



ISOLATION, EXTRACTION AND PURIFICATION OF LIPOPOLYSACCHARIDE FROM *SALMONELLA TYPHI* FOR DIAGNOSTIC APPLICATIONS

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ABSTRACT:

Typhoid fever remains a major public health concern in developing countries, particularly due to limitations in rapid and reliable diagnostic techniques. *Salmonella typhi*, the etiological agent of typhoid fever, possesses lipopolysaccharide (LPS) as a major outer membrane component that plays a crucial role in pathogenesis and host immune response. The present study was undertaken to isolate, extract, and purify lipopolysaccharide from *Salmonella typhi* using a salt extraction method followed by gel filtration chromatography, with the ultimate aim of producing a highly purified antigen suitable for diagnostic applications such as enzyme-linked immunosorbent assay (ELISA). The bacterial strain of *S. typhi* was cultured under controlled laboratory conditions and characterized using standard microbiological and biochemical methods. Bulk cultures were inactivated using formalin, followed by harvesting and disruption of cells through a temperature gradient method. The outer membrane fraction was obtained by differential centrifugation and ultracentrifugation. Lipopolysaccharide was extracted using sequential buffer treatments containing sodium dodecyl sulfate (SDS) and further purified by Sephadex G-100 gel filtration chromatography. Purity and molecular weight of the isolated LPS were assessed by 12% SDS-PAGE and silver staining. The purified LPS appeared as a single distinct band with an approximate molecular weight of 36 kDa, indicating high purity (>95%). UV spectrophotometric analysis revealed a single prominent elution peak during gel filtration, confirming successful separation of contaminating proteins. The findings demonstrate that the adopted methodology is efficient, reproducible, and suitable for obtaining diagnostically relevant LPS antigen. This purified antigen can serve as a valuable component in the development of sensitive and specific immunodiagnostic assays for typhoid fever.

Keywords *Salmonella typhi*, Lipopolysaccharide, LPS purification, Gel filtration, SDS-PAGE, Typhoid fever, ELISA antigen

INTRODUCTION:

Microorganisms are ubiquitous in nature and exist in virtually every environment, including soil, water, air, and living organisms. While many microorganisms are beneficial, several species are pathogenic and pose serious threats to human health. Contamination of food and water by pathogenic bacteria remains a major cause of infectious diseases, particularly in developing countries where sanitation and hygiene infrastructure are inadequate (Pelzar and Reid 1965).

Among food- and water-borne bacterial pathogens, members of the family *Enterobacteriaceae* are of significant concern. The genus *Salmonella* includes numerous pathogenic species responsible for severe gastrointestinal and systemic infections. *Salmonella typhi*, the causative agent of typhoid fever, remains one of the most important public health pathogens in tropical and subtropical regions, including India (Chin et al. 1998).

Typhoid fever is a systemic infection characterized by prolonged fever, abdominal discomfort,

hepatosplenomegaly, rose spots, intestinal ulceration, and, in severe cases, intestinal perforation. Globally, typhoid fever affects approximately 17 million individuals annually, resulting in nearly 600,000 deaths, predominantly in developing nations (Chin et al. 1998). Poor sanitation, unsafe drinking water, and inadequate sewage disposal systems significantly contribute to disease transmission.

Laboratory diagnosis of typhoid fever is challenging. Blood culture remains the gold standard but is time-consuming and often yields false-negative results due to prior antibiotic therapy. The Widal test, though widely used, lacks specificity and sensitivity and frequently produces false-positive or false-negative results, particularly in endemic areas or vaccinated individuals (Nandkumar and Muthukkaruppan 1993, Kristana et al. 2000, Debrah et al. 2001). These limitations necessitate the development of more reliable diagnostic tools.

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, plays a critical role in bacterial virulence and host immune response. In *S. typhi*, LPS acts as a potent antigen capable of inducing both humoral and cell-mediated immunity. Due to its antigenic specificity, LPS has emerged as a promising candidate for use in immunodiagnostic assays such as ELISA (Nguyen et al. 2002).

The present study focuses on the isolation, extraction, and purification of LPS from *Salmonella typhi* using a salt extraction method followed by gel filtration chromatography. The ultimate objective is to obtain highly purified LPS suitable for diagnostic applications.

MATERIALS AND METHODS

Study Design and Experimental Overview

The present investigation was designed as a laboratory-based experimental study aimed at the isolation, extraction, and purification of

lipopolysaccharide (LPS) from *Salmonella typhi*. The study followed a stepwise methodological approach that included bacterial culture and characterization, bulk cultivation, cell inactivation and harvesting, isolation of the outer membrane fraction, extraction of crude LPS, purification by gel filtration chromatography, and biochemical characterization of the purified LPS using SDS-PAGE and silver staining. Each experimental step was carried out under aseptic conditions to avoid contamination and to ensure reproducibility of results.

2. Bacterial Strain and Maintenance

A reference strain of *Salmonella typhi* was obtained from the Department of Biotechnology, New Arts, Commerce and Science College, Wardha. The strain was preserved on nutrient agar slants and stored at 4 °C. Periodic subculturing was performed to maintain viability and purity of the organism.

Before experimental use, the culture was revived by inoculation into nutrient broth and incubated at 37 °C for 18–24 hours. Purity of the culture was confirmed by streaking on selective and differential media.

3. Microbiological and Biochemical Characterization

3.1 Gram Staining and Morphology

Smears were prepared from overnight cultures, heat-fixed, and stained using the Gram staining technique. Microscopic examination revealed Gram-negative, short rod-shaped bacteria arranged singly or in pairs.

3.2 Motility Test

Motility was assessed using the hanging drop method and motility agar. The organism exhibited characteristic active motility, consistent with peritrichous flagellation.

3.3 Cultural Characteristics on Selective Media

The isolate was cultured on the following media to confirm identity:

- **MacConkey Agar:** Non-lactose fermenting pale colonies
- **Salmonella-Shigella Agar:** Colorless colonies with black centers due to H₂S production
- **Bismuth Sulphite Agar:** Black colonies with metallic sheen
- **Triple Sugar Iron (TSI) Agar:** Alkaline slant/acid butt with H₂S production

These results confirmed the identity of the isolate as *Salmonella typhi*.

4. Preparation of Bulk Culture

For large-scale LPS extraction, bulk cultures were prepared using tryptic soy (TS) broth. Initially, a seed culture was grown in 250 ml TS broth at 37 °C for 18 hours with gentle shaking. This seed culture was then used to inoculate a total volume of 5 liters of TS broth under aseptic conditions. The bulk culture was incubated at 37 °C for 16–18 hours to achieve optimal bacterial growth. Growth was monitored by turbidity measurement.

5. Inactivation of Bacterial Cells

To ensure biosafety and prevent viable pathogen handling during downstream processing, the bulk culture was inactivated by the addition of 1% (v/v) formalin. The culture was incubated overnight at room temperature with occasional mixing.

Complete inactivation was confirmed by inoculating aliquots of the treated culture onto nutrient agar plates and incubating for 48 hours at 37 °C. Absence of bacterial growth confirmed successful inactivation.

6. Harvesting and Washing of Cells

Inactivated cells were harvested by centrifugation at 5,000 rpm for 30 minutes at 4 °C. The resulting pellet was washed three times with sterile distilled water to remove residual media components and formalin. Each wash step involved resuspension followed by centrifugation under identical

conditions. The wet weight of the washed bacterial pellet was recorded for yield estimation.

7. Cell Disruption and Outer Membrane Isolation

Cell disruption was achieved using a temperature gradient method. The washed bacterial pellet was resuspended in distilled water and subjected to alternating temperature treatments of 20 °C and 160 °C. This process facilitated rupture of the bacterial cell envelope and release of cellular components. The disrupted cell suspension was centrifuged at 5,000 rpm for 30 minutes to remove unbroken cells and debris. The supernatant containing membrane components was then subjected to ultracentrifugation at 100,000 × g for 1 hour at 4 °C.

The resulting pellet, enriched in outer membrane fractions, was carefully collected and stored at 4 °C for further processing.

8. Extraction of Lipopolysaccharide

LPS extraction was carried out using the salt extraction method described by Arokiaswamy and Krishnaswamy (2000).

8.1 Buffer Treatments

The outer membrane pellet was sequentially treated with:

- **Buffer I:** To remove loosely bound proteins
- **Buffer II:** To solubilize membrane-associated components
- **Buffer III (containing SDS):** To extract LPS selectively

Each treatment involved incubation with gentle stirring followed by centrifugation to separate soluble and insoluble fractions.

The supernatant obtained after Buffer III treatment contained crude LPS.

9. Purification by Gel Filtration Chromatography

Crude LPS was further purified using Sephadex G-100 gel filtration chromatography.

9.1 Column Preparation

A Sephadex G-100 column was equilibrated with phosphate-buffered saline (PBS). The column dimensions and flow rate were standardized to ensure optimal resolution.

9.2 Fraction Collection

The crude LPS sample was applied to the column, and fractions were eluted at a flow rate of 0.3 ml/min. A total of 25 fractions were collected.

9.3 UV Spectrophotometric Analysis

Protein and LPS content of each fraction was monitored by measuring absorbance at 280 nm. Fractions corresponding to the major peak were pooled and used for further analysis.

10. SDS-PAGE and Silver Staining

Purity and molecular weight of the purified LPS were assessed using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gel was subjected to silver staining, a highly sensitive method suitable for detecting LPS. The molecular weight of LPS was estimated by comparison with standard molecular weight markers.

RESULT:

1. Morphological, Cultural, and Biochemical Identification of *Salmonella typhi*

The morphological and cultural characteristics were studied following standard microbiological

procedures (Cruickshank et al., 1975; Collee et al., 1996; Barrow and Feltham, 2003). Gram staining of overnight cultures revealed Gram-negative, short rod-shaped bacilli occurring singly and occasionally in pairs. No spore formation was observed. The organism demonstrated active motility when examined by the hanging drop method, confirming the presence of functional peritrichous flagella, a characteristic feature of *Salmonella* species. Biochemical identification was performed using standard diagnostic tests recommended for Enterobacteriaceae (Barrow and Feltham, 2003; Holt et al., 1994). Growth on selective and differential media further supported the identification of the isolate as *Salmonella typhi*. On MacConkey agar, the organism produced pale, non-lactose-fermenting colonies. On Salmonella-Shigella (SS) agar, colorless colonies with black centers were observed due to hydrogen sulfide production. Bismuth sulphite agar showed typical black colonies with a metallic sheen, while Triple Sugar Iron (TSI) agar demonstrated an alkaline slant and acidic butt with hydrogen sulfide production and no gas formation. These phenotypic characteristics were consistent with standard descriptions of *S. typhi* and confirmed the suitability of the isolate for LPS extraction studies. (Fig. 1)

Table 1. Morphological and cultural characteristics of *Salmonella typhi*

Parameter	Test / Medium	Characteristic of <i>Salmonella typhi</i>
Morphology	Gram staining	Gram's negative, short rod
	Motility test	Actively motile (peritrichous flagella)
	Spore formation	Non sporing
Cultural Characteristics	Nutrient agar	Small, smooth, circular, translucent colonies
	MacConkey agar	Pale/colorless colonies (non lactose fermenter)
	Salmonella-Shigella agar	Colorless colonies with black center (H ₂ S positive)

	Wilson & Blair agar	Jet black colonies
Biochemical Characteristics	Indole test	Negative
	Methyl Red test	Positive
	Voges-Proskauer test	Negative
	Citrate utilization	Negative
	Glucose fermentation	Positive (acid only, no gas)
	Lactose fermentation	Negative
	Sucrose fermentation	Negative
	TSI agar	Alkaline slant / Acid butt, H ₂ S positive, no gas



Fig.1 a. *S. typhi* on MacConkys Agar

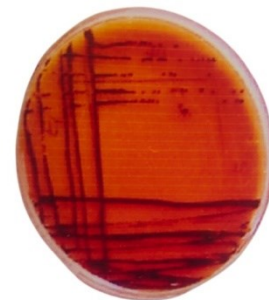


Fig. 1b. *S. typhi* on Salmonella Shigella



Fig. 1c. *S. typhi* on Bismuth Sulphite Agar



Fig. 1d. *S. typhi* on Triple Sugar Iron

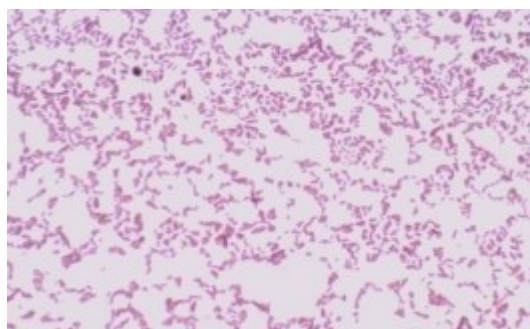


Fig. 1e. Gram's staining shows Small rod shaped, Gram negative, single or paired in arrangement of *Salmonella* spp.

Fig. 1. (1a-1e) Morphological and cultural characteristics of *Salmonella typhi*

Bulk Culture Growth and Biomass Yield

Large-scale cultivation of *S. typhi* in tryptic soy broth resulted in dense bacterial growth after 16–18 hours of incubation at 37 °C. The cultures displayed uniform turbidity, indicating successful proliferation of bacterial cells under the standardized growth conditions.

Following formalin inactivation and centrifugation, bacterial cells were harvested efficiently. The wet weight of the washed bacterial biomass ranged from 10 to 15 g per 5-liter batch of culture. The yield was consistent across independent batches, demonstrating reproducibility of the cultivation and harvesting process. Washing steps effectively removed residual culture medium components, as indicated by the clarity of wash supernatants.

3. Isolation of Outer Membrane Fraction

Cell disruption using the temperature gradient method resulted in effective rupture of bacterial cells, as evidenced by a significant reduction in intact cells following centrifugation. Initial low-speed centrifugation removed unbroken cells and large debris, yielding a clarified supernatant enriched with membrane components. Subsequent ultracentrifugation at $100,000 \times g$ for 1 hour resulted in the formation of a compact pellet containing outer membrane

fractions. The wet weight of the outer membrane pellet ranged from 0.75 g to 0.95 g across two experimental batches. The consistency of membrane pellet yield confirmed the effectiveness of the disruption and fractionation procedures.

4. Extraction of Crude Lipopolysaccharide

Protein profiling was analyzed using SDS-PAGE following the method of Laemmli (1970), and lipopolysaccharide bands were visualized by silver staining (Tsai and Frasch, 1982). Sequential buffer extraction was performed on the isolated outer membrane fraction to obtain crude LPS. Treatment with Buffer I and Buffer II resulted in the removal of loosely associated proteins and non-LPS membrane components, as indicated by proteinaceous material in the supernatants. The final extraction using SDS-containing Buffer III produced a visibly opalescent solution characteristic of lipopolysaccharide preparations. Centrifugation following SDS treatment yielded a clear supernatant containing crude LPS. The extraction process was reproducible and yielded sufficient quantities of LPS for downstream purification and analysis.(Fig.2)

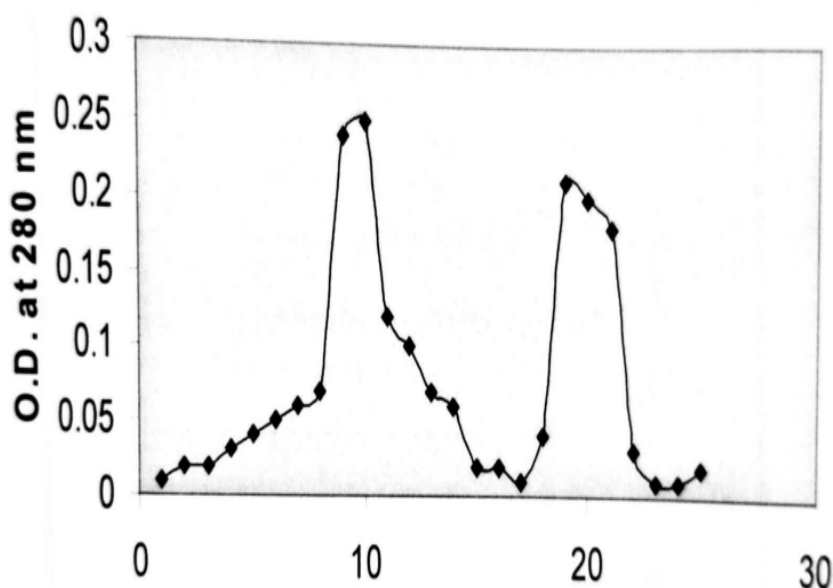


Fig. 2 Extraction of Lipopolysaccharides using Sephadex G-100

5. Gel Filtration Chromatography of LPS

Crude LPS preparations were subjected to gel filtration chromatography using Sephadex G-100 to remove contaminating proteins and achieve high-purity LPS. A total of 25 fractions were collected at a controlled flow rate of 0.3 ml/min. UV spectrophotometric analysis at

280 nm revealed a distinct elution profile. A single major peak was observed, corresponding to fractions containing purified LPS. The absence of multiple peaks suggested efficient separation of LPS from lower-molecular-weight proteins and other contaminants. Fractions corresponding to the major peak were pooled and concentrated for further analysis.

Table 2. Elution of optical density by UV visible spectroscopy

Fraction No.	Optical Density (at 280 nm)
1	0.01
2	0.02
3	0.02
4	0.03
5	0.04
6	0.05
7	0.06
8	0.07
9	0.240
10	0.250
11	0.120
12	0.10
13	0.07
14	0.06
15	0.02
16	0.02
17	0.01
18	0.04
19	0.210
20	0.200
21	0.180
22	0.06
23	0.03
24	0.01
25	0,01

SDS-PAGE Analysis and Purity Assessment

The pooled LPS fractions were analyzed by 12% SDS-polyacrylamide gel electrophoresis followed by silver staining, a highly sensitive method for detecting lipopolysaccharides. The electrophoretic profile revealed a single, well-defined band with an estimated molecular weight of approximately 36 kDa. No additional bands were detected, indicating the

absence of contaminating proteins or degraded LPS fragments. The sharpness and intensity of the band suggested a high degree of purity, estimated to exceed 95%. The molecular weight observed in this study was consistent with previously reported values for *Salmonella* LPS, confirming the structural integrity of the purified antigen (Fig.3).

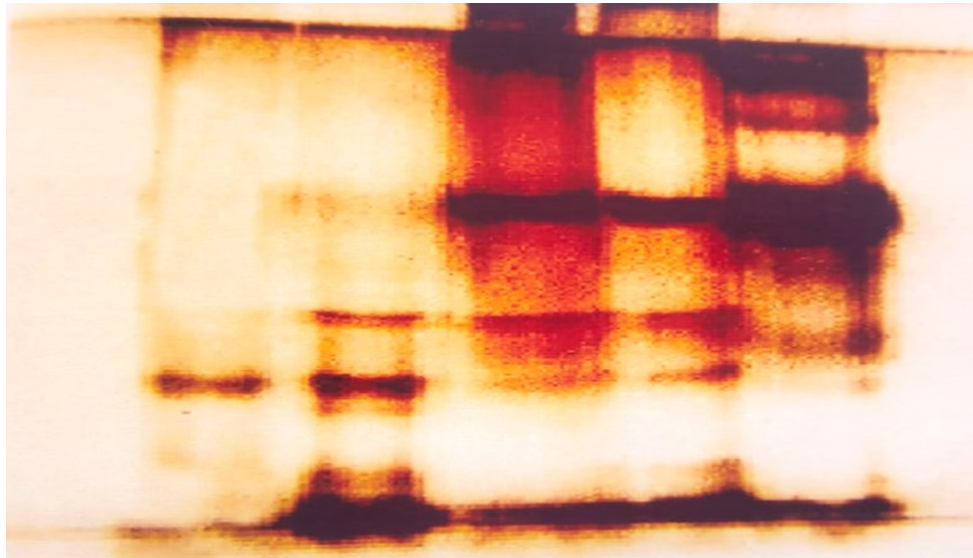


Fig. 3 Shows that single band of 36Kda Lipopolysaccharide was obtained after gel filtration using Sephadex G-100

Lane 1: Marker, BSA 66 KDA

Lane 2: Buffer 1 treatment

Lane 3: Buffer 2 treatment

Lane 4: Buffer 3 treatment

Lane 5: Purified Lipopolysaccharide(LPS) after gel filtration by SEPHADEX-100

Reproducibility of LPS Isolation and Purification

The entire isolation, extraction, and purification procedure was repeated in independent batches to assess reproducibility. Comparable yields of bacterial biomass, outer membrane fractions, and purified LPS were obtained in each batch. SDS-PAGE profiles from independent preparations were nearly identical, demonstrating consistency in molecular weight and purity.

The results of the present study demonstrate successful isolation and purification of lipopolysaccharide from *Salmonella typhi*. The combination of salt extraction and gel filtration chromatography yielded high-purity LPS with a defined molecular weight. The reproducibility of the method and the quality of the purified antigen support its suitability for downstream immunological

applications, particularly in the development of diagnostic assays for typhoid fever.

DISCUSSION

Typhoid fever continues to be a major public health concern in many developing countries, primarily due to inadequate sanitation, limited access to clean drinking water, and challenges associated with accurate laboratory diagnosis. The present study focused on the isolation, extraction, and purification of lipopolysaccharide (LPS) from *Salmonella typhi*, with the objective of obtaining a highly purified antigen suitable for diagnostic applications. The results demonstrate that the adopted salt extraction method combined with gel filtration chromatography is effective, reproducible, and capable of yielding LPS of high purity and defined molecular weight.

The initial identification and characterization of the bacterial isolate confirmed its identity as *Salmonella typhi* based on morphological, cultural, and biochemical characteristics. Accurate species identification is a critical prerequisite for antigen preparation, as cross-contamination or misidentification could compromise antigen specificity and diagnostic reliability (Wain et al., 2015). The consistent growth patterns observed on selective media and characteristic biochemical reactions align with earlier reports describing *S. typhi* phenotypic traits (Levine, 1999).

Bulk cultivation of *S. typhi* in tryptic soy broth yielded substantial bacterial biomass, providing sufficient material for downstream processing. The reproducibility of biomass yield across independent batches reflects the robustness of the cultivation and harvesting procedures. Formalin inactivation ensured biosafety during handling while preserving antigenic integrity, a method commonly employed in bacterial antigen preparation (Arokiaswamy & Krishnaswamy, 2000). Confirmation of complete inactivation was essential to prevent viable pathogen exposure and to comply with laboratory safety standards.

Isolation of the outer membrane fraction represented a critical step in enriching LPS prior to extraction. The temperature gradient method employed in this study effectively disrupted bacterial cells without extensive mechanical shear, thereby minimizing degradation of membrane components. Ultracentrifugation at high speed allowed selective recovery of outer membrane fractions, as reflected by consistent pellet yields. Similar membrane isolation strategies have been reported to be effective for Gram-negative bacteria, particularly when the goal is to preserve LPS structure and antigenicity (Raetz & Whitfield, 2002).

Extraction of LPS using the salt extraction method proved to be efficient and reproducible. Unlike classical hot phenol-water extraction methods, which

involve hazardous reagents and high temperatures, the salt extraction approach offers a safer and less destructive alternative (Westphal & Jann, 1965). Phenol-based methods, although widely used, may result in partial degradation of LPS or co-extraction of contaminating proteins, which can negatively affect antigen purity and immunological performance. The present study demonstrates that salt extraction combined with SDS treatment effectively solubilizes LPS while limiting protein contamination.

The appearance of an opalescent solution following SDS extraction is characteristic of lipopolysaccharide preparations and has been reported as an indicator of successful LPS solubilization (Arokiaswamy & Krishnaswamy, 2000). Sequential buffer treatments facilitated the removal of loosely bound proteins and non-LPS membrane components, thereby improving the quality of the crude extract prior to purification.

Gel filtration chromatography using Sephadex G-100 was a key step in achieving high-purity LPS. Size-exclusion chromatography separates molecules based on molecular size, allowing effective removal of lower-molecular-weight proteins and other contaminants. The UV absorbance profile obtained in this study revealed a single prominent peak, indicating successful separation and homogeneity of the purified LPS preparation. The absence of multiple peaks suggests minimal contamination, which is essential for diagnostic applications where antigen purity directly influences assay specificity (Crowley et al., 2016).

SDS-PAGE analysis followed by silver staining provided further confirmation of LPS purity and molecular integrity. The detection of a single band at approximately 36 kDa is consistent with previously reported molecular weights for *Salmonella* LPS and confirms the structural integrity of the purified antigen (Raetz & Whitfield, 2002). Silver staining is particularly suitable for LPS analysis due to its high sensitivity, allowing detection of minute quantities of

lipopolysaccharide that may not be visible using conventional protein stains.

The absence of additional bands on SDS-PAGE indicates that contaminating outer membrane proteins, such as porins, were effectively removed during purification. This is a significant achievement, as protein contamination is a common challenge in LPS preparation and can lead to cross-reactivity in immunoassays (Rietschel et al., 1994). High-purity LPS is therefore essential for developing reliable serological tests. From an immunological perspective, lipopolysaccharide plays a central role in host-pathogen interactions. The O-antigen component of *S. typhi* LPS is highly immunogenic and elicits strong antibody responses during infection. These antibodies form the basis of serological diagnostic tests. However, many existing assays rely on crude or partially purified antigens, which can compromise specificity and sensitivity due to cross-reactivity with other members of the Enterobacteriaceae family (Parry et al., 2002).

The limitations of conventional diagnostic methods such as blood culture and the Widal test highlight the need for improved antigen-based assays. Blood culture, while specific, lacks sensitivity and requires prolonged incubation, whereas the Widal test is plagued by poor specificity and variability in baseline antibody titers in endemic regions (Olopoenia & King, 2000). The use of highly purified LPS as an antigen offers a promising alternative, as it targets serovar-specific immune responses and minimizes nonspecific reactions.

The high purity and reproducibility of the LPS preparation obtained in this study suggest its suitability for use in enzyme-linked immunosorbent assay (ELISA) and other immunodiagnostic platforms. Previous studies have demonstrated that ELISA systems employing purified LPS exhibit improved sensitivity and specificity compared to assays using crude antigens (House et al., 2001). The present

findings support these observations and provide a methodological foundation for future diagnostic assay development.

In addition to diagnostic applications, purified LPS has potential utility in immunological and pathogenesis studies. Understanding the immune responses elicited by *S. typhi* LPS can provide insights into disease mechanisms, vaccine development, and host defense strategies. However, due to the potent endotoxic activity of lipid A, careful handling and characterization of LPS preparations are essential to ensure safety and consistency (Rietschel et al., 1994). While the present study successfully achieved its objectives, certain limitations should be acknowledged. The study focused primarily on biochemical purification and characterization of LPS and did not include immunological validation using patient sera. Future studies should evaluate the diagnostic performance of the purified LPS in ELISA or rapid test formats using well-characterized clinical samples. Additionally, comparative evaluation with commercially available antigens could further establish the utility of the purified LPS.

The findings of this study demonstrate that salt extraction followed by gel filtration chromatography is an effective approach for obtaining high-purity lipopolysaccharide from *Salmonella typhi*. The methodology is reproducible, avoids the use of hazardous chemicals, and yields antigen suitable for diagnostic and research applications. Given the persistent global burden of typhoid fever and the limitations of existing diagnostic tools, the development of reliable LPS-based diagnostics represents an important step toward improved disease detection and management.

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