finally, the isolates confirmed in the National Salmonella Center in Baghdad/ Ministry of public health.

Preparation of *S hadar* O antigen for immunization

Samples of the stock culture of *S. hadar* were used for preparation of the antigen. To produce the inoculate, bacteria were grown statically for 18 h at 37°C in brain heart infusion broth, harvested by centrifugation, and re-suspended in PBS. Bacteria was

washed extensively in phosphate-buffered saline (PBS) three times. Colony counts were performed for all inoculate to verify the number of viable bacteria at 1×10^8 CFU, then bacterial suspension was killed by heating at 100°C for 30 minutes (Smith et al., 1984). Protein content was determined by a biurat method. The antigen was tested for sterility and safety before use according to (OIE, 2004).

Preparation of potassium alum sulphate(adjuvant)

This solution prepared from 50% of potassium alum sulphate. Fifty gram of potassium alum sulphate was dissolved in distal water and the volume completed to 100 ml. sterilized by autoclave for 15 minutes at 15 Ibs with 121 C^o, then this solution used adjuvant at 1:1. The adjuvant was added to *S. hadar* according to (Goerge *et al.,* 1985).

Preparation of soluble antigen

Soluble antigen which used for DTH (skin test) prepared according (Mitov *et al.,* 1992) briefly; three to five colonies from the bacterial isolates on selective medium were inoculated into trypticase soy broth and incubated overnight. The cultures were harvested by centrifugation at 10.000Xg for 30 minutes. The sediment was sonicated for 50 minutes using ice at intervals in a water cooled sonicator oscillator at 40 MHZ per second full power. The homogenate was centrifuged twice by using a cooling centrifuge at 8000 Xg for 30 minutes each time to remove cellular debris. The supernatants were passed through a 0.22 μm Millipore filter and stored at (-20^{o}C) until used. Protein content was determined by biuret protein assay.

Immunization of mice with *S. hadar* O antigen

Seventy-five, (5-8) weeks old healthy mice (BALB/c) of both gender, obtained from National center of researches and monitor of drugs in Baghdad, adapted for two weeks before started experiment in separated clean and disinfected cages, they were fed on assorted pellets and clean water, then divided into 3 main groups (each 25 mice):

First group: Mice were immunized S/C with *S. hadar* O antigen at dose of 0.5 ml containing $1X10^8$ CFU (protein content 200μg/0.5ml).

Second group: Mice were immunized similarly with *S. hadar* O antigen mixed with adjuvant (0.5 ml mixed with 0.5 ml adjuvant. This mixture injected subcutaneously in two places.

Third group (control group): Mice were injected subcutaneouslywith 0.5ml PBS.

Detection of cellular immunity

1-**Delayed type hypersensitivity test DTH (Skin test)**

This test was done according to (Hudson and Hay 1980) after 21 days of immunization. Briefly, 0.1 ml of soluble antigen of *S. hadar* was injected intradermally in the right footpad of the mouse while the left side was injected by 0.1 ml of sterile PBS (pH=7.2). The thickness of the footpad was measured by vernier caliper before and 24, 48, and 72 h after Injection.

2-**E-rosette test**

This test was done according to (Braganza et al, 1975) with some modification. The test used for calculates the percentage of viable and non-viable T-lymphocytes and estimated activity of T-lymphocytes in three steps:

A. **Preparation of RBCs suspension** Three ml of blood was withdrawn from the jugular vein of a ram. The blood was mixed at once with equal volume of Al-severs - solution in order to prevent clotting or lyses of RBCs, the mixture was left for 18 hr at a refrigerator. Then the mixture was centrifuged at 1500 rpm for 5min and then 1 ml of the precipitate (cells) re-suspended in 100 ml (RPMI-1640).

B. **Lymphocytes suspension** was prepared by taken the spleen of a mouse and cut into tiny pieces, the pieces were crushed with a mortar on stainless steel seeped on Petri dish then washed twice with 3 ml (RPMI-1640). The suspension was centrifuged at (1200) rpm for (10) min.

C. **Test A** mixture of 0.25 ml RBCs suspension and 0.25 ml of lymphocytes suspension was prepared in a test tube, the test tube was incubated at 37°C for 15min and a drop was taken by pasture pipette and stained by Trypan blue stain on a slide and was examined by microscope, for T-lymphocyte – erythrocyte rosette forming shape (lymphocyte which attached to more than 3 or more RBCs forming the rosette shape).

Estimating the LD_{50}

Tenfold dilution (10^{-1},10^{-2}.10^{-3},10^{-4},10^{-5},10^{-6},10^{-7},10^{-8},10^{-9} and 10^{-10}) of *Salmonella hadar* were done. The viable counts of the bacteria in each diluent were made according to Quinn et al (2004)**.** The diluents which had these number of bacteria :(1×10^5 C.F.U./ml) , (1×10^6 C.F.U./ml cells) , (1×10^7 C.F.U./ml) , (1×10^8 C.F.U./ml) , (1×10^9 C.F.U./ml) , (1×10^{10} C.F.U./ml) and (1×10^{11} C.F.U./ml) were selected to injected groups of mice intraperitoneal. Forty-eight of healthy mice of both sexes were selected, the body weight was ranged from (25–30) gm and age range (6–8) weeks; they were divided into (8) groups, each group contained six mice. Seven groups out of eight groups of mice injected with (0.5) ml of the intraperitoneal calculated (C.F.U./ml) diluents and the last group injected with PBS (pH=7.2) and considered as a control group. All groups were observed for 30 days to calculate the live and dead mice and estimate the LD_{50} according to (Reed and Muench, 1938).

Statistical Analysis

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

Ethics Approval This study was approved by the ethical and research committee of Veterinary Medicine College/University of Baghdad

Results

Delayed type hypersensitivity test (DTH) Skin test

The first group showed increased in the thickness of skin footpads of the mice and the highest means of the thickness appeared after 24 and 48 hours and return back to normal after 72 hours post injection with soluble antigen of *S.hadar*, in the second group the means

of the thickness appeared after 24 hours and the maximum appeared at 48 hours and. Erythematous signs showed in the footpads of all immunized group. The control group didn't show any reaction or change in the thickness or color of footpads (Table. 1).

Table (1) Skin thickness (millimeters) of the immunized and control groups in DTH test

	Diameter of skin thickness	Diameter of skin thickness	Diameter of skin thickness
Times	First group O Ag	Second group O Ag +adjuvant	Third group Control
0 time	1.56±0.09	1.62±0.07	1.57±0.0597
24 hours	2.56±0.094	2.85±0.12	1.59±0.0640
48 hours	2.09±0.07	2.93±0.12	1.58±0.0611
72 hours	1.68 ± 0.08	2.33±0.13	1.58±0.0611

***E-rosette* test**

The first group which received O antigen without adjuvant showed a highest means of active E. rosette at 2nd and 5th weeks after injection with booster dose (59.0± 2.43&56.0 ±1.14) respectively. The second group which was received O antigen with adjuvant showed the highest increase in active lymphocyte in the 5th and 8th group, with a maximum activation at 5th weeks ranged (66.8 ± 1.83). The results of the second group appeared more increase than in first group. The control group showed a normal range of active lymphocyte (Table 2).

Table (2) Means of active E. rosette in immunized and control groups

	Time	First group	Second group	Control group	P value
Before immunization	0	20.0 ±0.70	19.2±0.86	19.6±0.67	**P> 0.05**
After immunization	2nd	29.8± 1.28	29.8± 1.28	19.8 ± 0.58	**P< 0.05****
After immunization	5th	28.8 ±0.58	33.4 ± 1.31	20.0±0.70	**P< 0.05****
After immunization	8th	26.6 ± 0.81	31.8 ± 0.91	19.4±0.24	**P< 0.05****

SE*:Standard error. **Means significant different (P< 0.05) between groups.

Result of LD_{50} dose

The estimation of *Salmonella hadar* LD_{50} in mice injected intraperitoneally have revealed that the LD_{50} is (1×10^8 C.F.U/ml) which estimated by calculating the dead and alive mice in each group during (30) days (table 3).the percentage of mortality was calculated according to the following equation :

Percent of Mortality = total dead / sum of (total a live + total dead).

Table (3) The results of estimating of LD_{50} of S. *hadar* in mice

(6 mice in	Dose	Alive	Dead	Total	Total	Percent
1	1×10^{11}	0	6	0	21	100 %
2	1×10^{10}	0	6	0	15	100 %
3	1×10^{9}	2	4	2	9	81 %
4	1×10^{8}	3	3	5	5	50 %
5	1×10^{7}	4	2	9	2	18 %
6	1×10^{6}	6	0	15	0	0 %
7	1×10^{5}	6	0	21	0	0 %
8	BPS	6	-	-	-	0%

No. of mice in each group = 6, Total No. of mice = 48 ,The dose calculated as (cells).

Experimental of the challenge

All groups of mice were challenged intraperitoneally with 4 LD_{50} (4 X 10^8) of virulent *S.hadar.* Reaction in the immunized mice were revealed mild signs of illness and depression for 2-3 days without signs of diarrhea and returned normal within 7 days without mortality. The post challenges reaction in non-immunized mice (control group) exhibited the following clinical signs: listlessness loss appetite, anorexia, severe diarrhea, increased respiration rate, severe dehydration, pregnant mice was aborted, and recumbent till death in 1-2 days after challenge.

Discussion

This study was aimed to evaluate the using of *S.hadar* O antigen with and without adjuvant in two doses to avoid the risk of live vaccines; the use of killed organisms was introduced as safer vaccines. This compatible with many researchers (Timms et al,1994; Gast et al, 1993;) using killed vaccine or subunit vaccine and the limitations of these kinds of vaccines are that their immunogenicity usually has to be enhanced by co administration with adjuvant, and, in any case, multiple doses are necessary for obtaining long-term protective immunity. The cellular immune response induced by *Salmonella hadar* which estimated by DHT-skin test in our study appeared resemble to the results recorded by (Yousif and al Naqeeb,2010) in mice infected with *S hadar*, also with a study of (Shallal, 2009) who used delayed-type hypersensitivity (skin test) as measure of cellular immunity in mice immunized with different types of salmonella antigens.

In the present study, E rossete used for detection of immunity, that gives significant positive results with O antigen of *S.hadar* and O antigen + adjuvant. This is resembling with a study of (Yousif and Almansory,2009) which used E-rosette test to detect cellular immunity against other *Salmonella* species (*Salmonella enteritidis*) in rabbits and proved its efficacy. Kumar, (2010) consider the E rosette test as one of the most important discoveries which showed T-lymphocytes form spontaneous E-rosettes with sheep erythrocytes (S RBCs), and proving it as one of the simplest biological markers for identifying T lymphocytes. Our results showed that injection of the O antigen with adjuvant produce more efficient cellular immunity more efficient than that of O antigen without adjuvant, this is in compatible with (Mazloomi *et al.*, 2012) who mix the adjuvant with killed antigen to increase the vaccine's efficacy, it shown lymphocyte proliferation in mice more than the killed vaccine alone. The role of adjuvant in vaccine development were Increase the total antibody titer or functional titers; Decrease the dose of antigen needed; Decrease the total number of doses of vaccine necessary for complete immunization; Overcome competition in combination vaccines and Induce potent cell-

mediated immunity (Dekker et al, 2008). The immunized groups in our study injected intraperitoneally with 4 $LD_{50\ were}$ resist the effect of lethal challenge and all mice were live, this may contribute to the immunity that induced after immunization with O antigen and O antigen with adjuvant and it its ability to reduce the appearances of sever clinical signs of salmonellosis. While the control group showed sever clinical signs of salmonellosis and dead within 1-2 days from challenge. These results are in agreement with many researcher reported that immunization of mice with *Salmonella* species induced strong cellular immunity and resist the lethal challenge of virulent *Salmonella* (Karasova, 2009; Yousif and Al-Mansory 2009; Simon,2011) .

In conclusion, the results of this study indicate that the *S.hadar* O antigen and O antigen with adjuvant can be used as safe and effective tool for prevention of *Salmonella* infection, due to its ability to induce the protective cellular immune response.

References

Antoine ST, Annaelle K, Anne B. (2008). Epidemiological analysis of *Salmonella* enterica from beef sampled in the slaughterhouse and retailers in Dakar (Senegal) using pulsed-field gel electrophoresis and antibiotic susceptibility testing. J Food Microbiol. 123: 191-197.

Berndt A, Wilhelm A, Jugert C, Pieper J, Sachse K and Methner U. (2007). Chicken cecum immune response to *Salmonella enterica* serovars of different levels of invasiveness. Infection and Immunity., 75(12): 5993–6007.

Braganza CM,Stathopoulos G Davies AJS Elliot, EV and Kerbel RS. (1975). Lymphocytes Erthrocytes (L.E) Rosettes as indicators of the heterogenecity of lymphocytes in variety of mammalian species:Cell. 4(1) 103-06.

Cailhol J, Lailler R, Bouvet P, Lavieille S. Gauchard F, Sanders P, Brisabois A. (2006) Trend in antimicrobial resistance phenotypes in nontyphoid Salmonella from human and poultry origins in France. Epidemiol Infect.134 (1):171-178.

Coburn B Grassl GA, and Finlay BB. (2007). *Salmonella*, the host and disease: a brief review. *Immunol Cell Biol* 85**:** 112–118.

Dekker CL, Gordon L and Klein J**. (2008).** Dose Optimization Strategies for Vaccines: The Role of Adjuvants and New Technologies. NVAC Subcommittee on Vaccine Development and Supply.

Gast RK, Stone HD, Holt PS. (1993). Evaluation of the efficacy of oil-emulsion bacterins for reducing fecal shedding of Salmonella enteritidis by laying hens. Avian Dis. 37(4):1085.

George FI, Graham HF, Mary JL, Reta AW. (1985). Production of potent *Salmonella* H antisera by immunization with polymeric flagellins. J.Clin. Microbiol. 22: 347-351.

Harab AA and Yousif AA. (2011). Isolation and serotyping of salmonella species in diarrheal children Thi-Qar Medical Journal (TQMJ). 5 (1):149-155.

Hirsch W, Gerichter CB, Bregman E, Lubling P, Altman G.(1954). A new Salmonella type (S. hadar). Acta Med Orientalia.4; 13:41.

Hudson L & Hay FC. (1980). Practical Immunology. 3rd ed. Black-Well Scientific Publications, Oxford London.

Jazani NH. Sohrabpour M. Mazloomi E. Shahabi S. (2011).A novel adjuvant, a mixture of alum and the general opioid antagonist naloxone, elicits both humoral and cellular immune responses for heat-killed Salmonella typhimurium vaccine. FEMS Immunol Med Microbiol. 61(1):54-62.

Karasova D; Sebkova A., Vrbas V., Avlickova H., Sisak F and Rychlik I (2009). Comparative analysis of salmonella enterica serovar enteritidis mutants with a vaccine potential. *Vaccine.* 27:5265-5270.

Kumar P, Ubhale P, Hari BabuY. (2010). E-rosette phenomenon for the early diagnosis of neoplastic conditions in buffaloes. Tamilnadu J. Veterinary & Animal Sciences 6 (6) 294-297.

Mastroeni P,Villarreal-Ramos B and Hormaeche C. (1993). "Adoptive transfer of immunity to oral challenge with virulent *Salmonella* in innately susceptible BALB/c mice, requires both immune serum and T-cells". Infection and Immunity. 61(9): 981-3984.

Mazloomi E, Jazani NH and Shahabi S. (2012). Anovel adjuvant, a mixture ofalum and the beta-adrenergic reseptor antagonist propranolol, elicits both humoral and cellular immune responses for heat-killedSalmonella typhimurium vaccine.Vaccine .30(16):2640--2646.

Mitov I, Denchev V and Linde K. (1992). Humoral and cell-mediated immunity in mice after immunization with live oral vaccines of S. typhimurium :auxotrophic mutants with two attenuating markers. Vaccine. 10. (1): 61-66.

OIE (2004). Manual of diagnostic tests & vaccines fnr Terrestrial Animal. Fifth Eidition Page 1018.

Quinn PJ, Carter ME, Markey B and Carter GR. (2004). "Clinical Veterinary microbiology". 6th ed. Mosby an imp. Wolf, London.

Reed LJ and Muench H. (1938). "A simple method of estimating fifty percent end point". Am. J. Hyg. 27(16): 8739-8744.

Schijns VE. (2003). Mechanisms of vaccine adjuvant activity:initiation and regulation of immune responses by vaccine adjuvants. Vaccine. 21: 829–831.

Shallal ZS. (2011). "A Clinical and immunological study of Salmonella mbandaka isolated from human in mice". M.Sc. thesis, Vet. Med. College / Baghdad University - Iraq.

Simon R, Tennant SM, Galen JE and Levine. MM (2011). Mouse models to assess the efficacy of non-typhoidal *Salmonella* vaccine: Revisiting the role of host innate susceptibility and routes of challenge. Vaccine. 29:5094-5106.

Smith B P, Reina-Guerra M, Hoiseth S K, Stockert A B, Habasha F, Johnson E and Merritt F. (1984). Aromatic dependent S. typhimurium as modified the vaccine for calves. Am. J. vet. Res. 45:181-89.

Snoussi S, El May A, Coquet L, Chan P, Jouenne T, Landoulsi A, and Emmanuelle DÉ· (2012). Adaptation of *Salmonella enterica Hadar* under static magnetic field: effects on outer membrane protein pattern. Proteome Sci. 10: 6.

Timms LM, Marshall RN Breslin MF. (1994). Laboratory and field trial assessment of protection given by a Salmonella enteritidis PT4 inactivated, adjuvant vaccine. Br Vet J. 150(1): 93-102.

Valdezate S, Echeita A, Diez R, Usera MA. (2000). Evaluation of phenotypic and genotypic markers for characterization of the merging gasteroenteritis pathogen Salmonella hadar. Eur. J Clinic Microbial.Infect Dis. 9:275–281.

Wilson-Welder JH, Torres MP, Kipper MJ, Mallapragada SK,Wannemuehler MJ & Narasimhan B .(2009). Vaccine adjuvants: current challenges and future approaches. J Pharm Sci 98: 1278–1316.

Yousif AA and Al-Mansory SH (2010) Immune response of *Salmonella enteritidis* antigens in rabbits. Research opinions in animals and Veterinary Research. 54: 1249-1255.

Yousif AA and Al-Naqeeb MMN (2010). Evaluation of the immune responses induced by experimental infection of (balb/c) mice with *salmonella hadar*. Basrah.J.Vet.Res, 9(2):119-126.

Yousif AA, Alshemmari IG M, Mahdi MS. (2011). Epidemiological study on *Salmonella* spp isolated from goat in some provinces in middle of Iraq. Iraqi Journal of Science.51: 3.