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Immune Response Elicited in Mice after Immunization with Flagellin from *Salmonella enterica* Serovar Enteritidis

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Abstract: Bacteria belonging to the species *Salmonella* are one of the major causes of intestinal diseases in humans and animals worldwide. This study investigated the immune response elicited in mice after S/C immunization with flagellin from *Salmonella enterica* serovar Enteritidis. Humoral immune response in the mice was analyzed using ELISA resulted in a significantly greater antibody response than in the control group. Peritoneal macrophages phagocytic activity exhibited higher value in the group immunized with flagellin. Serum lysozyme activity and nitric oxide were investigated at 24 hrs post-immunization showing elevation in their levels compared to the control group. Bacterial colony count in liver and spleen in mice vaccinated with flagellin and challenged orally with *Salmonella* showed no bacterial growth.

Key words: *Salmonella* · Flagellin · Macrophage · Nitric oxide and lysozyme

INTRODUCTION

Salmonella species produce many disease forms that range from a mild enteritis to severe systemic infection in a variety of animal hosts. The *Salmonella* serovars Enteritidis and Typhimurium are the most important causes of food-borne diseases [1].

The pathogenicity of *Salmonella* is linked to a number of virulence factors, including adhesions, flagella, enzyme, toxins and other bioactive factors, these facilitate adherence to the gut, invasion, systemic spread, survival and proliferation in systemic organ [2]. Flagellin is the protein component of the bacterial flagellum, the molecular motor necessary for motility in a wide variety of prokaryotes [3]. Flagella contribute to virulence of pathogenic bacteria through chemotaxis, adhesion to and invasion of host surfaces. The flagellin binds to Toll-like receptor (TLR5) which situated on epithelial cells, dendritic cells and macrophage to activate innate host defense genes and produce pro-inflammatory cytokines. Flagellin can trigger adaptive immune responses both by stimulating chemokine secretion by epithelial cells and subsequent migration and maturation of dendritic cells and by modulating T cell activation *in vivo* [4,5].

Macrophages detect and internalize bacterial pathogens in order to eliminate them from the host. Bacteria captured in phagosomes are usually killed by reactive oxygen species, nitrogen intermediates, acidification, starvation, lysosomal enzymes, antimicrobial peptides, or other activities [6].

Flagellin responsible for most of the antibody response that shown by Western blot analysis [7].

The current study was undertaken to evaluate the immunogenic properties of *S.* Enteritidis flagellin. Two steps were carried out. First one was extraction of flagellin and determination of flagellin protein profiles of *S.* Enteritidis by electrophoresis analysis (SDS-PAGE) and immunoblotting Second step was to evaluate the humoral and innate immune response of mice immunized with *S.* Enteritidis flagellin. Finally the immunity was evaluated by homologous challenge.

MATERIALS AND METHODS

Bacterial Strain: *S.* Enteritidis was isolated from chicks suffering from diarrhea in previous research. *Salmonella* isolate was identified by biochemical reaction [8] and serotype according to Kauffmann-White scheme as described by [9] in serology unit, Animal Health Research Institute.

Motility Test: To determine the swim phenotype, *S.* Enteritidis was inoculated into soft agar (1% tryptone - 0.7% Nacl-0.35% agar) and incubated over

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night at ambient temperature when appropriate tetracycline was included at final concentration of 20µg/ml. The ability to move through the agar was recorded [10].

Extraction of Flagellin: According to Ibrahim *et al.* [11] with some modification, bacterial cells were harvested by centrifugation at 8000 rpm for 30 min and then mixed with saline solution to form a moderately thick suspension. The suspension was adjusted to pH 2 with 1M HCl and maintained at that pH under constant stirring for 30 min at room temperature. The bacterial cells devoid of flagella were separated by centrifugation at 8000 rpm for 30 min. The supernatant which contained detached flagellin was further centrifuged at 33000 rpm for 1h at 4°C. The pH of the supernatant was adjusted to 7.2 with 1M NaOH. Ammonium sulfate was added slowly with vigorous stirring to achieve two-thirds saturation 2.67M. The mixture was centrifuged at 15000 rpm for 15 min at 4°C. The precipitate was dissolved in approximately 5ml of distilled water and then transferred to dialysis against phosphate-buffered saline (PBS) for 18h at 4°C. The dialyzed flagellin preparation was used. Protein concentration of flagellin was estimated by method of Lowry *et al*. [12].

Gel Electrophoresis: Electrophoresis of S. Enteritidis flagellin proteins was done in SDS-PolyAcrylamide Gels in the Tris/glycine discontinuous buffer system according to Laemmli [13].

Immunoblot analysis was carried out according to Feng *et al.* [14].

Hyperimmune serum against S. Enteritidis flagellin Strindelius *et al.* [7]. 20µg of extracted flagellin /100 µl saline was emulsified in an equal volume of Freund's complete adjuvant and injected S/C to female mice 8-12 week olds. The booster dose was given on day 14 followed by another dose at day 21. Blood sample was taken at day 28 and the serum was separated.

Immunization Assay: Protective immunity in mice was carried according to Muthukkumar and Muthukkaruppan [15]. Total of 40 mice 8-12 weeks old free from Salmonella were housed under hygienic condition. Mice were divided into two groups (20 mice/group). First group was injected subcutaneously with 20µg of flagellin /100 µl saline a booster dose was given after 3 weeks. The second group injected with physiological saline as a control group. Blood sample were taken from the eye at post vaccination weekly interval for six successive weeks and centrifuged to obtain the serum.

Indirect ELISA: Immunoglobulin G was measured with indirect ELISA according to Holt and Porter [16] with some modification. Briefly, plates were coated with flagellin antigen at a concentration of 10µg/ml and serum samples were diluted 1:20 in 1%. bovine serum albumin in PBS.

Isolation of Mouse Peritoneal Macrophages: According to Kreckler *et al.* [17] with some modification, mice were injected intra-peritoneally with 2 ml of 2% thioglycollate. After 4 days peritoneal cells were collected by lavage. Macrophages (10^7/ml) were seeded onto 24-well plates contain cover slip in RPMI 1640 medium with 10% calf serum for 4 hrs to allow the macrophages to adhere to the plates. Non-adherent cells were subsequently removed by washing with RPMI 1640 medium and re-incubated over night in the same condition then 1ml of C*andida albicans* (10^5/ml) was incubated for another one hour. Finally phagocytic percentage and phagocytic index were calculated.

Measurement of Lysozyme Activity: using Lysoplates technique according to Osserman and Lawlor [18].

Nitric Oxide Assay: It was carried out according to Green *et al.* [19].

Challenge Test: Immunized mice were challenged orally with 50µl of *S.* Entertidis (2×10^9 CFU per mouse) 3weeks after the booster vaccination. The spleens and livers were taken out 7 days after challenge to count the number of *Salmonella* colonies [20].

RESULTS

Motility Test: In the presence of tetracycline, the bacteria remained localized to the point of the inoculums and were unable to swim over an 18-24 hrs period from this site. However, in the absence of tetracycline, the bacteria expanded from the inoculums and rapidly migrate through the agar.

Characterization of S. Enteritidis Flagellin by SDS-PAGE Technique: SDS-PAGE analysis of flagellin of *S.* Enteritidis revealed up to 4 protein bands. The molecular weight of the polypeptide bands were

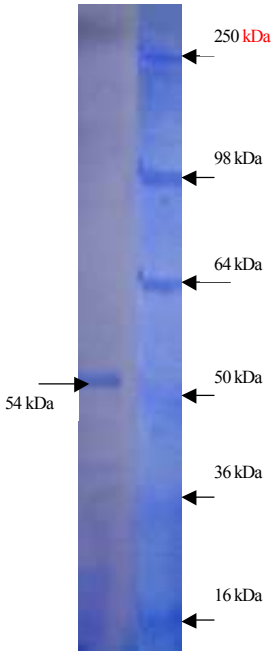

Fig. 1: SDS-PAGE of flagellin antigens

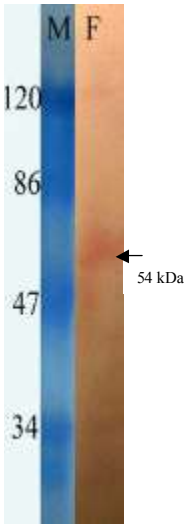

Fig. 2: Western immunoblot analysis

dominating one major band at about 54.11kDa which corresponds to the molecular mass of flagellin and 3 minor protein bands at 41 kDa,36.6 kDa and 25.7 kDa (Table 1 and Figure 1). 41kDa, 36.6 kDa and 25.7kDa were not clear due to it is minor band Where 54.11 it is major band.

Immunoblotting: Immunoblot analysis of flagellin using mice hyper immune serum indicated that (54.1 kDa) polypeptide was the main immunogen (Fig. 2).

Table 1: SDS-PAGE analysis of flagellin extracted from *S.* Enteritidis

	Flagellin proteins		Marker proteins	
Rows	(mol.w. /kDa)	(Amount)	(mol.w./kDa)	(Amount)
r1				
r2				
r3			250	3.7409
r4				
r5			98	1.9715
r6				
r7			64	2.8081
r8	54.111	5.0458		
r9			50	4.3446
r10	41.049	3.3412		
r11	36.689	2.8788	36	2.9329
r12	25.707	2.4576		
r13			16	14.175
Sum		15.0545		29.973
In Lane		100		100

Table 2: Overall mean of ELISA antibody levels among the vaccinated mice

Weeks post immunization	ELISA
Preimmunization	0.1954 ± 0.034^{A}
1	0.6117 ± 0.040^{B}
2	0.8202 ± 0.025^{C}
3	0.9494 ± 0.017^{D}
4	1.1104 ± 0.039^{E}
5	1.0673 ± 0.025^{E}
6	1.1698 ± 0.070^{E}

Means with different superscripts are significantly different at least $P<0.05$

Table 3: Phagocytic percentage, phagocytic index and lysozyme, in serum of vaccinated mice

	Phagocytic %	Phagocytic index	Lysozyme
Control	60.533 ± 2.853^{A}	0.590 ± 0.023^{A}	214.75 ± 13.553^{A}
vaccinated	74.667 ± 3.480^{B}	0.8167 ± 0.0698^{B}	288.33 ± 9.099^{B}

Means with different superscripts are significantly different at least $P<0.05$

Indirect ELISA: IgG antibody level in mice serum was increased significantly from the first week to six weeks after immunization with flagellin antigen (Table 2).

Measurement of Phagocytic Activity: Effect of flagellin on phagocytic activity presented in Table (3) and Figure (3) showed significant increased in both phagocytic percentage and index compared to control group.

Lysozyme Levels: Serum lysozyme level was shown in Table (3) revealed significant increased 24 hrs post vaccination compared to control unvaccinated.

Table 4: Nitric oxide (NO) in serum and supernatant of activated macrophages of the vaccinated mice.

Groups	NO in serum	No in supernatant of activated macrophages
Control	12.383±0.374^{A}	13.760±0.751^{A}
Vaccinated:		
1-24 hrs post 1st vaccination	15.591±0.301^{B}	16.900±0.443^{A}
2-24 hrs post 2nd vaccination	21.575±0.849^{C}	22.794±1.449^{B}

Means with different superscripts are significantly different at least P<0.05

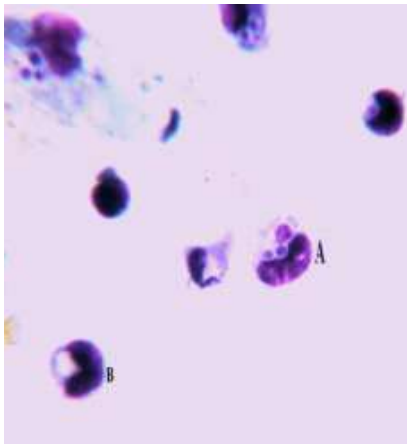

Fig. 3: Phagocytic assay, A phagocytic cell eating Candida, B phagocytic cell did not eat

Table 5: Protective efficacy of flagellin against *Salmonella* challenge in mice

Mice immunized with	CFU after 6 days post challenge	
	liver	Spleen
Flagellin	Negative for *Salmonella*	Negative
Phosphate buffer saline	$10x10^{6}$	$10x10^{5}$

Nitric Oxide Assay: Nitric oxide level was increased 24 hrs post the first and second vaccination with flagellin antigen in both serum and supernatant of activated macrophage cells (Table 4)

Re-isolation of *Salmonella* Enteritidis: Challenged vaccinated mice sacrificed after 7 days revealed no characteristic lesion of salmonellosis on necropsy and their organs (liver and spleen) were negative for *Salmonella* compared to control positive group Table (5).

DISCUSSION

Salmonellae are Gram–negative facultative intracellular bacteria caused wide range of infectious syndromes varied from mild enteritis to severe systemic infection in a variety of animal hosts. *S.* Enteritidis in poultry considered the major source of infection in man [21].

The general concept of our approach is to use conserved epitopes derived from the *S.* Enteritidis as immunogens. Each epitope is expressed in *Salmonella* flagellin; the resultant recombinant flagella can be easily cleaved and purified for administration.

The isolated *S.* Enteritidis remained localized to the point of the inoculums and were unable to swim over an 18-24 hrs period in the presence of tetracycline. However, in the absence of tetracycline, the bacteria expanded from the inoculums and rapidly migrate through the agar. This result indicated that tetracycline might influence the released of flagella [10].

SDS-PAGE analysis of isolated flagellin of *S.* Enteritidis have permitted the identification of protein component in 54.11 kDa (major band) and 3 minor band at 41 kDa, 36.6 kDa, 25.7 kDa (Table 1 and Figure 1). This result agree with Strindelius *et al.* [22, 20] who found that SDS-PAGE of the purified flagellin revealed one dominating band at about 56 kDa, which corresponds to the molecular mass of flagellin (50-60 kDa) They found also there is LPS contamination in minor bands.

Nmaba *et al*. [23] suggested that flagellin is a 55 kDa monomer obtained from bacterial flagella, a polymeric rode-like appendage extending from the outer membrane of Gram-negative bacteria that propels the organism through its aqueous environment.

Immunoblot analysis of flagellin revealed that 54 kDa polypeptide was the chief immunogen whereas other bands at 41 kDa, 36.6 kDa and 25.7 kDa detected in the gel electrophoresis but could not detected in immunoblot possibly it is not immunogenic as mentioned by Strindelius *et al.* [20].

The mice that vaccinated with flagellin subcutaneously had highly antibody levels (Table 2) up to six weeks post vaccination. So flagellin is a strong immunogen which considered a potent stimulator of adaptive immune responses this result concur with Parish [24] and Salazar-Gonzalez *et al.* [25].

The produced antibodies may be important in controlling bacterial replication by acting as opsonins [26].

The immunomodulatory behavior of bacterial flagellins has also been investigated by Braga *et al.* [27] as potential vaccine adjuvants, either for induction of humoral or cellular immune responses, to different target antigens.

Concerning the effect of flagellin on phagocytic activity our results in Table 3 And Figure 3 illustrate that both phagocytic percentage and index were significantly increased after vaccination with flagellin these results may be due to presence of Toll-like receptor 5 (TLR5) on different cell types including epithelial cells, dendretic cells and macrophages where flagellin binds to this receptor and activate it as recorded by Tsujimoto *et al.* [28] and Braga *et al.* [27].

Neutrophils and macrophages are the main cell types that harbor *Salmonella* during infection in mouse after recruitment to infected organs these cells amplify the inflammatory response initiated by resident cells by producing inflammatory mediators such as cytokines and chemokines [29]. They also exert the important function of phagocytosing and killing *Salmonella*. Even though *Salmonella* has evolved a number of mechanisms to evade killing, the ability of phagocytes to reduce the growth of bacteria through expression of Nramp1 (is a late endocytic/lysosomal protein that is situated in the membrane of *Salmonella*-containing vacuoles of monocytes/ macrophages and neutrophils) can be induced in phagosomes [30].

Lysozyme is a soluble bactericidal substance that increase during the infection it has the ability to lyse and kill bacteria by breaking down the mucopeptide of their cell wall [31] our results showed significant increased in serum lysozyme levels in mice vaccinated with flagellin twenty four hrs post vaccination (Table 3) these results coincided with Sano *et al*. [6] and El-Gayar *et al.* [30] who stated that lysozyme considered as one of innate immune response factors that elaborated during activation of phagocytic cells.

Nitric oxide (NO) is a biological molecule involved in physiological and pathological processes such as antimicrobial defenses and immunomodulation [32], In this study flagellin increased NO in serum and supernatant of macrophage twenty four hrs post vaccination (Table 4) our results agreed with Mizel *et al.* [33] who recorded that flagellin stimulates NO in macrophages via a pathway that requires Toll-like receptor 5 (TLR5) signaling pathway and TLR4. Flagellin induced NO synthesis in a murine macrophage cell line that expresses wild-type TLR4. Flagellin stimulated an increase in inducible NO synthase (iNOS) mRNA and activation of the iNOS promoter. In addition to Eaves-Pyles *et al.* [34] found that antiserum to flagellin blocks NO production in intestinal epithelial cells (IEC) induced by medium condition by a variety of motile Gram-negative enteric pathogens.

Mice vaccinated with flagellin were protected against homologous *S.* Enteritidis challenge showing no characteristic lesion of salmonellosis on necropsy and their organs (liver and spleen) were negative for isolation of *Salmonella* as shown in Table (5). This proved the role of flagellin in prevention of the multiplication of *Salmonella* in internal organs. In this aspect Makela *et al.* [35] found that the main sites for multiplication of *Salmonella* species during invasive infection were the liver and spleen. Schmitt *et al*. [36] found that a flagellate *S*. Enteritidis and *S*. Typhimurium strains were significantly less invasive to cells in culture and reduce capacity to cause lethal infection via the oral route in murine and chicks models.

It could be concluded that purified flagellin effectively trigger an immune response after s/c immunization so; we recommended flagellin vaccine for *S.* Enteritidis as the vaccine of the future.

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