Chemical Analysis of Lipopolysaccharide Extracted From *Salmonella enterica*serovarTyphi

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***Abstract*: Lipopolysaccharide (LPS) was extracted from *Salmonella enterica*serovarTyphi by hot phenol method. SDS-PAGE followed by silver staining of LPS showed a ladder like pattern of LPS molecules with molecular mass ranging from approximately 10 kDa to 55 kDa. Immunoblot of LPS showed that anti-LPS IgG in pooled sera from patients showing a positive Widal reaction reacted with the entire profile of LPS molecules. The extracted LPS was found to contain 50% w/w sugars when analysed for determination of total sugars in the LPS preparation. LPS was cleaved into lipid A and polysaccharide moieties by acetic acid hydrolysis. Thin layer chromatography of lipid A and O-polysaccharide regions showed that polysaccharide moiety had an R_f value of 0.65 while the lipid A moiety had an R_f value of 0.81. The purity of the extracted LPS was found to be similar to the commercial LPS when analysed by high performance liquid chromatography.**

Keywords: Lipopolysaccharide, O-polysaccharide, Thin Layer Chromatography, Silver staining, Lipid A, High Performance Liquid Chromatography.

I. INTRODUCTION

Typhoid (enteric fever) is a systemic bacterial infection caused by *Salmonella enterica*serovarTyphi. A similar but milder form of the disease called paratyphoid is caused by *Salmonella enterica*serovarParatyphi A, B, and C. Infants, children, and adolescents in south-central and south-eastern Asia experience the greatest burden of illness (Crump *et al*., 2010). Widal test remains the most widely used test for diagnosis of typhoid but owing to its suboptimal sensitivity and specificity, it is plagued with a controversy of its use as a serodiagnostic test for typhoid (Olopoenia and King, 2000).

Lipopolysaccharides (LPS) are a major component of the outer membrane of *S*. Typhi. They are endotoxic in nature and are also one of the main antigens against which antibody response is mounted during infection with *S*. Typhi (Raetz and Whitfield, 2000).

Lipopolysaccharides from gram negative bacteria may be isolated with high degree of purity by a variety of techniques. Such procedures include extraction with trichloroacetic acid at 4°C, extraction with aqueous ether at 6-12°C, extraction with water at 80°C, and extraction with aqueous phenol. Of these methods, extraction with aqueous phenol has acquired widespread usage because of its simplicity and application to many groups of gram negative bacteria. Nucleic acids and protein are the impurities that get separated along with LPS extracted by aqueous phenol. LPS extracted by hot phenol method may contain upto 60% RNA (Westphal and Jann, 1965).

Owing to the diagnostic significance of lipopolysaccharides in terms of their use as an antigen in developing serological tests for typhoid (Parry *et al*., 2011), the present study was undertaken to extract lipopolysaccharide from *S*. Typhi and characterize it.

II. MATERIALS AND METHODS

A. Culture of S. Typhi and extraction of LPS

*Salmonella enterica*serovarTyphi (catalogue no. MTCC 733) was obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. *S*. Typhi was grown in nutrient broth at 37 ºC for 24 hours. 37% formaldehyde was added in culture broth at final concentration of 1% and culture was kept at room temperature for 24 hours for cell inactivation. Cells were harvested by centrifugation and washed twice with phosphate buffered saline (pH 7.2). Cells were harvested by centrifugation and wet weight of cells was measured. Extraction of LPS was performed using the harvested cells by hot phenol method without any modifications (Iihara*et al*., 2006). The aqueous phases containing LPS were centrifuged to remove insoluble impurities and then lyophilized.

B. SDS-PAGE and Immunoblot of LPS

Lyophilized LPS was reconstituted in deionized water at a concentration of 1 mg ml^{-1}.LPS samples were mixed with equal volume of Laemmli buffer and heated at 100°C for 5 min in boiling waterbath. Samples were loaded in polyacrylamide gel containing 0.1% SDS, 12% acrylamide in resolving gel and 5% acrylamide in the stacking gel. Electrophoresis was done at 150 V till bromophenol blue reached the bottom of the gel. The LPS profiles were detected by sensitive silver stain (Tsai &Frasch, 1982).

LPS profiles were transferred to nitrocellulose membrane using western blot apparatus at 90 V for 1 hr 30 min. The membrane was kept for blocking in 2% BSA in phosphate buffered saline (PBS) (pH 7.2) for overnight at 4˚C. 10 ml pooled sera from patients showing a positive Widal reaction diluted 1:1000 in 1% BSA was added to the membrane and kept at room temperature for 1 hour. The membrane was washed thrice with PBS containing 0.01% Tween 20 (PBST). 10 ml anti-human IgG-HRP conjugate diluted 1:20,000 in 1% BSA was added to the membrane and kept at room temperature for 1 hour. The membrane was washed again as before. 10 ml substrate (50 mMTris-HCl pH 7.5, 20 µl 30% v/v H_2O_2 and one pinch 3, 3'-Diaminobenzidine) was added to the membrane followed

by addition of 10 µl 2 M $NiSO_4$. The membrane was allowed to develop in dark. When sufficient developing occurred, the membrane was washed with deionized water and air dried.

C. Estimation of total sugars in LPS

Estimation of total sugars in the LPS preparation was done by phenol sulphuric acid assay (Dubois *et al*., 1956) using glucose as standard.

D. Acetic acid hydrolysis and thin layer chromatography of LPS

Acetic acid hydrolysis and thin layer chromatography of LPS was done as per Hamidi*et al*. (2005).

E. HPLC of LPS

HPLC of LPS was done as per Rezania*et al*. (2011). Separation was done using HPLC equipment of Perkin Elmer 200 series at a flow rate of 0.8 ml/min and 210 nm UV wavelength detection. Separation was carried out over a C_{18} column with 4.6 mm diameter and 250 mm length with a mixture of water and acetonitrile (95:5) as mobile phase. LPS of *S*. Typhi extracted by hot phenol method was procured commercially and was used as standard LPS in HPLC. 25 µl sample was injected and analysed. The analysis was done at Sophisticated Instrumentation Centre for Applied Research and Testing (SICART) VallabhVidyanagar, Anand, Gujarat.

III. RESULTS

LPS was extracted from *S*. Typhi by hot phenol method and lyophilized. Lyophilized LPS was reconstituted in deionized water at concentration of 1 mg ml^{-1} and in all subsequent analysis, this LPS preparation was used.

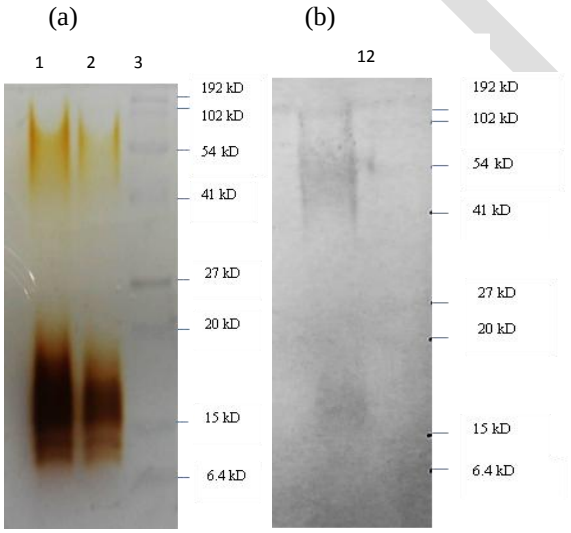


Fig. 1. SDS-PAGE and Immunoblot of LPS. (a) 12% silver stained SDS-PAGE polyacrylamide gel showing profile of LPS molecules. Lane 1 and 2 contains 5 µl and 2 µl LPS while lane 3 contains protein molecular weight marker. (b) Immunoblot of LPS showing reactivity of extracted LPS with anti-LPS IgG in pooled sera from patients showing a positive Widal reaction.

SDS-PAGE and Immunoblot of LPS

SDS-PAGE followed by silver staining of LPS showed a typical ladder like pattern of LPS molecules. Each band up the ladder shows an LPS molecule with an additional O antigen unit. The LPS molecules had a molecular mass ranging from approximately 10 kDa to 55 kDa (Fig. 1 a) .The anti-LPS IgG in patient sera reacted with the entire profile of LPS molecules (Fig. 1 b).

Estimation of total carbohydrates in LPS

To estimate total carbohydrate in LPS preparation, phenol sulphuric acid assay was done using glucose as standard. As determined from the standard graph, 1 mg LPS preparation contained 0.5 mg sugars. Thus, LPS contained approximately 50% w/w sugars.

Acetic acid hydrolysis and thin layer chromatography of LPS

LPS was cleaved into lipid A and O-polysaccharide by mild hydrolysis with 1% acetic acid. The acetic acid hydrolysate was centrifuged at 5500 g. The supernatant contained the polysaccharide while the pellet contained lipid A. Lipid A was extracted into solvent containing chloroform-methanol-water-triethylamine.

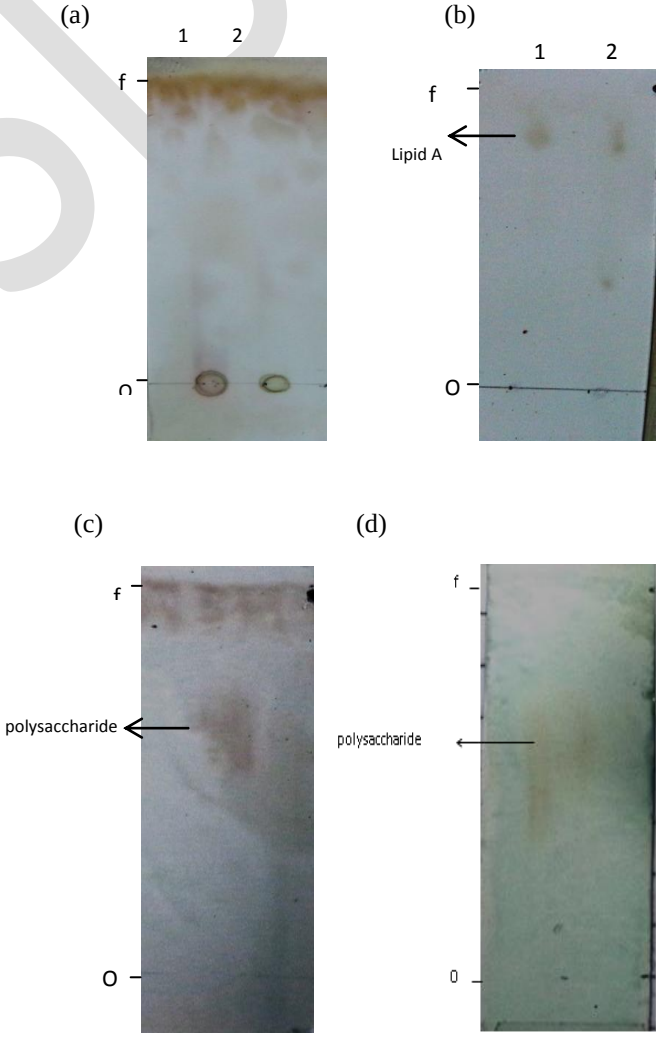
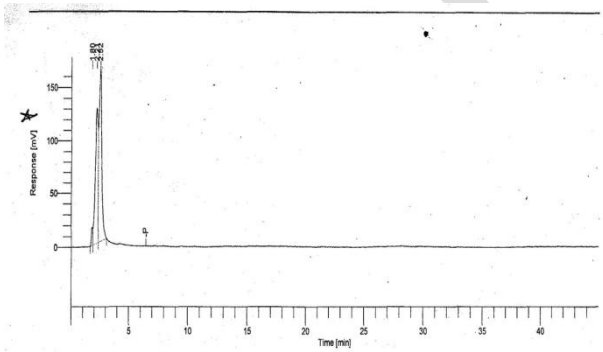


Fig. 2. Thin layer chromatography of LPS, O-polysaccharide and lipid A. (a) TLC of LPS. Lane 1: 10 µg extracted LPS, lane 2: 10 µg commercial LPS (b) TLC of lipid A. Lane 1: 5 µl lipid A (commercial LPS), 5 µl lipid A (extracted LPS) (c) TLC of O-polysaccharide of commercial LPS. 5 µl acetic acid hydrolysate of commercial LPS (d) TLC of O-polysaccharide of extracted LPS. 5 µl acetic acid hydrolysate of extracted LPS. O=origin, f= solvent front.

TLC of LPS, O-polysaccharide and lipid A was done using silica gel plates and the compounds were visualised by spraying the plates with 10% sulphuric acid in ethanol and charring. For TLC of LPS, no specific spot corresponding to LPS was obtained. However, pinkish smear originating from the point of spotting was visible after developing of plates (which probably represent LPS) along a spot with an R_f value of 0.27 for both, extracted and commercial LPS (Fig. 2 a). RNA in the LPS appeared as brown spot at the point of spotting. Similar results were obtained by Buttke and Ingram (1975) for LPS extracted from *S.* Typhimurium by hot phenol method. The polysaccharide from extracted and commercial LPS appeared as distinct brown spots after development of TLC plates and had an R_f value of 0.65 (Fig. 2 c and d). Lipid A was visible as a brown spot near the solvent front and had an R_f value of 0.82 (Fig. 2 b). An R_f value of 0.81 has been reported for lipid A from *S.* Typhimurium (Buttke and Ingram, 1975) and 0.82 for *Brucella pertussis* lipid A (Hamidi*et al*., 2005). An additional spot with R_f value of 0.33 was obtained for TLC of lipid A for extracted LPS (Fig. 2 b).

High Performance Liquid Chromatography of LPS

The purity of extracted LPS was assessed by HPLC. Commercial LPS was used as a standard for comparison of purity of both LPS preparations.



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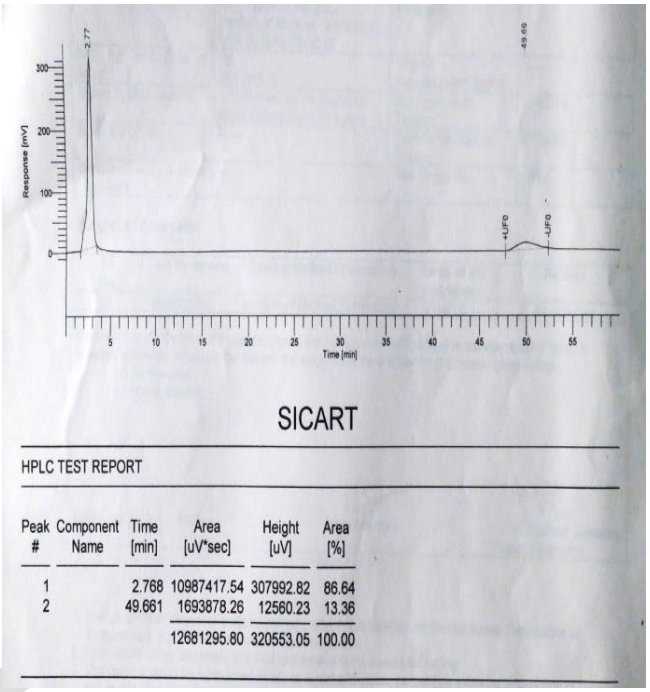
HPLC TEST REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Area [%]
1		1.803	129426.14	16653.21	2.52
2		2.209	2122109.18	127369.16	41.37
3		2.518	2878139.96	162178.38	56.11
			5129675.28	306200.75	100.00

Fig. 3. HPLC chromatogram of commercial LPS



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HPLC TEST REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Area [%]
1		2.768	10987417.54	307992.82	86.64
2		49.661	1693878.26	12560.23	13.36
			12681295.80	320553.05	100.00

Fig. 4.HPLC chromatogram of extracted LPS.

Three peaks at different retention times were obtained for commercial LPS. For extracted LPS, only a single sharp peak was obtained. No other peak was obtained for extracted LPS which showed that no impurity was present in the LPS preparation.

IV. DISCUSSION

Lipopolysaccharide was extracted from *Salmonella enterica*serovarTyphi by hot phenol method. SDS-PAGE followed by silver staining showed that LPS molecules had a molecular weight ranging from 10 kDa to 55 kDa. For LPS molecules separated by SDS-PAGE, the low molecular weight molecules of 15 kDa to 27 kDa stained heavily as compared to molecules of 40-55 kDa. LPS profiles were transferred to nitrocellulose membrane by immunoblotting for determining their immunoreactivity with patient sera. It was found that anti-LPS IgG in patient sera reacted with the entire profile of LPS molecules. LPS did not react with pooled sera from normal healthy individuals (data not shown). For TLC of LPS, no clear distinct spot was visible for LPS, howver, a pinkish smear originating from origin was observed. An $R_f$valueof 0.81 was obtained for LPS which was similar to the Rf value of lipid A of *S.* Typhimurium (Buttke and Ingram, 1975). Since the lipid A moiety among gram negative bacteria are highly conserved (Raetz and Whitfield, 2000) such results were expected to be obtained. High performance liquid chromatography of LPSs, commercial and extracted LPS was done. When the peaks obtained for the commercial LPS were compared with the peaks obtained for extracted LPS, it was found that no additional distinct peak was obtained for extracted LPS, thus the purity of extracted and commercial LPS were similar.

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