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Immune Efficacy of *Salmonella ohio* Somatic antigen in mice

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Abstract

This study was designed to evaluate the effect of *Salmonella ohio* Somatic antigen on humoral and cellular immunity in mice. Two groups of mice (thirty in each) were used, first group was immunized twice at two weeks' intervals subcutaneously (S/C) with 0.5 ml of somatic antigen (prepared by heat inactivation of S. ohio) containing 1×108C.F. U (protein content 200 µg); second group was injected S/C with phosphate buffer saline(PBS). Blood samples were collected at 2, 4, and 6 weeks post booster dose. Humoral immunity was detected by ELISA test, while cellular immunity detected by E. rosette and delayed type hypersensitivity test (DTH). The immunized and control mice groups were challenged with 5LD50 of virulent *Salmonella ohio* six weeks post booster dose. IgG was increased significantly ($P<0.05$) at 2, 4, and 6 weeks in the immunized group, and the maximum increase of antibody titers was determined at fourth week (651.7 ± 21.3) in comparison with the control group which remained within the normal value in all times of the experiment. E. rosette test showed a significantly increase in the mean of the activated lymphocyte of the immunized group at fourth week of immunization while control group gave normal range of active lymphocyte. In DTH test, immunized group showed a significant increase in footpad thickness after 24 hours post inoculation with soluble antigen in comparison with control group. Immunized mice were resist the challenge dose 5LD50 {5x (1.5x107)} of virulent *Salmonella ohio* and all mice of control group died within (3- 4) days. In conclusion, immunization of mice with somatic *S. ohio* antigen was induced humoral and cellular immune response against Salmonellosis.

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Introduction

Salmonella species are a leading bacterial cause of acute gastroenteritis. Although the global human health impact of *Salmonella* infections has not been estimated, gastroenteritis is a major cause of morbidity and mortality worldwide both in children under 5 years old and in the general population (Bern *et al.,*1992; Kosek *et al.,* 2003; Scallan *et al.,*2005). During the summer of 2005, an increase in reports of human cases of Salmonella enterica serovar Ohio infection was observed in Belgium. During 11 weeks, 60 cases of laboratory-confirmed Salmonella Ohio infection were reported to the National Reference Centre for Salmonella. All clinical isolates caused self-limiting gastroenteritis in both genders (males and females) and all age groups (children to adult) were affected.(Bertrand *et al.,* 2010).

In Iraq, Al Zubuidy and Yousif, (2012) isolated four *Salmonella* species (*Salmonella enteritidis*, *Salmonella newport*, *Salmonella anatum* and *Salmonella ohio*) from different organ of cows at slaughter house especially from which is used for human consumption.

S*almonella* generally exhibit an invasive potential and they can survive for extended periods within cells of the immune system. *In vivo Salmonella* infections are complex with multiple arms of the immune system being engaged. Both humoral and cellular responses can be detected and characterized, but full protective immunity is not always induced, even following natural infection. The murine model has proven to be a fertile ground for exploring immune mechanisms and observations in the mouse have often, although not always, correlated with those in other infected species, including humans. (Dougan *et al.,* 2011). Vaccination is potentially an effective tool for the prevention of Salmonellosis. Whole-cell killed vaccines and subunit vaccines were used with variable results for the prevention of *Salmonella* infection in humans and animals **(**Mastroeni *et al.,*2001). This study was designed to evaluate the humoral and cellular immune response in mice following exposure to somatic antigens of *S.ohio* against challenge with virulent strain.

Materials and Methods

Salmonella ohio was isolated from cows (bile and mesenteric lymph node specimens) at slaughter house in Iraq (Al zubaidy and Yousif, 2012), by culturing of on different selective media, biochemical and API tests (Quinn et al 2004). This isolate was confirmed in the National Center of Salmonella /Ministry of Public Health**.**

Preparation of somatic antigen for immunization

Samples of the stock culture of *S. ohio* were used for the preparation of somatic antigen. The culture was inoculated into brain heart infusion broth, and harvested during the early-logarithmic-growth phase, then the somatic antigen was prepared as followed:

Bacterial suspension was inactivated by heating at 100°C for 30 minutes. Then washed extensively in phosphate-buffered saline (PBS) before use (Smith *et al.,* 1984). Protein content of the antigen was determined by a method of (biurat). The antigen were tested for sterility and safety before use according to (OIE, 2004).

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 agar. after washing three times with PBS, the cultures were harvested by centrifugation at 10.000Xg for 30 minutes. The sediment was sonicated for 50 minute 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

To evaluate the efficacy of the prepared antigen, sixty adult healthy mice aged 4 to 6 weeks were selected. All mice had negative fecal bacteriological culture for salmonella. They were reared in separate cages in the Animal House of Veterinary College, University of Baghdad. The mice were divided equally into two groups.

i-The first group (immunized group) was Immunized with somatic Ag subcutaneously twice at two weeks' intervals at a dose of 0.5 ml containing 1x10^{8} CFU/ml and protein concentration 200μg.

ii- The second group (control group) was injected S/C with 0.5 ml of PBS at the same time. Blood samples were collected from all groups at 2nd, 4th and 6th week post-injection. Sera were separated and stored at -20^{O}C. This study was approved by the ethical and research committee of Veterinary Medicine College/University of Baghdad.

Estimating the LD_{50}

The viable count of the bacteria in eight-fold dilution (10^{-1},10^{-2}.10^{-3},10^{-4},10^{-5},10^{-6},10^{-7},10^{-8}) was made by bacterial plate count method according (Quinn et al 2004).

The LD50 was estimated according to (Reed and Muench, 1938). Forty-eight healthy mice of both sexes were divided into (8) groups (6 in each group). Seven groups of mice were injected intraperitoneal with 0.5 ml of calculated CFU diluents, and the eighth group was considered as a control group injected with PBS. All groups were monitored for 30 days to calculate total live and dead mice.

Immunological tests

1. **Enzyme-linked Immunosorbent assay (ELISA)** test for detection of IgG in the serum. This test was done according to manufacturer (immunological consultents laboratory, Inc.)

2. **DTH-skin** test was done 21 days after immunization as described by (Hudson and Hay 1980). Briefly, 0.1 ml of soluble antigen of *S. ohio* was injected intradermally in the right footpad of the immunized and control groups while the left side was injected by

0.1 ml of sterile PBS (pH=7.2). The thickness of skin was measured by vernier calliper before injection and at 24, 48 and 72 hours post injection.

3. **E –Rosette test:** It is a test that used for calculates the percentage of viable and non-viable T-lymphocytes and estimated activity of T-lymphocytes which formed after immunization of mice with the antigens. This test was done according to (Braganza *et al.*,1975).

a-Preparation of RBCs suspension: Three ml of blood was withdrawn from the jugular vein of a ram at the livestock of animals of the Veterinary Medicine College /Baghdad University. 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- Preparation of Lymphocytes suspension: It was prepared by taken the spleen of a mouse and cut to 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 tube of mixture of 0.25 ml RBCs suspension and 0.25 ml of lymphocytes suspension was prepared, the precipitate was incubated at 37° c for 15min and a drop was taken by pasture pipette and mixed with a drop of Trypan blue stain on a slide and was examined, unstained lymphocytes connected with 3 or more RBCs forming the rosette shape was calculated (200 cells).

Challenge of immunized mice

At 6 weeks after the second immunization (booster dose), all mice were challenged intraperitoneal with 5 LD^{50} of virulent *S. ohio* in 0.5-ml PBS. The relative degree of protection afforded by the antigen was assessed by the number of mice surviving 30 days after infection.

Statistical Analysis

Statistical package for social science (SPSS) version 17 was used to calculate the means, standard error and ANOVA test was conducted to test the significance of effects of groups and periods post injection on the examined traits.

Results

The results of ELISA test

All mice before immunization (at zero time) showed the same means of IgG titers (191± 11.3). After two week of immunization with the booster dose the serum IgG titers of immunized group was (383.4 ± 55.3) and the peak appeared at fourth and six weeks (651.7 ± 21.3; 533.4± 40.1) respectively. The results showed a significant increase of antibody titers ($P<0.05$) at (2, 4, and 6) weeks, as compared with the control group (Table 1). The results of delayed type hypersensitivity have showed increases in the thickness of the foot pad skin of the immunized mice and the highest means of the thickness

appeared after 24 hours post immunization. DTH tests indicated that the values were significantly high ((P< 0.05) in the immunized group compared to the control group and there is a significant effect of the antigen injected on the thickness of the foot pad skin of mice after 24 and 48 hours as shown by table (2). The E. rosette test showed the maximum reaction with mean (68.80 ± 2.02) after 4 weeks from booster dose in the immunized group while active lymphocyte remained within the normal range during experiment in the control group (Table 3).

Results of estimating LD_{50}

The results of estimating LD_{50} of *Salmonella ohio* in mice injected intraperitoneal with bacteria have revealed that the LD_{50} is (1.5×10^7 cells), which estimated by calculating the dead and alive mice in each group during (30) days (table, 4). The calculation of mortality percent as followed in this equation: - Percent Mortality = total dead / sum of (total a live + total dead).

Clinical signs post challenge

All mice were challenged with 5 LD50 ($5\times1.5\times10^7$) 6 weeks post immunization, the immunized group exhibited moderate signs for 2-3 days while the control group exhibited these signs included listlessness, anorexia, severe diarrhea, rough coat, hunched posture and crowding near the water supply. Death occurred within 3 to 5 days after the challenge

Table (1): Means of the antibody (IgG) titers in the immunized and control groups of mice.

Time(weeks)	Immunized group with somatic Ag Mean ± SE*	Control group Mean ± SE*	P.value
0 time	191± 11.3	191± 11.3	P> 0.05
2nd	383.4 ± 55.3	203± 11.1	P< 0.05**
4th	651.7 ± 21.3	189.1± 12.1	P< 0.05**
6th	533.4± 40.1	191± 11.3	P< 0.05**

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

Table (2): Showing the thickness of skin reaction in mice before and 24,48 &72 hours after injection with *S.ohio* antigen.

Periods after injection of soluble antigen	Immunized group Footpad Skin thickness Mean ± SE*	Control group Footpad Skin thickness Mean ± SE*
Befor test/mm	1.65±0.129A	1.58±0.011A
After 24hours/mm	2.66 ± 0.19^A	1.59±0.013B
After 48hours/mm	2.43±0.211A	1.57±0.012B
After 72hours/mm	1.916 ± 0.098A	1.58±0.008B

***SE=standard error. A-B Means in the same row with different (capital letter) superscripts differed significantly at P<0.05**

Table (3) : Active E. rosette means of immunized and control groups

Time (weeks)	Immunized group	Control group
	Mean ± SE	Mean ± SE
0 time	21.1 ± 1.11A	20.8 ± 1.478A
2nd	32.5 ± 1.88A	21.1 ± 1.11B
4th	27.9 ± 3.70A	21.2 ± 1.314B
6th	26.70 ± 1.04A	20.8 ± 1.478B

A-B/ Means in the same row with different (capital letter) superscripts differed significantly at P<0.05

Table (4): Results of LD^{50} of *S. ohio* in mice.

(6 mice in each	Dose	Alive	Dead	Total alive	Total dead	Percent mortality
1	1.5×10^{10}	0	6	0	21	100 %
2	1.5×10^{9}	0	6	0	15	100 %
3	1.5×10^{8}	2	4	2	9	81 %
4	**1.5×10^{7}**	**3**	**3**	**5**	**5**	**50 %**
5	1.5×10^{6}	4	2	9	2	18 %
6	1.5×10^{5}	6	0	15	0	0 %
7	1.5×10^{4}	6	0	21	0	0 %
8	BPS	6	-	-	-	0%

No. of mice in each group = 6, Total No. of mice = 48

Discussion

The important role of antibody producing B cell in protection against salmonellosis has been reported in many studies (Smith *et al.*, 1993; Lindberg *et al.*, 1993; Mastroeni *et al.*, 2000). In the current study, immunization of mice with somatic Ag of *S. ohio* resulted in stimulation of significant antibody titers in the immunized group compared with control group. This is in agreement with study of (Yousif and Al-Mansory, 2011) reported that immunization with *Salmonella enteritidis* somatic Ag resulted in increasing of antibody titers. Our result is agreed with that mentioned by (Shallal, 2011) which noticed that the experimentally infected mice were able to induce humoral immune response which represented by producing antibody against Salmonella after two weeks and reached the peak after four weeks post infection.

Also our result were agreed with result mentioned by Matsiota –Bernard *et al.*, (1993) who reported that IgG in mice during (7 to 35) days, raised on day 15 and continued to increase slightly until day 35, and with Kusumawati *et al.*, (2006) who measured IgG titers from serum samples of mice at 2 weeks after infection with *Salmonella typhimurium*. Similar results obtained by Hur *et al*., (2011) indicate the effective of live and killed salmonella vaccine in inducing IgG titers in the serum of mice. It is obvious that *Salmonella ohio* is able to induce cellular immune response during experimental infection with somatic antigen. the result of the skin test in our study is in agreement with

(Strindelius *et al.,* 2002) used delayed-type hypersensitivity – skin test as a measure of cellular immunity in mice immunized with different types of *salmonella* antigens, the immunized mice showed a significant increase in the skin test.the positive result of skin test in this study is in agreement also with result of others (Mitov *et al.,* 1992; Yousif and Al-Naqeeb, 2010; Yousif and Al-Mansoryo, 2011). Many investigations have led to the conclusion that cellular immunity is the primary mechanism of protection against Salmonellosis, especially when vaccines are employed (Mastroeni *et al.,* 1993). The results of the present study have showed that antigen of *Salmonella Ohio* induce a high cellular immunity, this is compatible with other studies used E. rosette test to detect cellular immunity against other intracellular organism (Talal,2007). E rosette test is considered as one of the most important discoveries that T-lymphocytes form spontaneous E-rosettes with sheep erythrocytes (S RBCs), proving one of the simplest biological markers for identifying T lymphocytes (Kumar, 2010). The LD_{50} dose of *S. ohio* ($1.5x10^{7)}$ is similar to *Salmonella hadar* LD_{50} dose mentioned by (Al Naqeeb, 2009) isolated from goat in Iraq. In contrast to (Al-Hashimi, 2005) who recorded the LD_{50} of *S. enteritidis* in mice was (1.4×10^6 C.F.U./ml). The immunized groups in our study resisted the effect of lethal challenge and all were live after immunization with somatic antigen and due to its ability to reduce the appearances of severs clinical signs of salmonellosis while the control group showed sever clinical signs of salmonellosis and died within 3-4 days after challenge. These results are in agreement with Karasova, (2009) who reported that mice with *S. enteritidis* induced strong cellular immunity and resisted the lethal challenge.

In conclusion, our results in the present study indicate that the *S.ohio* antigen can be a safe and effective tool for prevention of *Salmonella* infection. It can induce a protective cellular and humoral immune responses.

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