

## CHARACTERIZATION OF SALMONELLA TYPHIMURIUM FLAGELLAE ISOLATED FROM DIARRHOEIC CALVES

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### SUMMARY

The present study was undertaken to define the role of the flagella proteins, bactrine and lipopolysaccharides (LPs) of *Salmonella enterica* serovar Typhimurium in the induction of protective immunity against a challenge with homologous *S. Typhimurium* and heterologous *S. Typhi*. Flagellae bactrine, and LPs of *S. typhimurium* were extracted and characterized using Immunoblotting analysis.

In the active immunization assay, flagellae or bactrine 50µg conferred 100% protection to mice challenged with up to 500 LD50 of *S. Typhimurium*. Even at 90 days post-immunization a substantial level of protection (78%) was observed in the immunized mice, while bactrine was capable of inducing long lasting protection (100%). Immunization with LPs 50 µg was considerably inferior to the flagellae or bactrine in the induction of short duration immune status.

The results indicated that flagellae, probably bactrine are valuable immunogenes in the protection against *Salmonella* infection. In addition, cross-protection against two *Salmonella* species can be obtained. Flagellae of *salmonella typhimurium*, was found to be a suitable antigen for eliciting delayed-type hypersensitivity in mouse salmonellosis.

### INTRODUCTION

Salmonellosis is a disease of major economic importance and to be continuously increasing as a public health problem (McEvoy et al 2003). The major obstacle in developing a suitable vaccine was the poor understanding of the nature of immunogenic moieties associated with protection (Surz and Amon (2003).

Attention has been addressed to the role of the flagellae of Gram-negative bacteria in the induction of specific immunity The production of flagellae

(H) antisera for Salmonella serotyping is usually done by the intravenous injection of rabbits with live or formalinized broth cultures of highly motile Salmonella organisms at intervals of several days (Eaves-Pgles et al 2000). This procedure gives sera containing not only flagellar antibodies but also somatic (O) antibodies. The H antisera titer in this case is frequently low, and the O antisera titer is often so high that it interferes with H agglutination results. Consequently, the O antisera are usually removed by (Angerman and Eisenstein 1980, Gray et al., 2006).

Therefore, current research efforts have focused on the delineation of the precise immunologically relevant antigen(s) from the pool of flagellae. The initial step in this work was to extract and determine the flagellae protein, as well as bactrine and lipopolysaccharides (LPs) of Salmonella Typhimurium by electrophoretic analysis and immunoblotting. The role of flagella and LPS immune rabbit serum in the passive transfer of immunity was assessed. The availability of such information should provide rational experimental approaches for the development of effective vaccines against Salmonella infections. And Delayed type hyper sensitivity (DTH) elicitation.

## **MATERIALS AND METHODS**

### **1. Bacterial strains :**

Strains of Salmonellae used in this study, S. Typhimurium was offered from Bacteriology Department, Animal Health Research Institute. S.

Typhimurium was originally isolated from diarrheic calves. S. Typhi was originally isolated from a patient with typhoid fever. These isolates were identified by biochemical reactions (Krieg and Holt, 1984).

S. Typhimurium was used for extraction flagellae, bactrine and LPs. S. Typhi and S. Typhimurium were used for challenge. The 50% lethal dose, was calculated.

### **2. Experimental animals:**

#### **2.1. New Zealand rabbits:**

White New Zealand rabbits were used in this study. weight (1Kg). Before the experimental work, faecal samples were collected from all rabbits for bacteriological examination by culture methods to exclude Salmonella infection. No antibiotics were given to the rabbits before and during the work.

#### **2.2. Mice:**

Mice aged 8 weeks were used. They were fed commercial feed and water was given.

### **3. Preparation of flagellae**

#### **Antigen preparation according to Hassan et al (1990)**

Three different antigen preparations a whole bacterial cell flagellae protein (bactrine), and lipopolysaccharide antigen. To prepare the bactrine 1 litre of a 24 h broth culture of S. Typhimurium

which had been shaken overnight was centrifuged and the pellet resuspended and washed three times in 10 ml phosphate buffered saline (PBS). This suspension was sonicated for two minutes and centrifuged at 5000 g for 60 minutes. The supernatant was stored at -20°C until used.

Flagellae antigen was prepared by centrifuging of 24 hour broth culture of *S. Typhimurium* and resuspending the pellet in 200 ml PBS. The flagellae were removed by homogenizing the bacterial suspension for one minute in a mechanical blender and the suspension was then centrifuged at 1500 xg for 15 minutes to remove bacterial cells. The supernatant was further centrifuged at 75,000 g for 45 minutes at 4°C. After the pellet was resuspended in 3 ml PBS, 0.15ml of 1M hydrochloric acid was added and the mixture incubated at room temperature for 30 minutes and then centrifuged at 80,000g for 60 minutes. The supernatant fluid was neutralized with 1M sodium hydroxide and the flagellae precipitated by adding an equal volume of saturated ammonium sulphate solution followed by incubation for 16 hours at 4°C. the precipitate was pelleted by centrifugation at 20,000 g for 15 minutes. The pellet was resuspended in 5 ml distilled water and the ammonium sulphate removed by dialysis against PBS.

**Extraction and characterization of lipopolysaccharide (LPs):** LPs was extracted and purified

from proteins and other contaminant by phenol-chloroform, petroleum ether method. Harvested bacteria were washed with water and treated successively with ethanol, acetone and twice with ether.

Lipopolysaccharide antigen was prepared as follows. A suspension of *S. Typhimurium* cells in distilled water at a concentration of approximately 10 mg/ml dry weight at 0°C was mixed with 10 volumes of acetone at -20°C and left for 60 minutes at this temperature. The yellow supernatant was removed and the residue washed with five volumes of cold acetone dried and ground in a mortar. It was then suspended at 6 per cent w/v in distilled water at 65°C and an equal volume of 90 percent for five minutes, cooled to 4°C and centrifuged at 2000g for 60 minutes at 0°C. The aqueous upper layer was removed and dialysed against water for 48 hours.

#### **4. Biassay of the extracted flagellae, bactrine and LPs in rabbits according to Ibrahim et al. (1985):**

our groups of white New Zealand rabbits, first, second and third group were immunized by subcutaneous (S/C) injection of the flagellae, bactrine and LPs preparation, respectively. The injections were given on day 0, 14, 28, 42. 100µg protein indicated antigen was determined in the presence of Freund complete adjuvant. The fourth group

was kept as unvaccinated control. Serum was collected before immunization and 10 days after the last injection. Hyperimmune serum was used for determination of antibody profile (immunoblotting) and for passive immunization of mice.

#### **Gel electrophoresis SDS-PAGE and western blots immunoblots Towbin et al (1979):**

lagellae, bactrine and LPs of *S. Typhimurium* were electrophoresed using 10% SDS-PAGE under reducing conditions. The fractionated antigens were determined by staining with 5% coomassie blue or were transferred to nitrocellulose sheet (NC) for subsequent immunologic analysis.

#### **6. Immunization assay:**

##### **a. Protective immunity induced by flagellae, bactrine and LPs to *S. Typhimurium* infection in mice according to George et al (1985) :**

Four groups of mice, each comprised of 20 mice. First, second and third groups were immunized with flagellae, bactrine and LPs, respectively. Ten mice of each group were injected with 10 $\mu$ g of antigen and the remaining mice (10) were injected with 50  $\mu$ g of antigen subcutaneously S/C twice at 15 day intervals. The fourth group was injected with Tris-EDTA buffer (control group). Ten days after the last injection, each group of mice was challenged with 500 LD<sub>50</sub> of *S. Typhimurium* or challenged with 100 LD<sub>50</sub> of *S. Typhi*. The number of survivors at the end of 14 days post-challenge were recorded.

#### **Long-term immunization of mice with flagellae, bactrine, bactrine and LPs of *S. typhimurium* .**

To determine whether flagellae, bactrine and LPs induced long lasting protection, three groups of mice (1st, 2nd and 3rd) group were immunized S.C. with two doses of 50 $\mu$ g of Flagellae, bactrine and LPs, respectively at 15 day interval 30, 60, 90 days before challenge with 500 LD<sub>50</sub> of *S. Typhimurium*. For each time point one control group was injected with Tris-EDTA buffer and challenged similarly. Protection was assessed by determining the number of survivors at 14 days after challenge.

#### **Passive immunization of mice according to George et al (1985):**

Four groups of mice, the first, second and third groups were injected in the central tail vein with 0.2ml rabbit antiserum against flagellae, bactrine and LPs, respectively. The fourth group was injected with normal rabbit serum diluted with 0.9% NaCl. All groups of mice were challenged intraperitoneally 1 hour later with 100 LD<sub>50</sub> of *S. Typhimurium* or with 50 LD<sub>50</sub> of *S. Typhi*. The daily death count was recorded and after 14 days the percentage of survival in each group was calculated.

## **RESULTS**

### **Characterization of *S. Typhimurium* flagellae, bactrine and lipopolysaccharide (LPs) by SDS-**

### **PAGE technique and immunoblotting:**

(Table 1 and Photo 1) Western blot analysis of the anti Flagellae and anti bactrine immune rabbit serum appears to be reacting with greater intensity with the protein bands located between 60.9 kDa and 62.2 kDa. The bactrine immunoblotting revealed also 2 band of M.W. 30.4 kDa and 28.5 kDa.

Immunoblotting analysis of LPs of *S. Typhimurium* revealed two protein bands. MW 22.4 kDa and 19.6 kDa (Photo (1)).

### **Protective immunity induced by flagellae, bactrine and LPs to *S. typhimurium* infection in mice:**

Immunization with as little as 10 µg of flagellae, bactrine conferred 80-100% protection, respectively to mice challenged with up to 500 ID<sub>50</sub> of *S. Typhimurium*. All the mice immunized with 50 µg of flagellae or bactrine induced a strong protective immunity as evident by 100% survivors against challenge with 500 LD<sub>50</sub> of *S. Typhimurium*. The results were different when the mice were challenged with *S. Typhi* 100 LD<sub>50</sub>, since immunization with 50 µg of Flagellae or bactrine gave a protection of about 60-80% respectively. However only 20-40% protection when mice were immunized with 10 µg of flagellae and bactrine, respectively. Immunization with 50µg of LPs gave a protection of only 40% against challenge with 500 LD<sub>50</sub> of *S. Typhimurium* whereas no protection at all was elicited against 100 LD<sub>50</sub>

of *S.typhi*. All control and LPs 10 µg immunized mice died. analysis of the results indicated that the protection in the flagellae and bactrine immunized mice were significantly ( $P < 0.05$ ) whereas the LPs the protection was not significant (Table 1 and fig. 1).

Photo (1): Immunoblot profile flagellae, bactrine and LPs (L) were analysed by immunoblotting using hyperimmune rabbit serum

### **Long-term immunization of mice with Flagellae, bactrine and LPs of *S. Typhimurium*:**

Even at 90 days post-immunization a substantial level of protection (78%) was observed in the immunized mice while bactrine was capable of inducing long-lasting protection (100%). None of LPs immunized and control mice survived, analysis of the results indicated that the duration of protection in the flagellae and bactrine immunized group was significantly different at  $P < 0.05$  compared to the control (Table 2).

### **Results of protection with rabbit anti flagellae anti-bactrine and anti LPs serum:**

The results as shown in Table (3) and Fig. (1) indicated that flagellae, bactrine

Immune rabbit serum were able to confer 100% protection against challenge with 100 LD<sub>50</sub> of *S. Typhimurium* and 50%, 70% protection respectively against challenge with 50 LD<sub>50</sub> *S. Typhi*. LPs immune rabbit serum was able to confer 14%

Table (1): Ability of flagellae, bactrine and LPs to induce protective immunity to *S. Typhimurium* or *S. Typhi* infection in mice:

Immunization	Immunization dose ( $\mu\text{g}$ )	<i>S. Typhimurium</i> (500LD <sub>50</sub> )		<i>S. Typhi</i> (100 LD <sub>50</sub> )	
		No. of survivors /Total No. of mice	% survivors	No. of survivors /Total No. of mice	% survivors
Flagellae	10	4/5	80	1/5	20
Bactrine	10	5/5	100	2/5	40
LPs	10	0/5	0	0/5	0
Control*	-	0/5	0	0/5	0
X <sup>2</sup>			4.562#		5.685#
Flagellae	50	5/5	100	3/5	60
Bactrine	50	5/5	100	4/5	80
LPs	50	2/5	40	0/5	0
Control	-	0/5	0	0/5	0
X <sup>2</sup>			9.541#		8.754#

\* Control mice were injected with tris-EDTA buffer.

protection against *S. typhimurium* but no protection at all against *S. Typhi*. Control mice injected with normal rabbit serum showed no protection at all.

The results of Table (4) groups of BALB/c mice were immunized by injecting  $5 \times 10^4$  live *S. Ty-*

*phimurium*. Control mice were treated similarly with phosphate-buffered saline. At weekly intervals DTH was tested by injecting  $5\mu$  of the flagellae, bactrine, LPS extract into the hind footpad. The other hind footpad served as the buffer control.

Table (2) : Results of long-term immunization of mice with flagellae, bactrine and LPs of *S. Typhimurium* (Duration of protection).

Immunization dose	Time of challenge after last injection (days)	No. of survivors/No. of mice in group	% survivors
50µg			
Flagellae	30	9/9	100
Bactrine	30	9/9	100
LPs	30	0/9	0
Control*	30	0/9	0
X <sup>2</sup>			7.822#
Flagellae	60	8/9	89
Bactrine	60	9/9	100
LPs	60	0/9	0
Control	60	0/9	0
X <sup>2</sup>			8.965#
Flagellae	90	7/9	78
Bactrine	90	9/9	100
LPs	90	0/9	0
Control	90	0/9	0
X <sup>2</sup>			8.754#

# significant at P < 0.05 using Chi square test.

Table (3): Results of passive transfer of protection with rabbi anti-flagellae, anti-bactrine and anti-LPs serum.

Immunization serum	<i>S. Typhimurium</i> (100LD <sub>50</sub> )		<i>S. Typhi</i> (50 LD <sub>50</sub> )	
	No. of survivors/Total No. of mice	% survivors	No. of survivors/Total No. of mice	% survivors
Anti-Flagellae	7/7	100	5/9	55.5
Anti-Bactrine	7/7	100	7/9	77.7
Anti-LPs	1/7	14.28	0/9	0
Control*	0/7	0	0/9	0
X <sup>2</sup>		16.541#		27.652#

\* Control mice were injected with normal rabbit serum

# significant at P < 0.05 using Chi square test.

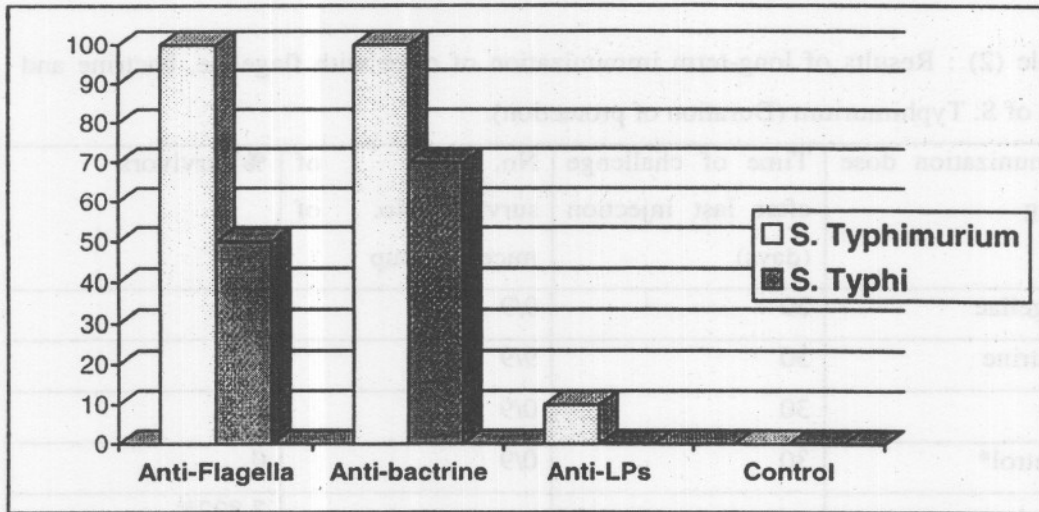


Fig. (1): Passive immunization assay of protection with rabbit anti-flagellae, bactrine and anti-LPs serum.

Table (4): DTH response elicited by various antigenic preparations in *S. typhimurium*-infected mice.

Immunization	Elicitation (days postimmunization)	Mean increase in footpad thickness (0.1-mm units) $\pm$ SE <sup>a</sup> after elicitation with:		
		Flagellae	Bactrine	LPS
Phosphate-buffered saline (control) M525 ( $5 \times 10^4$ )	None	2.5 $\pm$ 0.3	2.1 $\pm$ 0.2	0.9 $\pm$ 0.1
	7	7.4 $\pm$ 0.6	7.5 $\pm$ 0.5	2.4 $\pm$ 0.3
	15	12 $\pm$ 0.7	11 $\pm$ 0.7	6.1 $\pm$ 0.4
	21	16 $\pm$ 0.9	14 $\pm$ 0.5	7.2 $\pm$ 0.7
	30	8.2 $\pm$ 0.4	9.2 $\pm$ 0.6	5.4 $\pm$ 0.4



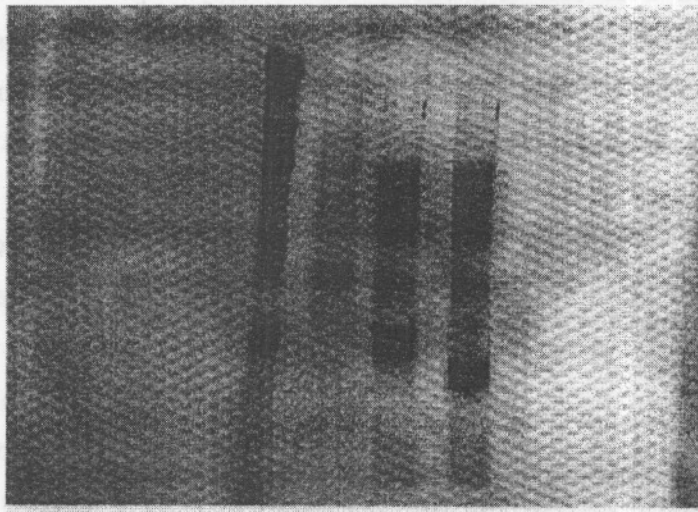


Fig. (2): Immunblotting of salmonella Typhimurium. lane M . Marker, lane 1. S. typhimurium flagella, lane, 2. S. typhimurium bactrine, lane, 3. S. typhimurium LPs.

## DISCUSSION

Flagellin represents the most active bacterial immunogenes described thus for both innate and adaptive mammalian immune system (Eaves-pyles et al., 2001) salmonella flagellin. The presence of flagella may act as a cloak to partially protect salmonellae from the microbicidal enzymes, pH and other defenses of the macrophages (Wyant et al., 1999). The current study was undertaken to evaluate the protective immunity induced by flagellae, bactrine, and LPs to test, the efficacy of these antigen in the protection against. S. Typhimurium infection.

Highly purified polymeric flagellin preparation from Salmonella were used to produce specific Salmonella H antisera with high titers by the im-

munization of rabbits. Antigen emulsions in complete Freund adjuvant were administered at the rate of 50 µg per rabbit by multiple intradermal injection. Booster injections were given 110 days after the primary immunization. Moreover, in most cases the characteristics of the antisera produced, such as titers and levels of contaminating O antibodies, have not been reported. (George et al., 1985).

The production of antisera with high titers and specificity to Salmonella flagellins is of current interest. This is because of the need to develop rapid immunoassay procedures for the detection of salmonellae. (Gray et al. 2006).

A study by strogio and Ferreira (2001) found that flagellae extracted form both smooth and rough

strains of *S. Typhimurium* were able to induce protective immunity to salmonellosis. Also, Qureshi and Takayama 1982. suggested that immunization with LPs at doses equivalent to those found in the flagellae was considerably inferior to the Flagellae in.

In this concern Ikeda et al (2001) suggested that flagellae from rough strain of *S. Typhimurium* protect mice against long infection as long as these Flagellae are linked to polysaccharide which function, as an adjuvant. Sbrogio, et al, (2001) indicated that O-specific polysaccharide-porin conjugate vaccine, protects mice against challenge with *S. Typhimurium*.

The results indicate that the immune response is directed at shared epitopes between these *Salmonella* species. Sant Anna et al., (1982) found that by gene sequencing it has been found that there is extensive homology among the flagellae specifically the porin of different gram negative bacteria despite some polymorphism. This result could also have valuable applications in the future for the development of vaccine against more than one species of Gram-negative pathogens.

Verma et al., (1995) found that good protection elicited by the live vaccine was correlated with a cellular immunity directed both against O-antigen.

Interestingly, flagellae and bactrine induced long-lasting protection which persisted at least up to 90 day after immunization. Whereas non of LPs immunized mice withstood the challenge.

This result is of Importance because other non-viable preparations like acetone-killed cells and LPs were shown to afford immunity for only a short duration MeEwen et al., (1992). Also, suggested that flagella is capable of inducing a substantial level of protection many months post-vaccination. To determine the role of humoral immunity in the protection of mice against *S. Typhimurium* by Flagellae, bactrine and LPs, we performed passive immunization assay.

The protection results agree with Yokoyama et al (1998) who concluded that antibodies specific for *Salmonella* flagella, bactrine may protect mice from experimental salmonellosis when passively administered orally. Of these antibodies, anti-flagella exhibited the highest level of protection in-vivo and in-vitro. In the present study, LPs Immune rabbit serum was able to confer protection 14% against *S. Typhimurium* but no protection at all against *S. Typhi*. This result agree with that of Stocker and Maklia (1986) who suggested that antibodies against the somatic antigen (LPs) correlate well with previous infection but not with protection.

It is interesting that the western blot analysis of

flagellae rabbit antiserum showed that it reacted mainly against the polypeptide band migrating between 60 and 62. These results suggested that this protein may be the main target of the immune response and the humoral immunity directed against flagellae probably the porin play an important role in the immunity against *S. Typhimurium* Sbrogio- and Ferreira. 2001.

Among the three antigenic preparations. only flagellae was able to elicit a typical DTH reaction, we suggesting that flagella could be used as an eliciting antigen in mouse salmonellosis. It is also important to point out here that in human typhoid there is no suitable assay system at present to detect the CMI reaction in vivo or in vitro. Which may be due to the lack of a suitable test antigen to be used either for DTH elicitation without side effects or for proliferative response. Cho et al, (1983).

It can be concluded that flagellae probably bac-  
trine are good nrununogens in the induction of protective immunity against the relevant *S. Typhimurium* as well as closely related bacteria of the genus *Salmonella*, *S. Typhi*. In addition, this protective immunity is with long lasting' immunogenic memory.

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