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# 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

Slamonellosis is a disease of major economic importance and to be continuously to 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 fla gellae of Gram-negative bacteria in the inductio of specific immunity The production of flagella

(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 Bactariology Department, Animal Health Research Institute. S. Typhimurium was originally isolated from diarrheic calves. *S. Typhi* was originally isolated form 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 a (1990)**

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

which had been skaken 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 blendor 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 4oC. After the pellet was reuspended 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 sub cutaneous (S/C) injection of the lagellae, bactrin and LPs preparation, respectively. The injection were given on day 0, 14, 28, 42. 100µg protein indicated antigen was determined in the presen of Freund complete adjuvant. The fourth gro

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. bactine 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 flogellae, bactrine and LPs, respectively. Ten mice of each group were injected with 10µg of antigen and the remaining mice (10) were injected with 50 µg of antigen subcutaneously S/C twice at 15 day intervals. The fourth group was injected with Tris-EDTA buffer (control group). Tend days after the last injection, each group of mice was challenged with 500 $LD_{50}$ of S. Typhimurium or challenged with 100 $LD_{50}$ 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, bactnne 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 50µg of Flagellae, bactrine and LPs, respectively at 15 day interval 30, 60, 90 days before challenge with 500 $LD_{50}$ of S. Typhimurium. For each tiem 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 $LD_{50}$ of S. Typhimurium or with 50 $LD_{50}$ 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 Photol) 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.

Immoblotting 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 $ID_{50}$ fo 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 $LD_{50}$ of S. Typhimurium. The results were different when the mice were challenged with S. Typhi 100 LD50, since immunizatioon 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 prootection of only 40% against challenge with 500 $LD_{50}$ of S. Typhimurium whereas no protection at all was elicited against 100 $LD_{50}$ of S.typhi. All control and LPs 10 µg immunized mice died. analysis of the results indicated that the protectionin 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 flagella 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 $LD_{50}$ of Typhimurium and 50%, 70% protection respectively against challenge with 50 $LD_{50}$ S. Typh 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 (μg) | S. Typhimurium (500$LD_{50}$) | | S. Typhi (100 $LD_{50}$) | |
|---|---|---|---|---|---|
| | | No. of survivors /Total No. ofmice | % survivors | No. of survivors /Total No. ofmice | % 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 |
| $X^2$ | | | 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 |
| $X^2$ | | | 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 x $10^4$ live S. Typhimurium. Control mice were treated similarly with phosphate-buffered saline. At weekly intervals DTH was tested by injecting 5μ 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 50µg | Time of challenge after last injection (days) | No. of survivors/No. of mice in group | % survivors |
|---|---|---|---|
| Flagellae | 30 | 9/9 | 100 |
| Bactrine | 30 | 9/9 | 100 |
| LPs | 30 | 0/9 | 0 |
| Control* | 30 | 0/9 | 0 |
| $X^2$ | | | 7.822# |
| Flagellae | 60 | 8/9 | 89 |
| Bactrine | 60 | 9/9 | 100 |
| LPs | 60 | 0/9 | 0 |
| Control | 60 | 0/9 | 0 |
| $X^2$ | | | 8.965# |
| Flagellae | 90 | 7/9 | 78 |
| Bactrine | 90 | 9/9 | 100 |
| LPs | 90 | 0/9 | 0 |
| Control | 90 | 0/9 | 0 |
| $X^2$ | | | 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 | S. Typhimurium ($100LD_{50}$) | | S. Typhi ($50\ LD_{50}$) | |
|---|---|---|---|---|
| | 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 |
| $X^2$ | | 16.541# | | 27.652# |

* Control mice were injected with normal rabbit serum

# significant at $P < 0.05$ using Chi square test.

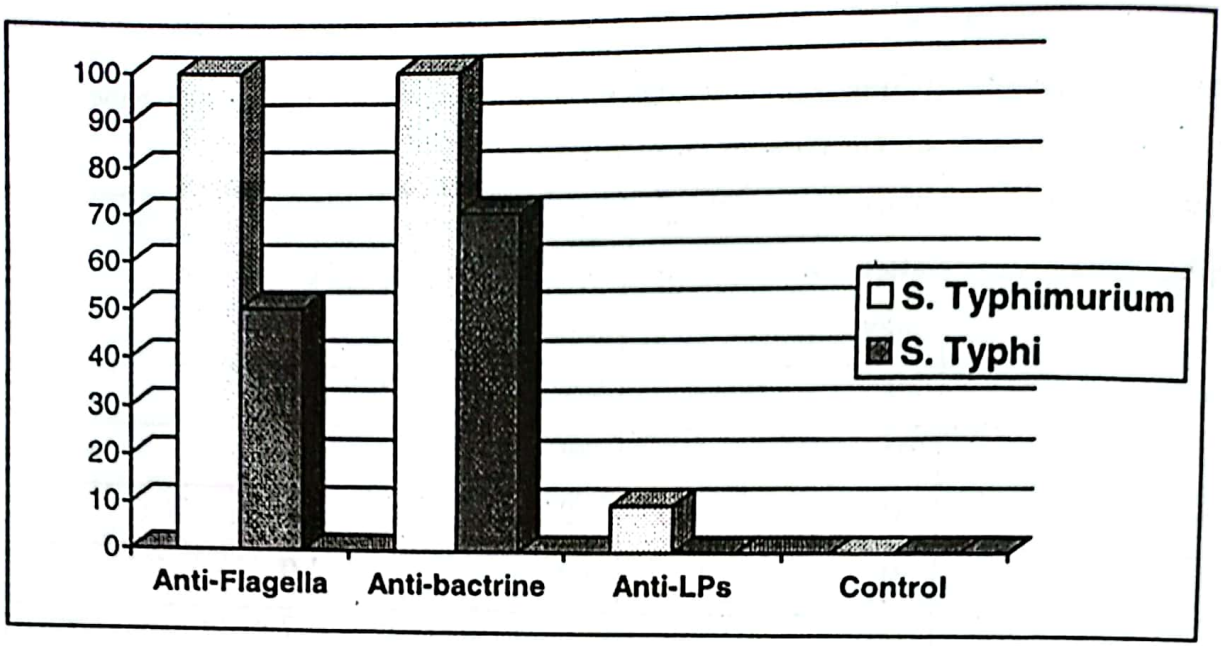


Fig. (1): Passive immunization assay of protection with rabbit anti-flagellae, bactrine and anti-LPs serum.

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) ± SE[a] after elicitation with: | | |
|---|---|---|---|---|
| | | Flagellae | Bactrine | LPS |
| Phosphate-buffered saline (control) M525 ($5 \times 10^4$) | None | 2.5±0.3 | 2.1±0.2 | 0.9±0.1 |
| | 7 | 7.4±0.6 | 7.5±0.5 | 2.4±0.3 |
| | 15 | 12±0.7 | 11±0.7 | 6.1±0.4 |
| | 21 | 16±0.9 | 14±0.5 | 7.2±0.7 |
| | 30 | 8.2±0.4 | 9.2±0.6 | 5.4±0.4 |

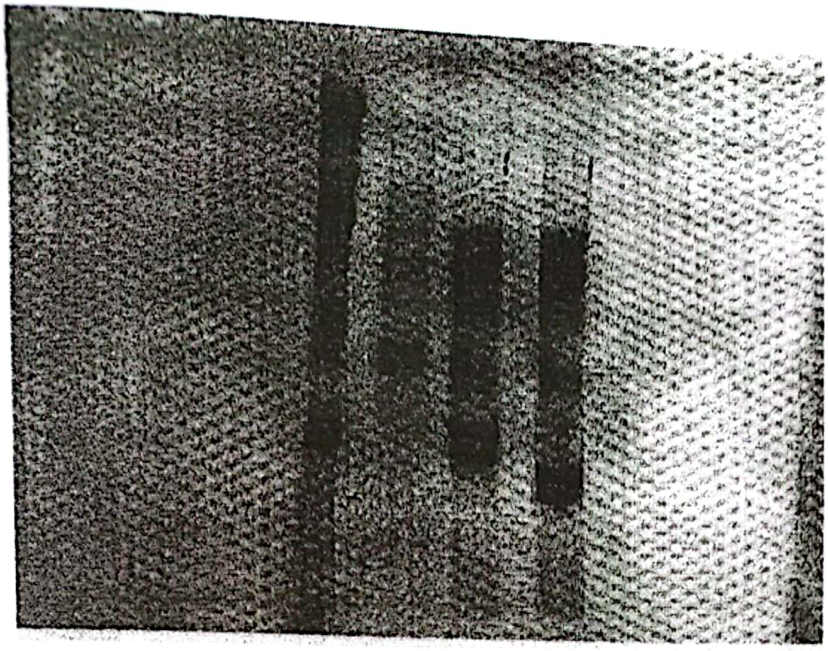

Fig. (2): Immunbloting of salmonella Typhimurium. lane M . Marker, lane 1. S. typhimurium flagella, lane, 2. S. typhimurium bactrine, lane, 3. S. typhimurium LPs.

## DISCUSSION

Flagellin represents the most active bacterial immunogenes described thus for both innate and adaptive mammalian immune system (Eavespyles 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 immunization 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 slamonellosis. 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 salmomllosis 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 Stockcr 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

gellae rabbit antiserum showed that it reacted inly against the polypeptide band migrating be- een 60 and 62. These results suggested that this otein may be the main target of the immune re- onse and the humoral immunity directed against agellae probably the porin play an important le in the immunity against S. Typhimurium brogio- and Ferreira. 2001.

among the three antigenic preparations. only fla- ellae was able to elicit a typical DTH reaction, ve suggesting that flagella could be used as an liciting antigen in mouse salmonellosis. It is also mportant to point out here that in human typhoid here is no suitable assay system at present to de- tect 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. Typhi- murium as well as closely related bacteria of the genus Salmonella, S. Typhi. In addition, this pro- tective immunity is with long lasting' immuno- genic memory.

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