RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

ISSN 2221-1896 (PRINT) www.roavs.com ISSN 2223-0343 (ONLINE)

Immune response of *Salmonella enteritidis* antigens in rabbits

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Abstract

This study aimed to examine the effect of two inactivated *Salmonella enteritidis* antigens (O and H Ag) on humoral and cellular immunity in rabbits. Twelve rabbits of matching age and weight were divided into 3 equal groups. The first group was injected subcutaneously with 1 ml of O Ag, the second group was injected similarly with H Ag and the third group, (control) was injected with 1 ml phosphate buffer saline (PBS). Blood samples were collected at 2, 4, and 8 weeks after injections. Two-way analysis of variance was used to test the significance of the effects of treatments and period on hemagglutination test and delayed type hypersensitivity skin test (DTH). Eight groups of rabbits (4 rabbits per group) were used to determine the lethal dose 50 (LD_{50}). All rabbits of the treated and control groups were challenged with 4 LD_{50} (8×10^{10}) of virulent *Salmonella enteritidis*. Passive hemagglutination test showed significant increase in antibody titers in the two experimental groups compared with the control. Cellular immunity estimated by DTH gave higher response, while control group did not show any reaction. The post challenge with LD_{50} of *S. enteritidis* revealed that all rabbits in control group suffered from severe clinical signs of salmonellosis and died within 3-4 days. The present study indicated that injection of O and H Ag improved the immune response in rabbits.

Keywords: *Salmonella enteritidis*, O and H Antigens, Humoral immunity, Cellular Immunity

Introduction

Salmonella enteritidis remains one of the main causes of food-borne disease. It is considered as the most important pandemic zoonotic disease that spreads under natural conditions (Rodrigue et al., 1990). *Salmonella enterica* serovar *enteritidis* has emerged during the last 20 years as the major causative agent of food-borne gastro-enteritidis in humans (Toyofuku, 2008). There is a need to develop new vaccines and therapeutics (Mastroeni et al., 2001; Mastroeni and Grant, 2011) due to the difficulty in the prevention of salmonellosis by implementation of hygiene measures. In rabbits, salmonellosis is characterized by septicemia and rapid death with diarrhea and abortion. Mortality is the highest in young rabbits and pregnant does. Bacteria are shed in the faeces of carrier rabbits and clinically ill animals (Patton et al., 2008). The rabbit can be used as a model for diarrheal disease and sequel associated with salmonellosis (Hanes et al., 2001).

Salmonella are Gram-negative, flagellated, facultative anaerobic bacteria. The bacilli possess three major antigens: H (flagella antigen), O (somatic antigen) and Vi (virulence antigen) possessed by only a serovars (Duguid et al., 1989; Rubin and Weinstein, 2004). Ibebuike et al. (2008) showed that O and H antigens have the possibility of producing high Salmonella antisera against antigens. Also, Nalbantsoy et al. (2010) isolated and purified O and H antigens from *S. enteritidis.*

This study was designed to evaluate the humeral and cellular immune response in rabbits following exposure to O and H antigens of *S. enteritidis*.

Materials and Methods

Salmonella enteritidis was isolated from Iraqi goats by culturing on different selective media and by biochemical tests (Quinn et al 2004; AL-Shemmari, 2008). The bacterial confirmation was done in Salmonella Centre in Baghdad, Ministry of Health.

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tests was prepared according to Mitov et al. (1992). Briefly, a bacterial suspension of *S. enteritidis* obtained from overnight agar culture was sonicated at 50 minute intervals in a water cooled sonicator oscillator at 40 MHZ per second and the homogenate was centrifuged twice by using a cooling centrifuge at 8000 rpm per 30 minutes each time to remove cellular debris. The supernatants were passed through a 0.22 µm Millipore filter and stored at (-20°C) until used. Protein content was determined by biuret protein assay.

The somatic antigen (O Ag) was prepared by heating bacterial suspension of *S. enteritidis* on water bath at 100°C for 30 minutes. The flagller antigen (H Ag) was prepared by adding 3% formaldehyde solution to the bacterial suspension of *S. enteritidis* and left overnight (Smith et al., 1984). The two antigens were tested prior for sterility and safety before use according to OIE (2004).

To evaluate the efficacy of the prepared antigens, 12 adult healthy rabbits aged 6 to 8 months were selected. All rabbits had negative faecal bacteriological culture for salmonella. They were reared in separate cages in the Animal House of Veterinary College, University of Baghdad. The animals were divided equally into three groups. The first group (Immunized with O Ag) was injected twice at two weeks intervals S/C with the prepared O antigen at a dose of 1 ml containing $1x10^8$ CFU/ml. The second group was injected with the prepared H antigen at same dose. The third group (control) was injected S/C with 1 ml of PBS. Blood samples were collected from all groups at 2nd, 4th and 8th week post-injection. Sera were separated and stored at -20°C.

The humoral immunity was evaluated by passive haemagglutination test (PHA) as described by Boyden (1951). DTH-skin test as described by Hudson and Hay (1980). Briefly, 0.1 ml of soluble antigen of *S. enteritidis* was injected intradermally in the right flank of the immunized and control groups while the left flank was injected by 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.

The lethal dose 50 (LD_{50}) was estimated by using eight fold dilution for counting bacteria by viable bacterial plate count method (Quinn et al., 2004). Thirty two healthy rabbits of both sexes were divided into (8) groups (4 per group). Seven groups of rabbits were injected subcutaneously with 1 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 the total live and dead rabbits, and also to estimate the LD_{50} according to Reed and Muench (1938). After 8th weeks, all immunized and control rabbits were challenged intra-peritonealy with 4 LD_{50} virulent *S. enteritidis* to evaluate the efficacy of prepared the antigens in inducing immune response.

Statistical Analyses

Using statistical package for social science (SPSS) version 13.0, two-way analysis of variance was conducted to test the significance of effects of groups and periods post injection on the examined traits. The statistical differences among means of the different treatments were tested By Duncan's multiple range test.

Results

The PHA test showed a significant increase ($P<0.05$) in antibody titre in the groups injected with O and H Ag compared to the control group. Also, the titre was significantly high on 4th weeks compared to 2nd and 8th week (Table 1). DTH tests indicated that the values were significantly high in the experimental groups compared to the control group. Again, the thickness (mm) increased ($P\leq0.05$) after 48 hours and then returned to normal (Table 2&3). The results of mortality are shown in Table 4.

Table 1: Antibodies titre (Mean ± SE) of the experimental and control groups by PHA test

Weeks of injection	O Ag injected group	H Ag injected group	Control group
2nd	$^{b}38.00\pm6.25^{A}$	$^{c}28.00\pm4.00^{A}$	0.00^{B}
4th	$^{a}88.00\pm24.00^{A}$	$^{a}72.00\pm20.13^{A}$	0.00^{B}
8th	$^{b}48.00\pm9.23^{A}$	$^{b}64.00\pm8.23^{A}$	0.00^{B}

[a-c]means in the same column with different (small letter) superscripts differed significantly at P<0.05; [A-B]Means in the same row with different (capital letter) superscripts differed significantly at P<0.05

Table 2: Mean skin thickness (Mean ± SE) of experimental and control groups in DTH test

Periods after injection of soluble antigen	Diameter of skin thickness (mm)		
	O Ag injected group	H Ag injected group	Control group
24 hours	$^{b}0.42\pm0.06^{A}$	$^{b}0.52\pm0.08^{A}$	$^{a}0.27\pm0.07^{B}$
48 hours	$^{a}0.70\pm0.08^{A}$	$^{a}0.90\pm0.16^{A}$	$^{a}0.40\pm0.08^{B}$
72 hours	$^{b}0.50\pm0.168^{A}$	$^{b}0.52\pm0.08^{A}$	$^{a}0.20\pm0.04^{B}$

[a-c] Means in the same column with different (small letter) superscripts differed significantly at P<0.05; [A-B] Means in the same row with different (capital letter) superscripts differed significantly at P<0.05

Table 3: Mean skin redness (Mean ± SE) of experimental and control groups in DTH test

Periods after injection of soluble antigen	Diameter of skin redness(mm)		
	O Ag injected group	H Ag injected group	Control group
24 hours	$^{b}0.67 \pm 0.13^{A}$	$^{b}0.42 \pm 0.10^{B}$	0.00^{C}
48 hours	$^{a}1.00 \pm 0.05^{A}$	$^{a}0.97 \pm 0.04^{A}$	0.00^{C}
72 hours	$^{c}0.37 \pm 0.02^{A}$	$^{b}0.42 \pm 0.06^{A}$	0.00^{C}

$^{a-c}$ means in the same column with different (small letter) superscripts differed significantly at P<0.05; $^{A-B}$ means in the same row with different (capital letter) superscripts differed significantly at P<0.05

Table 4: Mortality rate (%) of LD_{50} of *S. enteritidis* in rabbits

Rabbits group	*Calculated dose	Alive	Dead	Total Alive	Total Dead	Percent mortality
1	2×10^{12}	0	4	0	10	100 %
2	2×10^{11}	1	3	1	6	81 %
3	2×10^{10}	2	2	3	3	50 %
4	2×10^{9}	3	1	6	1	18 %
5	2×10^{8}	4	0	10	0	0 %
6	2×10^{7}	4	0	14	0	0 %
7	2×10^{6}	4	0	18	0	0 %
8	PBS	4	-	-	-	0%

No. of rabbits in each group = 4, Total No. of rabbits = 32, *The dose calculated as (cells).

Clinical signs post challenge

All rabbits injected with O and H Ag and control group challenged with 4 LD_{50} ($4\times2\times10^{10}$) 8 weeks post immunization exhibited moderate elevation in the body temperature, which persisted for 2-3 days with mild signs of illness and depression without diarrhoea and returned to normalcy within 7 days.

The control group also exhibited the clinical signs post challenge. These signs included listlessness, anorexia, severe diarrhoea, fever, rough coat, hunched posture and crowding near the water supply. Increase in the respiration rate, abortion of the pregnant does, severe dehydration and recumbency appeared at the later stage and death occurred within 3 to 5 days after the challenge.

Discussion

Since *Salmonella* organisms are ubiquitous pathogens of human and animal species, an understanding of events during the immune response is of paramount importance in developing an effective prophylactic agent (Collins, 1974). 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, inoculation of rabbits with O and H Ag of *S. enteritidis* resulted in stimulation of significant high titres of antibody in the experimental groups compared with control. This is in agreement with George et al. (1985), who reported that immunization of rabbits with H and O. S*almonella* antigens resulted in high yield of antisera titre. Weidans et al. (1964) recorded that the rabbits injected with bacterial cells or isolated somatic antigen of *S. enteritidis* and *Escherichia coli* produced higher cellular and humoral immune response. Mittrucker and Kaufmann (2000) mentioned that the response to *S. typhimurium* involves both T and B cell-mediated immunity which are important for control of primary infection and protection against secondary infection attack.

In the present study, the first group inoculated with O Ag evoked antibody response, and this is compatible with Ibebuike et al. (2008) and Saxon et al. (1986) who demonstrated the possibility of producing high *Salmonella* antisera by inoculating rabbits with salmonella antigens. Also, the second group immunized with H Ag revealed significantly higher titres of antibody compared with the control group. The significance of such immune response has been highlighted in several studies (Stocker and Newton, 1994; Ibebuike et al., 2008).

Many investigations have concluded that cellular immunity is the primary mechanism of protection against salmonellosis, especially when vaccines are employed (Mastroeni et al., 1993). Our study showed significant increase in the antibody titre against O and H antigen, indicating the possibility of the stimulation of cellular immunity. This result is in agreement with reports which recorded cellular immune against attenuated or heat killed *S. typhimurium* or with outer membrane proteins or flagellin (Ogunniyi et al., 1994; Thatte et al., 1995; Cookson and Bevan 1997; Aderem and Ulevitch, 2000).

In this investigation, delayed type hypersensitivity skin test showed significantly higher value in experimental animals. This result is in agreement with the study of Strindelius et al. (2002) who used delayed-type hypersensitivity – skin test as a measure of cellular immunity in mice immunized with different types of *S. enteritidis* antigens and record a significant increase in ear thickness of all immunized groups. Also, Mitov et al. (1992) detected positive DTH test in immunized

mice with salmonella and their results revealed development of long lasting cell mediated immunity that showed striking correlation to the protection.

The immunized groups in our study resisted the effect of lethal challenge and all were live after immunization with O and H antigens 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 Uchiya et al. (1991) and Karasova (2009) who reported that of mice with *S. enteritidis* induced strong cellular immunity and resisted the lethal challenge. Also, Simon et al. (2011) reported that the mice immunized with flagellin alone, or flagellin conjugates were protected from lethal challenge with wild-type *S enteritidis*.

In conclusion, immunization of rabbits with O and H *S. enteritidis* antigens induced humoral immune response as detected by the studied parameters.

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