Vol.4,No.1, July, 2015
224 - 229,ISSN:0975-5446 [VNSGU JOURNAL OF SCIENCE AND TECHNOLOGY]

Extraction, Characterization and Determination of Immmunoreactivity of Lipopolysaccharide of *Salmonella typhi*

ZANKHARIA Urvi S.
Senior Research Fellow (CSIR)
Institute of Medical Technology
Udhna, Surat (Gujarat)
urvi.zankharia@gmail.com

Abstract

Lipopolysaccharide (LPS) was extracted from Salmonella Typhi by hot phenol water method and concentration of protein and RNA in the extract was determined. SDS-PAGE followed by silver staining of LPS showed a characteristic ladder like pattern indicating that the extracted LPS is a heterogeneous mixture of molecules of molecular weight ranging from ~ 10 kD– 55 kD. Western blot of LPS showed that antibodies in patient sera reacted mainly with molecules of 40 kD – 55 kD. There was no significant reaction with low molecular weight molecules indicating that antibodies in patient sera are mainly directed against the O antigen and that there were no considerable antibodies to react with core and lipid A regions of LPS.

Keywords: Lipopolysaccharide, Heterogeneous, O antigen.

1. Introduction

Lipopolysaccharide (LPS) is a major component of outer membrane of gram negative bacteria. It consists of three regions namely lipid A, core oligosaccharide and the O polysaccharide. Lipid A imparts endotoxicity to LPS while O polysaccharide imparts serological specificity and immunogenecity [1]. LPS can be extracted from bacteria by variety of techniques. These include extraction with trichloroacetic acid (TCA) [2], EDTA-Mg^{2+} [3], chloroform petroleum-ether [4] and hot phenol [5]. The quantity of LPS obtained depends on the choice of method and strain used for extraction. For eg., When extracting LPS by EDTA-Mg^{2+} method, *E.coli* releases 50% of cell's LPS while *Salmonella* spp. releases only 15-45% LPS under same conditions [3]. Hot phenol water method has been most widely used for extraction of LPS because it can be applied to many groups of bacteria and is relatively simple.

LPS extracted by hot phenol water method has been reported to contain upto 50% RNA [5]. Here, extraction of LPS from *Salmonella* Typhi by hot phenol water method is reported along with its characterization in terms of the level of RNA and protein impurities and immunoreactivity with sera from presumptive typhoid cases showing a positive Widal reaction.

GlcNAc, N-acetylglucosamine; P, phosphate; KDO, 2- keto-3-deoxyoctulosonic acid; P-ETN, phosphorylethanolamine; Hep, heptose; Glc, glucose; Gal, Galactose; Rha, rhamnose; Man, mannose; Tyv, tyvelose. Wavy lines signify fatty acids.

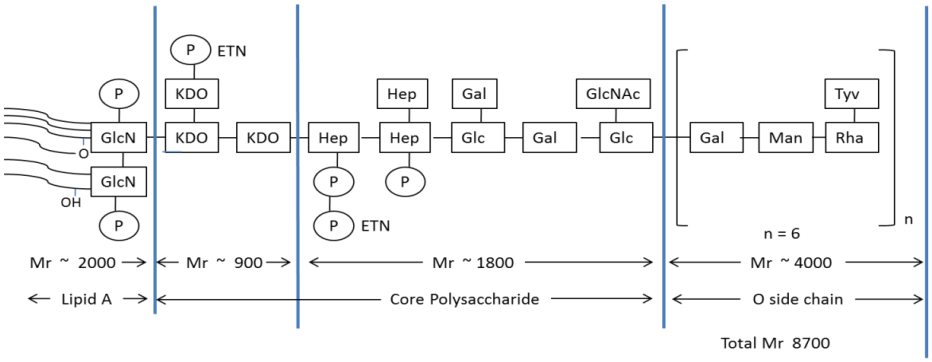


Figure 1: Structure of *S.* Typhi lipopolysaccharide.

2. Materials and methods

2.1 Bacteria and culture conditions

Salmonella enterica serovar Typhi was obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. It was maintained as pure culture on Medium 3 as recommended by MTCC and was revived periodically by successive transfers on same medium. A single colony of *S.* Typhi was inoculated in 10 ml Luria Bertanni Broth and incubated overnight at 37 °C. 5 ml overnight grown culture was inoculated in 1 litre flask containing 500 ml LB broth and incubated at 37 °C on incubator shaker at 180 rpm for overnight. 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.

2.2 Sera

Serum samples from presumptive typhoid cases showing a positive Widal reaction were obtained from Desai Metropolis Laboratory, Surat. The serum samples had anti-O titers of ranging from 1:30 to 1:240 while an anti-H titer of ≥1:120. Sera from healthy blood donors were collected from SPAN Diagnostics Ltd., Surat. Serum samples were stored at -20ºC until use.

2.3 Extraction of LPS

Extraction of LPS was done by hot phenol water method without any modifications [10]. 12 g (wet weight) of cells was suspended in 105 ml distilled water prewarmed to 65-70 °C. Equal volume of 90% aqueous phenol prewarmed to 65-70 °C was added to the cell suspension. The mixture was stirred vigorously at 65-70 °C for 15 min. The mixture was chilled on ice for 15 min. Mixture was centrifuged at 8000 rpm (Remi cooling centrifuge C-24 BL) at 4°C for 15 min for separation of aqueous and phenol phase. The upper aqueous phase containing LPS was carefully collected (aqueous phase 1). To the left phenol layer and cell debris, equal volume of distilled

water prewarmed to 65-70 °C was added and extraction was repeated and aqueous phase was collected (aqueous phase 2). The two aqueous phases were dialyzed against distilled water in dialysis membrane of MWCO 3.5 kD till absorbance at 260 nm of water outside dialysis membrane became zero [7]. The aqueous phases were centrifuged at 8000 rpm for 15 min to remove insoluble impurities and stored at 4 °C.

2.4 Estimation of LPS

Concentration of LPS in aqueous phases was determined by thiobarbituric acid assay [9]. A standard curve was prepared using LPS extracted from *S.* Typhi (Sigma). LPS analysis was performed on 25 µl LPS solutions each containing 5, 10, 15, 20 and 25µg LPS. Absorbance was measured at 548 nm against blank containing all other reagents except LPS.

2.5 Estimation of RNA

Absorbance of aqueous phase 1 and 2 was measured at 260 nm in UV visible spectrophotometer (Spectrascan UV visible spectrophotometer, Chemito) against distilled water as blank. The absorbance was multiplied by 40 to get concentration of RNA in µg/ml [9].

2.6 Treatment with RNase A

In order to confirm that RNA was the nucleic acid present in LPS, it was treated with RNase A. Ethanol precipitation of LPS was done as follows. Sodium acetate was added to LPS at final concentration of 0.15 M. Tube was placed on ice and four volumes of chilled 96% v/v ethanol was added and kept at -20 °C for 24 hours [7]. Precipitates of LPS were collected by centrifugation at 5500 g. 10 mg LPS pellet was reconstituted in 2 ml milli Q water. RNase A was added at final concentration of 0.4 mg/ml and incubated at 37 ˚C for overnight [6]. Following incubation, RNase A treated LPS was subjected to agarose gel electrophoresis to check degradation of RNA.

2.7 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in 1.8% agarose gel containing 0.5 µg/ml ethidium bromide. The samples were run at 75 V till tracking dye migrated till the bottom of gel. The gel was visualized under UV transilluminator to detect presence of RNA in LPS samples.

2.8 Estimation of protein

Estimation of protein was done as per Bradford's microassay method (BIORAD) as given in the instructions manual.

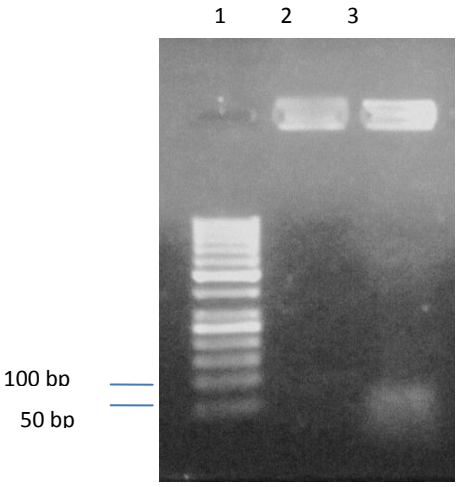
2.9 SDS-PAGE and Silver staining

10 µl of LPS preparations were mixed with 10 µl Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% β mercaptoethanol) and heated at 100 °C for 5 min. Samples were loaded in gel consisting of 12% resolving gel and 5% stacking gel. Electrophoresis was carried out using BIORAD's Mini Protean Tetra Cell apparatus at 150 V till bromophenol blue reached the bottom of the gel. LPS was visualized by silver staining as per Tsai and Frasch[12].

2.10 Western Blot

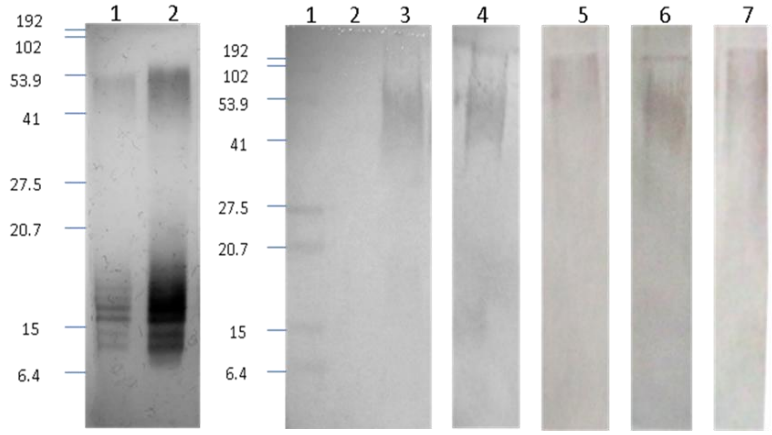
LPS profiles were transferred to nitrocellulose membrane using BIORAD's Transblot apparatus at 90 V for 1 hr 30 min. The membrane was kept for blocking in 2% BSA in PBS (pH 7.2) for overnight at 4˚C. 10 ml serum diluted 1:1000 in 1% BSA was added to the membrane and kept at room temperature for 1 hour 30 min. The membrane was washed thrice with phosphate buffered saline plus 0.01%Tween 20 (PBST).10 ml anti-human IgG-HRP conjugate (Sigma A8792) or anti-goat IgG-HRP conjugate (Sigma A5420) diluted 1:20,000 in 1% BSA was added to the membrane and kept at room temperature for 1 hour 30 min. The membrane was washed again as before. 10 ml substrate (50 mM Tris-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 milli Q water and air dried.

3. Results



Photograph 1: Agarose gel electrophoresis of RNase A treated LPS.

Lane 1: 50 bp DNA ladder; Lane 2: 20 µl RNase A treated LPS; Lane 3: 20 µl LPS without RNase A treatment.



(A) **(B)**

Photograph 2: A) Silver stained SDS-polyacrylamide gel showing LPS isolated from *S.* Typhi MTCC 733. Lane 1: 10 µl second aqueous phase; Lane 2: 10 µl first aqueous phase. B) Western blot of LPS. Lane 1: molecular weight marker; Lane 2: 5 µg BSA; Lane 3-7: Western blot of LPS showing reactivity of LPS with sera from five different patients.

Two aqueous phases were obtained following LPS extraction. The first aqueous phase contained 2.17 mg/ml of LPS, 0.122 mg/ml RNA and 0.022 mg/ml protein while the second aqueous phase contained 0.77 mg/ml LPS, 0.120 mg/ml RNA and 0.012 mg/ml protein. Thus, LPS in aqueous phase 1 contained 5% RNA and 1% protein by weight and LPS in aqueous phase 2 contained 14% RNA and 1% protein by weight. LPS in aqueous phase 1 and 2 had purity of 94% and 85% respectively.

Agarose gel electrophoresis of LPS showed presence of RNA of ~ 50 bp molecular weight. In order to confirm that RNA was the only nucleic acid present in LPS, it was treated with RNase A. LPS not treated with RNase A showed a band corresponding to approximately 50 bp. No such band was present in LPS treated with RNase A indicating that RNase A had degraded RNA present in the extract confirming that RNA was the nucleic acid present in LPS.
SDS-PAGE followed by silver staining of LPS showed that the aqueous phases contained LPS of molecular weight ranging from ~10 kD – 55 kD. For western blotting, LPS was fractionated by SDS-PAGE and was transferred to nitrocellulose membrane. The membrane was cut into 5 strips and each strip was incubated with serum of patient showing a positive Widal reaction, one strip was incubated with normal human serum which served as negative control. It was observed that antibodies in the patient sera showed a prominent reaction with ~40 kD - 55 kD molecules, there was no significant reaction with low molecular weight molecules. No reaction was observed with normal human serum (data not shown).

4. Discussion

Lipopolysaccharides are a major component of the outer membrane of gram negative bacteria. They are endotoxic in nature and are also one of the main antigens against which antibody response is mounted during infection.

In this study, LPS was extracted from *S.* Typhi MTCC 733 culture by hot phenol method and RNA and protein impurities in the aqueous phases was quantified. Estimation of LPS in the first and second aqueous phase showed that concentration of LPS was higher in the first aqueous phase than second indicating that major portion of LPS is extracted during the first extraction while the remaining is extracted in subsequent extraction. LPS in aqueous phase 1 and 2 were found to have purity of 94% and 85% respectively.

Such a purity of LPS preparation (upto 94%) has been reported to be obtained only when LPS extracted by hot phenol method is enzymatically or chemically treated or repeatedly ultracentrifuged to remove nucleic acid impurities and subject to proteinase K treatment for removal of proteins [5,6,7]. LPS molecules were separated according to their molecular weight by SDS-PAGE and silver stained. A characteristic ladder like pattern of LPS molecules of 10 kD-55 kD molecular weight was obtained. The low molecular weight molecules (~10 kD-20kD) stained heavily in silver staining while the intensity of staining of high molecular weight molecules (40 kD-55 kD) was comparatively less. This showed that in a mixture of LPS molecules, concentration of low molecular weight molecules is more as compared to high molecular weight molecules.

Western blot was done using sera from presumptive typhoid cases showing a positive Widal reaction. It is known that the immunological specificity and antigenicity of LPS is due to the sugar residues of the O antigen. The low molecular weight molecules have less number of O antigen repeats than high molecular weight molecules. From western blot of LPS, it was found that high molecular weight molecules (~40 kD-55 kD) showed very strong reactivity as compared to low molecular weight molecules. The binding of antibodies to high molecular weight

molecules in patient sera indicated that antibodies were directed against the O antigen which is evident from the observation that maximum reactivity of antibodies is observed with 40 kD-55 kD molecules only. The concentration of 10 kD -15 kD molecules was more as compared to 40 kD-55 kD molecules as was observed in silver staining. If there had been sufficient antibodies in the patient sera against the lipid A- core moiety, even molecules of 10 kD-20 kD (with less no. of O antigen repeats) would have got stained. However, this has not been observed. Thus, it can be said that antibodies in the patient sera were mainly directed against the O antigen.

Acknowledgements

I am thankful to the Council of Scientific and Industrial Research (CSIR), New Delhi for providing me financial assistance in form of Senior Research Fellowship and SPAN Diagnostics Ltd., Surat for laboratory facilities for experimentation.

References

[1] C. R. H. Raetz, C. Whitfield, Lipopolysaccharide endotoxins, Annual Reviews Biochemistry, 71 (2002) 635-700.

[2] A. M. Staub, Bacterial Lipido-protinopolysaccharides ('O' Somatic Antigens) Extraction with Trichloroacetic Acid, Methods in Carbohydrate Chemistry. 5 (1965) 92-93.

[3] L. Leive, & D. C. Morrison, Isolation of Lipopolysaccharides from Bacteria, Methods in Enzymology. 28 (1972) 254-262.

[4] C. Galanos, 0. Luderitz, and 0. Westphal, A newmethod for the extraction of R. lipopolysaccharides, European Journal of Biochemistry. 9 (1969) 245-249.

[5] O.Westphal, K. Jann, Bacterial lipopolysaccharides, Extraction with phenol-water and further applications of the procedure, In: Whistler RL, Wolfan ML, editors. Methods in Carbohydrate Chemistry. New York: Academic press, 83–91, 1965.

[6] M. Luchi, D.C. Morrison, Comparable endotoxic properties of lipopolysaccharides are manifest in diverse clinical isolates of gram-negative bacteria, Infection and Immunity. 68-4 (2000) 1899-1904.

[7] R. Perdomo, V. Montero, Purification of E. coli 055:B5 lipopolysaccharides by size exclusion chromatography, Biotecnología Aplicada. 23 (2006) 124-129.

[8] J.S. Evans, M.C.J. Maidens, Purification of meningococcal lipo-oligosaccharide by FPLC techniques, Microbiology. 142 (1996) 57-62.

[9] S. Nakamura, Y. Higashiyama, K. Izumikawa, M. Seki, H. Kakeya, Y. Yamamoto, K. Yanagihara, Y. Miyazaki, Y. Mizuta, S. Kohno, The roles of the quorum-sensing system in the release of extracellular DNA, lipopolysaccharide, and membrane vesicles from Pseudomonas aeruginosa, Japanese Journal of Infectious Diseases. 61-5 (2008) 375-378.

[10] H. Iihara, T. Niwa, M.M. Shah, P.H. Nhung, S.X. Song, M. Hayashi, K. Ohkusa, Y. Itoh, S. Makino, T. Ezaki, Rapid multiplex immunofluorescent assay to detect antibodies against Burkholderia pseudomallei and taxonomically closely related nonfermenters, Japanese Journal of Infectious Diseases, 60-4 (2007) 230-234.

[11] O. Westphal, K. Jann, Bacterial lipopolysaccharide. Extraction with phenol-water and further applications of the procedure, Methods in Carbohydrate Chemistry. 5 (1965) 83-91.

[12] C.M. Tsai, C.E. Frasch, A sensitive silver stain for detecting lipopolysaccharides in poly-acrylamide gels, Analytical Biochemistry, (1982) 115–119.