Original Article

Extraction and Purification of *Haemophilus influenzae* Type b Lipooligosaccharide by Modified Phenol Method

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Abstract:

Introduction: *Haemophilus influenzae* type b (Hib) is a Gram negative bacterium and one of the causative agents of acute bacterial meningitis, especially in infants and children less than 5 years old. Lipooligosaccharide (LOS), one of the virulence factors which plays an important role in pathogenesis of Hib, has multiple applications in diagnosis and conjugate vaccines. In this study, LOS extracted from two Hib standard strains (ATCC 39930 and ATCC 10214) were compared. **Methods:** LOS was extracted by a modified hot phenol method from the aqueous and phenol phase and its concentration and purity assayed. Protein contaminations of the samples were determined spectrophotometrically and their endotoxin contents were assayed by the *Limulus* amebocyte lysate (LAL). **Result:** The yield of the extracted LOS from strain ATCC10214 was about 475 µg/ml and the protein contaminations of the samples were approximately 0.07 mg/ml, whereas strain ATCC39930 yielded 520 µg/ml of LOS with protein contamination of 0.08 mg/ml. The results showed that the production of LOS by both strains was similar and the variation observed was not statistically significant ($p < 0.001$). *Vac Res, 2014, 1 (1): 28-30*

Keywords: *Haemophilus influenzae* type b, Lipooligosaccharide, Meningitis.

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INTRODUCTION

Haemophilus influenzae is a Gram-negative, non-motile and fastidious bacterium classified into two groups of capsular and non-capsular strains [1]. The encapsulated strains are serologically subdivided into 6 (a – f) distinct types based on the capsular antigen. H. *influenzae* type b (Hib) is an important cause of serious invasive infections in children less than five years old and meningitis is a frequent consequence of invasive Hib *infection* [2, 3].

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Several factors such as the membrane proteins HMW1, HMW2, P5, Hap, P2 and P6 contribute to the pathogenesis of *H. influenzae*. Although the capsular polysaccharide is the key virulence determinant, this pathogen also expresses an O-deacylated lipooligosaccha-ride (LOS) consisting of a mainly conserved triheptose backbone attached to a core region, covalently linked to lipid A [4-8]. Lipid A is a natural ligand for the MD2- Toll-like receptor 4 (TRL4) complex with a pivotal role in producing inflammatory responses [8, 9]. The observed inter- and intra-strain heterogeneity of LOS has been attributed to the oligosaccharide extensions stemming from the triheptose core region [9, 10]. Lipid A constitutes about 60% of LOS while the remainder of this molecule consists of hydrophilic polysaccharides [11]. It appears that the structure of lipid A in different strains of Hib is relatively conserved as it has

been shown that nearly 50% of a large collection of Hib strains shared at least one epitope in lipid A region [12, 13].

The ability of LOS to up and down-regulate the production of inflammatory cytokines and proinflammatory markers, albeit to a lesser extent than LPS, and its ubiquitous presence in all *H. influenzae* strains makes this molecule an attractive vaccine candidate. The detoxified LOS, conjugated with outer membrane proteins strains lacking the capsule, has been used as a candidate for vaccination against the non-typable strains [10]. In addition, the immunostimulatory properties of LOS might be considered of value for potential adjuvant developments as a TLR4 agonist [8, 9]. Moreover, LOS could be used as a good antigen for diagnostic applications. In this study, LOS was extracted from two different strains of Hib (strains ATCC39930 and ATCC10214) and their concentrations and purities compared.

MATERIALS AND METHODS

Bacterial strains and the cell biomass production. Standard strains of Hib (Pasteur Institute of Iran, Department of Bacterial Vaccine production)

Were cultured overnight at 36.6+- .5°C in Brain Heart Infusion agar (Merck, Germany) supplemented with 1% hemin (Sigma-Aldrich, Germany) and 0.01% Nicotinamide Adenine Dinucleotide (NAD; Mast Group, UK). This culture was used to inoculate 2 l Casamino acids and yeast extract (CY) medium (in a 5 l flask), supplemented with 0.02 g/l of NAD and 0.04 g/l of hemin (pH 7.3), incubated for 24 h at 37°C, to obtain the seed culture [14]. The seed culture was added to a 60 l industrial fermenter containing 35 l CY medium and after 12 h, the fermentation process was stopped by addition of 10% phenol and the biomass was harvested by centrifugation at 4000 rpm for 45 min at 4˚C.

LOS purification. LOS was extracted by a modified Westphal and John method [15]. Briefly, the bacterial biomass was suspended in 170 ml distilled water, followed by addition of 190 ml 90% (v/v) hot phenol (66°C) and the mixture was stirred then for 14 min at 66°C. The bacterial suspension was kept on ice and the temperature was lowered to 0°C and then centrifuged (8500×g, 4°C) for 15 min. LOS was extracted by the addition of 25% and 100% (v/v) cold ethanol (4˚C) to the aqueous and phenol phase, respectively. One hundred ml of 75% (v/v) cold (4˚C) ethanol was added to the supernatant of the aqueous phase and kept at 4˚C for 6 h, then centrifuged and LOS-containing pellet dissolved in distilled water. The pellet obtained from phenol phase was dissolved in distilled water and centrifuged as above for 1 h and the LOS-containing supernatant was kept for the final purification. To reduce the nucleic acids and protein contaminations, DNase, RNase and proteinase K were used and for precipitation, 1 g trichloroacetic acid (TCA) per 20 ml of the extracted sample was added and the solution was kept at 4°C for 3 h. Finally, the TCA-treated samples were centrifuged (2000×g 4°C for 10 min) and dialyzed against deionized water for 72 h.

Physicochemical analysis. The dialyzed samples were analyzed by 10% SDS-PAGE and stained by silver nitrate [15]. LOS concentration was assayed colorimetrically and the absorbance was measured at 595 nm. The concentration of LOS in the samples was calculated against a curve drawn by using *E. coli* LPS as standard [16]. Protein content of the extracted samples was measured spectophotometrically using NanoDrop (Thermo Scientific, USA). The endotoxin levels of the samples were assayed by choromogenic Limulus amebocyte lysate (LAL) method [15].

RESULTS

In batch fermentation under submerged culture condition, the yield of the biomass was 120 g for Hib strain ATCC39930 and 107 g for Hib strain ATCC10214. LOS concentration was calculated based on the standard curve obtained by the linear equation of y = 0.001x-0.0003 (Fig. 1A).

The optical density (OD) for Hib strain ATCC39930 was 0.520 and its calculated concentration was 520 µg/ml, whereas the OD of Hib strain ATCC10214 was 0.475 equaling to a concentration of 475 µg/ml (Fig. 1A). The silver-stained SDS-PAGE of the LOS from both strains showed no detectable protein contamination (Fig. 1B).

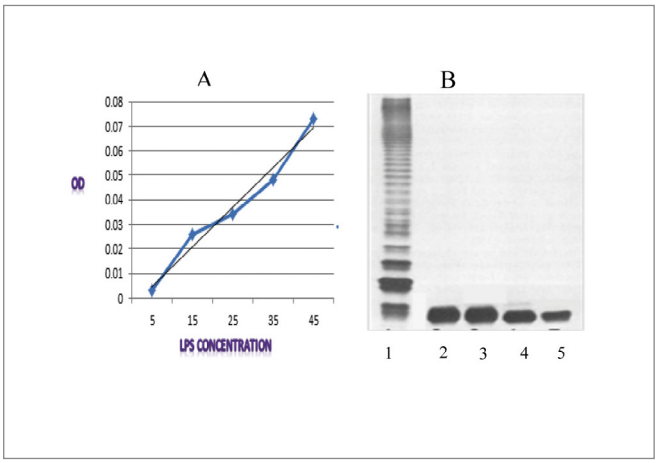

Fig. 1: A) Standard curve representing OD vs. standard LPS concentration. **B)** Silver-stained SDS-PAGE of the extracted LOS from strains ATCC39930 and ATCC10214. Lane 1: standard LPS, lanes 2 and 3: Hib strain ATCC39930, lanes 4 and 5: Hib strain ATCC10214.

The amount of protein contaminations in the extracted samples were 0.08 mg/ml for Hib strain ATCC39930 and 0.07 mg/ml for Hib strain ATCC10214. The endotoxin levels of the extracted samples were measured by chromogenic LAL test and were 1420 EU/ml for Hib strain ATCC39930 and 1480 EU/ml for Hib strain ATCC10214.

DISCUSSION

LOS of *H. influenzea* is a heterogenous and complex glycolipid which plays a critical role in the virulence of Hib [17]. The difference between LOS and LPS is that in LOS, the variable branched region or O-antigen is a non-repeating unit. This structure can be isolated from a number of bacteria such as

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Haemophilus ducreyi, Branhamella catarrhalis, Neisseria meningitidis, Neisseria gonorrhoeae and *Bordetella pertussis* [18]. LOS could induce both innate and acquired immune responses by two component polysaccharide and lipid moiety separately. With the increasing application of LOS, establishing a protocol for culturing Hib and purification of LOS could be of value for research and development of subunit vaccines. Although the optimized phenol and butanol extraction methods are the two well-established procedures for extraction and purification of LPS and LOS but modifications to these methods have been reported [15, 18]. However, in comparison the optimized phenol extraction method has given the highest LPS yield and the lowest protein contamination, although proteinase K method has been reported as less cumbersome [19]. In this study, a modified hot phenol method followed by the use of proteinase K digestion of the bacterial proteins and nucleases, resulted in preparation of a high quality LOS, free of protein and nucleic acids contaminations. The main aim of this study was the extraction of purified LOS for further application as an antigenic macromolecule. The use of highly-purified LOS can lead to the induction of specific immune responses including the innate and acquired immunity [9].

In conclusion, although the yield of the biomass production by two strains of Hib have been shown equal in batch fermentation, extracted LOS were 1420 EU/ml for Hib strain ATCC39930 and 1480 EU/ml for Hib strain ATCC10214. According to chemical analysis of the extracted sample, we finally conclude that optimized phenol extraction is the best method with the least protein and nucleic acid contaminations and would be recommended to extract and purity Hib LOS for further application.

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CONFLICT OF INTEREST

No potential conflicts of interest were disclosed.

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