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# Characterization and Lethality of Endotoxic Lipopolysaccharides (LPS) of *Salmonellae*

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**Abstract**
*Salmonellae* Lipopolysaccharides (LPS) could be extracted from different serovars in amounts varying from 1.5% to 5% of bacterial weight. No clear influence of motility on the amount of extracted LPS was detected. These LPS were studied by Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) and all of them gave one major band representing the core antigen with a molecular weight varying from 4 to 12 while some of them showed extra minor bands. The LD50 of different LPS preparations varied from 150 to 300 µg. The highest value was obtained by motile and non-motile *Salmonella Dublin* (300 µg each). Sensitization of mice at time of LPS injection by galactosamine resulted in the exaggeration of lethality induced by LPS preparation for up to 300 times.
**Keywords:** Lipopolysaccharide – LPS – Salmonella– SDS-PAGE – electrophoresis

## 1. INTRODUCTION

*Salmonellae* are thought to be major pathogens leading to serious economic losses in animal industry. Salmonellosis is an important endemic disease of calves. Hardman et al., (1991) documented the increase incidence of salmonellosis, associated with the development of intensive rearing system, especially that caused by *Salmonella Typhimurium* in calves. Pathogenicity of *Salmonellae* in animals and human beings reflects their virulence and this is due to or depends upon several factors related to the host and to the bacteria. These factors are named the virulence factors or components of virulence. Virulence in microorganisms is associated with the capacity to attach and colonize at the site of infection, with subsequent damage to the host and is promoted by aggressions that interfere with the host defense (Burrows, 1985).

Recently the bacterial lipopolysaccharide (LPS) which is a major component of cell surface has received much attention as a factor of pathogenicity in Gram negative infections. It's thought to induce a large number of pathophysiology activities that may lead to shock and death within the host (Weinbaum et al., 1971). Bacterial lipopolysaccharides (endotoxins, LPS) belong to the most potent immunostimulators in mammals (Ulrich et al., 2003).

Endotoxin or bacterial lipopolysaccharide (LPS) causes various inflammatory symptoms and pathophysiological disorders, including fever, disseminated intravascular coagulation, multiple organ failure, and septic shock (30, 33). The septic shock induced by bacteremia caused by gram-negative bacteria is thought to be due to the massive release of endotoxin from infecting organisms by spontaneous release or bacterial lysis (Teruo et al., 1998). Endotoxins, also called lipopolysaccharides (LPS), are major contaminants found in commercially available proteins or biologically active substances, which often complicate study of the biological effects of the main ingredient. The presence of small amounts of endotoxin in recombinant protein preparations can cause side effects in host organism such as endotoxin shock, tissue injury, and even death. (Pérola et al., 2007)

During infection with gram-negative bacteria, exposure of immune cells to lipopolysaccharide (LPS) from the bacterial cell membrane induces a rapid cytokine response which is essential for the activation of host defenses against the invading pathogens. Administration of LPS to mice induces a state of hyporesponsiveness, or tolerance, characterized by reducing cytokine production upon subsequent LPS challenge (Martin et al., 2001).

Therefore, the present work was planned to extract, purify and characterize LPSs of different *Salmonella* serovars using SDS-PAGE. Lethality of LSPs in mice was also an objective of this study.

## 2. MATERIAL AND METHODS

Fifty six *Salmonella* isolates were obtained from newly born cow and buffalo calves (apparently healthy, diarrheic and dead) during the period from May 2013 to August 2013. All isolates were identified morphologically, culturally, biochemically and serologically according to Kreig and Holt (1984) and Collee et al., (1996). The *Salmonella* isolates belonged to 6 serovars: *Typhimurium*, *Anatum*, *Dublin, Enteritidis*, *Meleagridis*, *Infantis*, *Paratyphi* B and Larochell. Serotyping was carried out using polyvalent O, H and monovalent antisera. Motility was assured by stab cultivation of the organism into tube of semisolid penassay agar (Carsiotis et al., 1984). From each serotype one motile and non-motile (if found) were used for the following experiments.

**1- Extraction and characterization of lipopolysaccharides**

LPS was extracted and purified from proteins and other contaminant by phenol-chloroform-petroleum ether method described by Galanose et al., (1969) and modified by Qureshi and Takayama, (1982).

 

a- Preparation of bacteria: Bacteria were cultivated as described by Schulte et al., (1968): Hrvested bacteria were washed with water and treated successively with ethanol, acetone and twice with ether.

b- Extraction: a mixture containing liquid phenol (90 g phenol + 11 ml water) chloroform and petroleum ether in a volume ration of 2:5:8 respectively was prepared. Dried bacteria were placed in a centrifuge vessel and extraction mixture was added in a ratio of 1:4. The suspension was then homogenized with homogenizer for 2 minutes at 20ºC. The bacteria were centrifuged at 5000 rev/min. supernatant which contained LPS was withdrawn by sterile pipette. The bacterial residue was extracted once mere with the same amount of extraction mixture, stirred and centrifuged as above mentioned and supernatant was added to the first extract. The extraction was repeated for a third time. Petroleum ether and chloroform were then removed on a rotary evaporator 30ºC - 40ºC and remaining phenol was crystalized. Six volumes of diethyl ether/acetone (1:5 V/V) were added to one volume of the LPS phenol solution. The precipitated LPS were centrifuged (3000 rev/min for 10 minutes).

The supernatant was decanted and the tube was allowed to stand for 2 to 3 minutes upside down. It was then wiped inside with filter paper. The precipitate was washed 2 to 3 times with 80% phenol and the inside of the tube was siped with filter paper after decantation of the supernatant. The precipitate was washed 3 times with ether to remove the remaining of phenol. LPS was taken in distilled water at 45ºC and vacuum applied to remove air. LPS were then centrifuged at 100000xg for 4h. Resulting sediment each *Salmonella* serovar, tubes were weighted before and after extraction and purification. The difference between the two weights represented the amount of extracted LPS was expressent in mg/gram of dry bacteria.

**2- Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

It was performed according to method described by Lugfenberg et al. (1975).

**3- Silver stain**

Staining of LPS in the gel was according to Tsai and Fragsch (1982). The LPS in the gel was stained dark brown in 2-5 minutes.

**4- Lethality and calculated lethal dose (LD50) of LPS**

Lethality of LPS was carried out as Galaonse et al. (1979) and Shnrya et al. (1993). One and 350µg of LPS of LPS was administrated I/P in 0.5 ml pyrogen free PBS in sensitized and non-sensitized mice by 8 mg galactosamine respectively. In control experiment, mice were given either 1µg of LPS I/P or 8 D-galactosamine.

## 3. RESULTS

Results illustrated in Table (1) showed that LPS could be extracted from *Salmonella* serovars in amounts varying from 1.5% of the bacterial weight (as in non-motile *S. Anatum*) up to 5% in motile *S. Dublin* . The percentages of extracted LPS from motile versus non-motile bacteria were 4% vs 2.4%, 3% vs 1.5%, 5% and 3% and 3% vs 3% in *S. Typhimurium, S. Anatum, S. Dublin and S. Meleagridis respectively.*

Results indicated that one major band in SDS-PAGE was detected after staining with silver nitrate method and the molecular weight (MW) ranged between 5 and 12 KDa.

There were some extra minor bands detected with non-motile *S. Typhimurium,* motile *S. Anatum*, motile and non-motile serovar *Dublin* , non-motile serovar *Meleadridis*, motile serovar *Infantis*, motile serovar *Paratyphi* B and serovar *Larochelle*.

LD50 of different LPS extracted from *Salmonellae* Varied from 150µg to 300 µg. No clear influence for motility of the LD50 values in examined *Salmonella* strains was observed (Table 2).

Administration of 1 µg LPS/sensitized mice with galactosamine resulted in 100% lethal effect in *Salmonella* serovars, *Typhimurium, Enteridis, Meleagridis, Infantis*, *Paratyphi B and Larochelle* while it was 90% in mice injected with non-motile serovar *Anatum* as well as motile and non-motile *S. Dublin* (Table 3).

## 4. DISCUSSION

Lipopolysaccharide plays an important role in the outer membrane providing it with selective permeability and determines its interaction with the host cells bacteriophages (Rycroft, 1984). Weinstein et al. (1985) Suggested that the bacterial survival within the host mucosa may depend on the presence of smooth LPS.

The percentages of extraction that represent 1.5 - 5% of bacterial body weight obtained by phenol chloroform ether method described by Qureshi and Takayma (1982) are in agreement with those obtained with different *Salmonella* serovars by Galanose et al. (1969) who recommended this method of extraction. Although there was unexpectedly increase in the amount of LPS extracted from motile versus to non-motile serovars was different especially in *S. Anatum* yet (Table 1). This variation was not constant indicating that it may be accidental. Generally there is no clear influence of motility in the amount of extracted LPS. In this concern, Carsiotis et al. (1984) found no quantitative difference in LPS extracted from motile and non-motile *Salmonellae.*

Extracted LPS preparations obtained in almost pure from indicated one major band in SDS-PAGE as detected after staining with silver nitrate method. This band presumably represents the core region as reported by Bradury

 

et al. (1984). This core polysaccharide of different LPS is structurally similar in most lipopolysaccharides as reported by Siber et al. (1985). SDS-PAGE of *Salmonella* LPS extracted showed different mobilities that ranged from 5 to 12 kDa molecular mass regions which are similar to that reported by Bogard et al. (1987). Some other multiple bands of various molecular weights represent clusters of polysaccharide side chains. Similar observation was recorded by Ludertiz et al. (1984) and Martin et al. (2001).

LD50 of extracted LPS (Table 2) indicated that there was no distinct variation correlated with motility or with the amount of extracted LPS. The values of LD50 of LPS of different *Salmonella* serovars varied from 150 µg to 300 µg. this result is in agreement with that reported by Galanose et al. (1979).

The lethal effect of LPS was studied in galactosamine sensitized and non-sensitized mice. In mice sensitized with galactosamine, the lethality reached 90 to 100% after I/V injection of 1 µg of LPS compared with 80 to 100% after I/V injection of 350 µg in non-sensitized mice. These results run parallel to those obtained with Galanose et al. (1979) who reported increased susceptibility with galactosamine several thousand fold, and shnyra et al. (1993) who reported to 100 fold increase.

It's hopped that data obtained will lead to more study about the pathogenesis of *Salmonellae* in calves.

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Table (1): Amount of LPS extracted from *Salmonella* serovars isolated from newly born cow and buffalo calves

| *Salmonella* serovars | Weight of bacteria in g | Weight of extracted LPS in mg | % of LPS from extracted bacteria |
|---|---|---|---|
| Tm | 6 | 240 | 4 |
| Tn | 7.5 | 185 | 2.46 |
| Am | 6.5 | 195 | 3 |
| An | 5 | 75 | 1.5 |
| Dm | 7 | 350 | 5 |
| Dn | 8.5 | 255 | 3 |
| Em | 6 | 240 | 4 |
| Mm | 5.5 | 165 | 3 |
| Mn | 7 | 210 | 3 |
| Im | 8 | 240 | 3 |
| Pm | 6 | 240 | 4 |
| Lam | 7.5 | 225 | 3 |

Tm= *Typhimurium* motile
Dm= *Dublin* motile
Tn= *Typhimurium* non- motile
Dn= *Dublin* non- motile
Em= *Enteritidis* motile
Pm= *Paratyphi* B motile
Am= *Anatum* motile
Mm= *Meleagridis* motile
An= *Anatum* non- motile
Mn= *Meleagridis* non- motile
Im= Infantile motile
Lam= Larochelle motile

Table (2): Calculated LD50 of different LPS extracted from *Salmonella* strains

| Strains | Mortality in mice after injection with | | | | | | Calculated LD50 µg |
|---|---|---|---|---|---|---|---|
| | 100µg | 150µg | 200µg | 250µg | 300µg | 350µg | |
| Tm | 0/10 | 2/10 | 3/10 | 5/10 | 7/10 | 10/10 | 250 |
| Tn | 1/10 | 2/10 | 5/10 | 7/10 | 7/10 | 9/10 | 200 |
| Am | 1/10 | 3/10 | 3/10 | 5/10 | 8/10 | 9/10 | 250 |
| An | 0/10 | 1/10 | 3/10 | 4/10 | 7/10 | 8/10 | 260 |
| Dm | 0/10 | 1/10 | 3/10 | 3/10 | 5/10 | 8/10 | 300 |
| Dn | 0/10 | 2/10 | 3/10 | 3/10 | 5/10 | 8/10 | 300 |
| Em | 3/10 | 5/10 | 7/10 | 7/10 | 9/10 | 10/10 | 150 |
| Mm | 0/10 | 2/10 | 4/10 | 7/10 | 7/10 | 9/10 | 230 |
| Mn | 0/10 | 1/10 | 2/10 | 5/10 | 8/10 | 9/10 | 250 |
| Im | 2/10 | 3/10 | 5/10 | 6/10 | 8/10 | 8/10 | 250 |
| Pm | 1/10 | 2/10 | 5/10 | 7/10 | 7/10 | 9/10 | 200 |
| Lam | 2/10 | 3/10 | 6/10 | 8/10 | 9/10 | 10/10 | 190 |

Refer to footnote of table (1)
Dead / Total
No deaths were recorded in control mice

 

Table (3): Lethal toxicity of LPS extracted from different *Salmonella* strains in galactosamine sensitized mice compared with non-sensitized mice.

| Strains | sensitized mice (1 µg) | | non-sensitized mice (350 µg) | |
|---|---|---|---|---|
| | Mice deaths | Lethality% | Mice deaths | Lethality% |
| Tm | 10/10 | 100 | 10/10 | 100 |
| Tn | 10/10 | 100 | 9/10 | 90 |
| Am | 10/10 | 100 | 9/10 | 90 |
| An | 10/10 | 90 | 8/10 | 80 |
| Dm | 9/10 | 90 | 8/10 | 80 |
| Dn | 9/10 | 90 | 8/10 | 80 |
| Em | 9/10 | 100 | 10/10 | 100 |
| Mm | 10/10 | 100 | 9/10 | 90 |
| Mn | 10/10 | 100 | 9/10 | 90 |
| Im | 10/10 | 100 | 8/10 | 80 |
| Pm | 10/10 | 100 | 9/10 | 90 |
| Lam | 10/10 | 100 | 10/10 | 100 |

Refer to footnote of table (1)
( ) = Dose of LPS injected I/V

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