Quantitative Detection of the Partially Purified Endotoxin Extracted from the Locally Isolated *Salmonella typhimurium* A3

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Abstract

A total of 95 stool samples were collected from pediatric patients suffering from diarrhea in order to isolate bacteria belonging to ***Salmonella typhimurium*** species. After performing microscopic examination, cultural characterization, biochemical and Api 20E identifications, only 9 isolates showed positive ***Salmonella typhimurium***. Following the separation of endotoxin from each isolate the biological activity of endotoxin was estimated by using E-TOXATE kit (*Limulus* amebocyte lysate test) and the isolate ***Salmonella typhimurium*** A3 showed the lowest concentration that gave positive result (0.5 µg/ml). Partial purification of endotoxin using sepharose Cl-6B gel chromatography for A3 endotoxin was applied and after purification two peaks were obtained. Chemical characterization involving the estimation of carbohydrate and protein contents showed that the first peak contained the higher carbohydrate contents (38.6%) and lower protein contents (1.15%) as compared with the second peak which showed carbohydrate and protein contents 13.2 and 5.75% respectively. In addition, the first peak showed maximum activity of (≥0.1µg/ml), the second peak and the crude endotoxin showed 2.5 and 0.5µg/ml respectively.

Keywords: Bacterial Endotoxin, Lipopolysaccharide, *Salmonella.*

Introduction

In general terms, endotoxin refers to any cell-bound bacterial toxin–a characteristic that distinguishes it from exotoxins which are secreted by bacterial cells [1]. The terms endotoxin and lipopolysaccharide (LPS) are often used synonymously in the literature, and for all practical purposes are the same. The term endotoxin was first coined in 1893 by Pfeiffer to distinguish the class of toxic substances released after lysis of bacteria from the toxic substances (exotoxins) secreted by bacteria [2]. Lipopolysaccharides (LPSs) are the components of the external membrane of almost all gram-negative bacteria. They usually constitute a hydrophilic moiety consisting of the *O* -specific chain (*O*-antigen) and the core oligosaccharide, covalently linked to a lipophilic moiety (Lipid A) that anchors LPS to the outer membrane [3]. Some bacteria in nature lack the *O*-antigen of the LPS molecule, and mutants which do not contain the 0-antigenic side chain are easily obtained by selecting for resistance to certain bacteriophages [4]. Bacteria which contain LPS that lacks the 0-antigenic side chain often are referred to as rough owing to their colonial morphology, whereas bacteria which have this LPS component are referred to as smooth, this diversity affect the procedure for extracting LPS [5].

LPS elicits an extraordinary variety of distinct biological effects, such as pyrogenicity, adjuvanticity, macrophage activation, Blymphocyte mitogenicity, and tumor regression [6]. Endotoxin has been shown to induce many of the harmful manifestations include: fever, hypoglycemia, dissemination intravascular coagulation, multiple organ failure and septic shock [7, 8]. Some of the effects of endotoxin are beneficial, i.e., endotoxin can stimulate immune response (Immunogenic). Bashar *et al*., [9] showed that endotoxin has an adjuvant effect, enhancing B-cell response to a variety of antigens. It has mitogenic effects on B lymphocytes that increase resistance to viral and bacterial infection, induction γ-interferon productions by T-lymphocytes, activation of

complements and induction of the formation of interleukin-1 [10].

The pathophysiological activity of LPS depends on the chemical structure of the hydrophobic portion lipid A, the biologically active center of LPS [11], which generally consists of a β(1-6)-linked 2-amino-2-deoxy-D-glucose (GlcN) disaccharide carrying phosphate and fatty acid residues; many fine structural variations are observed in different bacterial families [12]. Due to the importance of bacterial endotoxin as a membrane barrier, bacterial recognizing site, induction of immune system and therapeutic activity [6], many procedures have been developed for bacterial endotoxin extraction depending on the chemical nature, bacterial type and purpose of extraction [13].

In spite of that wild type *Salmonella typhimurium* are smooth form, the present work describes a simple procedure for LPS extraction which is useful for both smooth and rough Gram negative *Salmonella typhimurium* with a high yield, detecting the biological activity of extracted endotoxin and compared with the partially purified endotoxin.

Materials and Methods

Bacterial Isolation and Identification:

A total of 95 stool samples were collected from pediatric patients (under 5 years old), suffering from diarrhea, in Al-Elwyia Pediatric Hospital in Baghdad. Stool samples were taken randomly from both male and female pediatric patients using wooden stick applicators and transferred to test tubes containing 10ml sterile peptone water. Samples were incubated at 37°C for 24 hours. After incubation, 5ml of cultured stool sample were transferred to 250 ml flasks containing 50ml tetra-thionate broth (an enrichment media for the selection of *Salmonella*), and incubated at 37°C for 24 hours. After incubation a loopful from each flask was streaked on selective medium SS agar plates, incubated at 37°C for 24 hours [14]. The suspected colonies were subjected to further microscopic, biochemical [15] and API 20E tests.

Lipopolysaccharide (Endotoxin) Extraction from *Salmonella typhimurium* Isolates:

It involved two steps, first cell preparation by growing each isolate in flask containing 25 ml of LB broth supplemented with 10mM glucose (for bacterial activation) at 37ºC for 18 hours. The fresh cultures were used to inoculate 3-3.5L of LB broth suspended in 500-ml conical flasks filled with 200ml broth. The inoculated flasks were incubated at 37ºC for 24 hours with shaking at 150rpm. Cultures were centrifuged (3000rpm, 15 min), the pellet washed twice with phosphate buffer. Cells were suspended in phosphate buffer containing 0.5% formalin (pH= 7.2) and were kept at 4ºC for18 hours. After that the cells were centrifuged (3000rpm, 15 min) and washed with phosphate buffer. Finally, cells were dried using cold acetone by ten times the sample's volume [16]. Second, the dried cells then used for LPS extraction using a procedure described by Chandan and Fraser [17].

Detection and Semiquantitation of Bacterial Endotoxin

The detection and semiquantitation of endotoxin extracted from different *S. typhimuirum* isolates was done by using E-TOXATE Kit supplied by Sigma-Aldrich Company. Procedures regarding the kit were used according to the leaflet supplied by the company.

Partial Purification of Lipopolysaccharide by Using Gel Filtration Chromatography

This was preformed according to the method described by Johnson and Perry [18]. The gel was prepared according to the manufacturer instruction in which the gel was washed several times with 0.025 M phosphate buffer pH: 7.2. Gas bubbles were degassed using vacuum pump. Then the gel was slowly poured into the inner side of the column (3 × 50 cm) and equilibrated with the phosphate buffer (0.025 M, pH: 7.2) with a flow rate 30 ml/hour.

Crude endotoxin (30 mg/ 5 ml) was added gently through the sepharose Cl-6B column that has been previously equilibrated with phosphate buffer using a pasture pipette. Fractions have been collected at a flow rate 30 ml/hour and with fraction size of 3 ml/tube.

Chemical Analysis of Partially Purified Lipopolysacharride

The collected fractions were examined by measuring the absorbance at 280 nm for detecting the contaminated proteins within fractions [19], estimating the carbohydrate concentration according to method described by Dubois *et al* [20], measuring the absorbance at 260 nm for detecting the nucleic acids [21] and determined the protein concentration according to the method described by Bradford [22].

Results and Discussion

Isolation and Identification of *Salmonella typhimurium*

Out of 95 stool samples were collected from 95 pediatric patients (under 5 years old) suffering from diarrhea, *Salmonella typhimurium* was isolated and identified from only 9 stool samples. Isolation of *Salmonella typhimurium* was performed by cultivating the stool samples in tetra-thionate broth medium, which was used as a selective enrichment for the cultivation of *Salmonella* spp. that may be present in small numbers and compete with intestinal flora. Selectivity is accomplished by the combination of sodium thiosulfate and tetra-thionate, which suppresses common intestinal organisms. In addition, bile salt inhibits coliform and Gram-positive organisms [23]. For pure cultures, 100µl aliquots from tetra-thionate broth cultures were transferred and spread onto the surface of *Salmonella-Shigella* agar plates (S-S agar). S-S agar is highly selective and differential media formulated to inhibit the growth of most coliform organisms and permit the growth of species of *Salmonella* and *Shigella* from environmental and clinical specimens. The growth of *Salmonella* spp. in S-S agar shows colorless colonies with black centers owing to H_2S production while *Shigella* spp. does not blacken [24]. As a result, 29 isolates which showed black colonies in SS agar were suspected to belong to *Salmonella* spp. and subjected for further identifications.

Biochemical tests were achieved on the suspected isolates, for this purpose seven biochemical tests were achieved. Results showed that only 9 isolates gave negative reactions for indole, urease, and Voges-Proskaur tests, but positive reactions for citrate utilization, methyl red and glucose fermentation. In triple sugar iron test, all of the isolates were H_2S and gas producers and most of isolates turned the color of the butt to yellow. Results indicated that the suspected 9 isolates were identified as *Salmonella typhimurium* by the criteria of Bergey's Manual of Systematic Bacteriology [25]. These results were further proved by using AP 20E kit, indicating that all of the nine isolates belonged to the species *Salmonella typhimurium*.

Isolation of Lipopolysaccharide (Endotoxin) from *Salmonella typhimurium* Isolates

The isolates were grown in LB broth at 37ºC with shaking. LB broth has been effectively employed for cultivation of *Salmonella typhimurium* isolates besides the supplementation of 10mM glucose results in higher biomass yield and recovery of LPS (20 gram dry weight bacteria) [26]. Lipolysaccharides were extracted from *Salmonella typhimurium* by using a hot water and enzymatic treatment [17]. Results in Fig.(1) shows that, the LPS recovery from the *Salmonella typimurium* isolates ranged between 130-198 mg dry weights following lyophilization.

LPS extraction method described by Chandan and Fraser [17] can be considered as simple and easy to run procedure with higher yield of LPS. Many reports used different methods for LPS extraction, however the overall yield of LPS was reported to range between 100-500mg of LPS from 20g dry weight cells [27], while Kato *et al*., [12] gave a proportion that LPS (7.1 mg/g dry weight of cells) could be extracted. It was noticed that the suspension of dried bacterial cells in EDTA solution and the application of autoclaving result in destruction of bacterial cells and denaturation of bacterial proteins. Following the centrifugation at 10000 rpm two phases were separated, the upper phase (aqueous phase) contains the LPS and nucleic acids and the lower phase contains denatured proteins and cell debris [28]. The aqueous phase was aspirated off and dialyzed against distilled water in order to remove salts and other impurities [29]. Due to the amphopathic nature of the LPS, this procedure takes the

advantage that the majority of bacterial LPS show hydrophilic ability and hence become soluble within the aqueous phase [13, 30] in addition this procedure is suitable for extraction of both smooth and rough bacteria with low amount of contaminated proteins (due to autoclaving and proteinase K application) and nucleic acids (due to nucleases treatment).

Semi-quantitative Detection of Endotoxin Activity

A series of endotoxin concentrations were prepared for each isolate ranged between 100-2.5 µg/ml. Results in Table (1) indicated that *Salmonella typhimurium* A3 isolate showed the lowest concentration (0.5 µg/ml) that gave a positive result than other isolates. The biological activity of endotoxin can be expressed either as mg and µg or endotoxin unit EU [31]. There are more than 20 assays for the detection of endotoxin, three of which considered to be highly used for clinical and research purposes: the rabbit progeny assay, the *Limulus* amebocyte lysate (LAL) bioassay and immunoassays [10]. The advantages of LAL assay are high in sensitivity, potential for quantization and reactivity with the biologically active component lipid A [32].

Many studies reported that LPS from Gram-negative bacteria can produce a positive LAL assay at concentrations as low as pg/ml [33]. However, this is highly dependent on the *Limulus* amebocyte lysate that is provided from the manufacturer and its ability to form a hard clot, E-TOXATE reagent from Sigma has a limit of sensitivity: 0.05-0.1 EU. Endotoxin from *Salmonella typhimurium* A3 showed to be biologically active at concentration 0.5 µg/ml, which was lower than those obtained from other isolates. Therefore *Salmonella typhimurium* A3 endotoxin was candidate for purification process. The chemical characterization of A3 endotoxin showed that the percentage of carbohydrates and proteins of crude endotoxin were 29.2% and 2.37% respectively.

Partial Purification by Gel Filtration of *S.typhimurium* A3 Endotoxin

The crude endotoxin was applied to a sepharose Cl-6B column previously equilibrated with 0.025 M phosphate buffer, fractions were collected and endotoxin was recovered by detecting the carbohydrate contents for each fraction according to the method of Dubois *et al* [20] at 490 nm, also the contents endotoxin-associated proteins were detected at 280 nm. Results shown in Fig.(2) indicated the separation of two peaks, one large mostly consisting of carbohydrates and the other is small containing endotoxin-associated proteins. This result is in agreement with many studies which noticed the presence of the endotoxin-associated proteins which cannot be separated after one purification step [34, 8]. Kirikae *et al* [8] and Johnson and Perry [18] indicated the resolved of two distinct peaks revealing the presence of two LPS molecules: a relatively board peak with a higher molecular weight and a small peak with a lower molecular weight, were subsequently separated following purification by gel filtration of endotoxin from *Salmonella*. Chemical analysis and biological activity of the partially purified endotoxin in the large peak (peak 1) and a small peak (peak 2) were determined.

Table (1)
Detection of endotoxin activity extracted from S.typhimurium isolates using E-TOXATE kit.

Salmonella Isolates	*Lowest Conc. of Endotoxin Give +ve Reaction (µg/ml)*
A1	5
A2	10
A3	0.5
A4	15
A5	15
A6	20
A7	15
A8	5
A9	10

Chemical analyses involve determination of carbohydrate, protein and nucleic acid contents for the active fractions from 14^{th} to 20^{th} appear in the large peak and fractions from 27^{th} to 34^{th} appear in the small peak. Results indicated that the percentage of carbohydrates was 38.6% in peak 1and 13.2% for peak 2, while the biological activity was (0.1 µg/ml) for peak 1

and 2.5 µg/ml for peak 2. Many studies indicated that the percentage of carbohydrates following endotoxin purification may be widely varied [35, 36]. These differences in calculations are generally attributed to the types of bacterial species from which LPS was extracted, method of extraction and purification process. The biological potency of LPS following purification was highly increased (peak 1), indicating that purification protocol did not affect biological activity of LPS. The increase in potency can be explained because of the purer product that contains a higher proportion of LPS per mass unite. Perdomo and Montero [37] obtained potency from a partially purified LPS a ten-fold increase as compared to the potency of the crude LPS using LAL assy.

On the other hand, the protein content was also investigated and results showed that the protein percentage was 1.15% and 5.75% for peak 1 and peak 2 respectively. This indicated to the efficiency of purification by gel filtration (peak 1), and this result is in agreement with Dooley *et al* [38] which estimated the percentage of proteins following purification in 1.4%-0.1%.

A decrease in biological activity within peak 2 may be attributed to the higher contents of proteins that either interferes with endotoxin activity [39] and Hiki *et al* [40] revealing that endotoxin binding proteins can significantly reduce the harmful action of circulating endotoxin

In addition the application of nucleases enzymes results in 0% of nucleic acids after gel filtration purification of endotoxin.

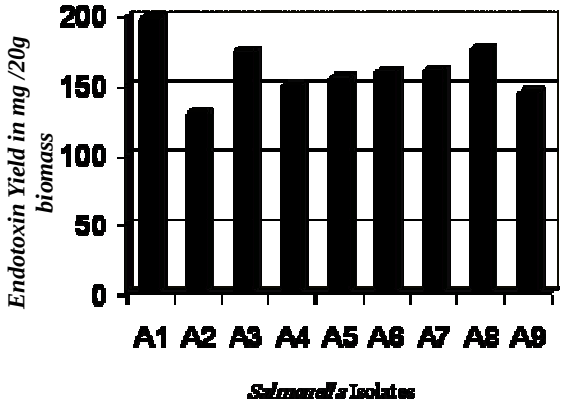

Fig.(1) Yield of LPS from Salmonella isolates following extraction.

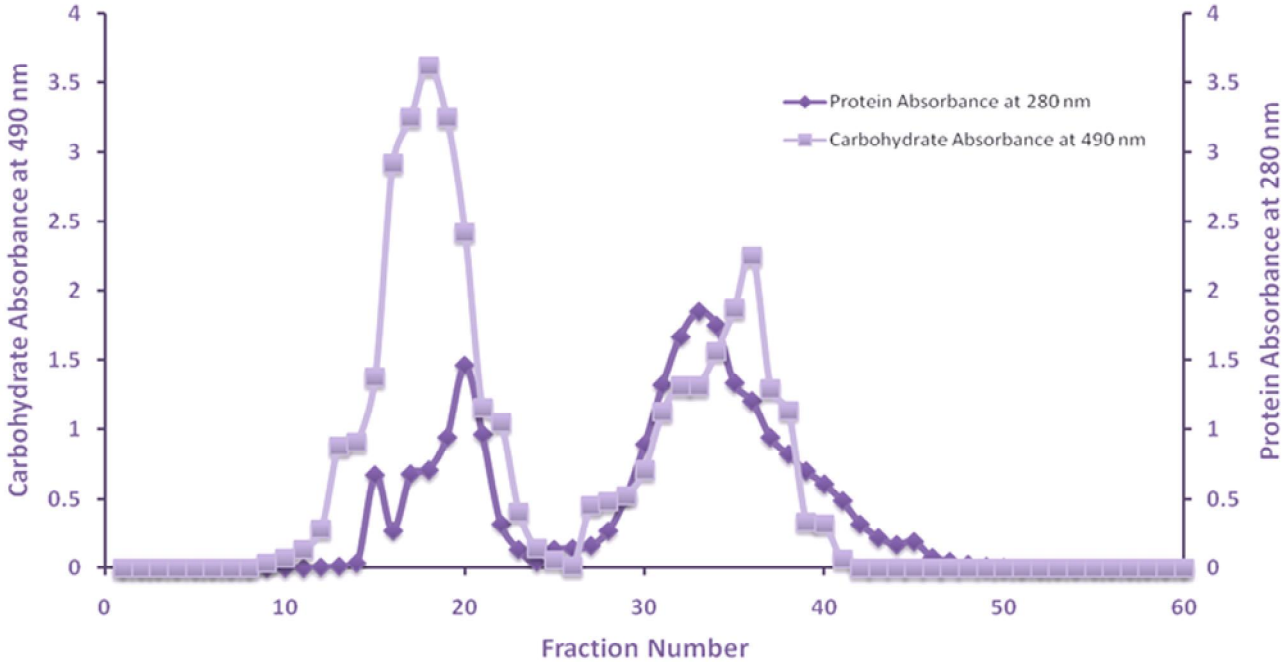

Fig. (2) Purification of endotoxin by gel filtration chromatography (Sepharose Cl-6B) column (3×50 cm) equilibrated with 0.025M phosphate buffer pH 7.2, flow rate 30ml/hour.

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الخلاصة

تم جمع 95 عينة خروج من اطفال مصابين بالاسهال لغرض عزل بكتريا المنتمية الى جنس السالمونيلا. بعد اجراء الفحص الفحص المجهري و التشخيص الزرعي و الفحوصات الكيموحيوية و استخدام العدة التشخيصية AP 20E, شخصت 9 عزلات على انها *Salmonella typhimurium*. تم تقدير الفعالية الحيوية للذيفان بعد استخلاصه من العزلات المشخصة و باستخدام العدة E-TOXATE و بعد استخدام هذا الفحص اظهر اقل تركيز من الذيفان المستخلص من العزلة *Salmonella typhimurium* A3 الذي اعطى نتيجة موجبة (0.5 µg/ml) و على هذا الاساس تم اختيار الذيفان المستخلص من *Salmonella typhimurium* A3 في التنقية و الفحوصات الكيميائية. اظهرت التنقية الجزئية للذيفان المعزول من *Salmonella typhimurium* A3 باستخدام كروماتوغرافيا الترشيح الهلامي باستخدام هلام (sepharose Cl-6B) انفصال قمتين رئيستين. تم تشخيص القمتين تشخيصا كيميائيا و ذلك بتقدير محتوى البروتينات و الكاربوهايدرات و تبين ان القمة الاولى تكون ذات محتوى كاربوهايدراتي عالي (38.6%) و محتوى بروتيني واطئ (1.15%) مقارنة بالقمة الثانية التي اظهرت محتوى كاربوهايدرتي و بروتيني كالاتي (13.2% و 5.75%) على التوالي. بالاضافة الى ذلك اظهرت القمة الاولى فعالية حيوية عالية (0.1 µg/ml) بالمقارنة مع القمة الثانية و السم الداخلي الخام و التي كانت كالاتي 2.5 و 0.5 مايكروغرام/مل.