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Research article

Extraction and Purification of *Pseudomonas aeruginosa* Lipopolysaccharide Isolated from Wound Infection

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ABSTRACT

Samples were obtained from patients suffering from burns and wounds and then identified using the biochemical tests and the VITEK 2 fluorescent system. The tests confirmed the presence of *Pseudomonas aeruginosa*. Antibiotic susceptibility testing showed that the isolate was sensitive to piperacillin, ceftazidime, cefepime, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin and levofloxacin while resistant to ampicillin, cefazolin, ceftriaxone, tigecycline and trimethoprim/sulfamethoxazole. The lipopolysaccharide (LPS) was extracted by the hotEDTA method and partially purified by gel filtration chromatography using the Sephadex G-200.

Keywords: : Gel filtration chromatography, hotEDTA, Lipopolysaccharide, *Pseudomonas aeruginosa*, Sephadex G-200, VITEK 2 system

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INTRODUCTION

Infection is considered one of the dangerous complications of burns. The most infecting organism is likely to be *Pseudomonas aeruginosa* [1]. During this infection, treatment can be very difficult and the death rates among infected patients can reach up to 40–50% [1,2]. It is an opportunistic pathogen found in the soil and surfaces in aqueous environments and due to its adaptation and antibiotic resistance, it can influence a wide spectrum of other natural and artificial settings, such as surfaces in medical facilities. Severe *P. aeruginosa* infections are usually nosocomial and all are related to compromised host defenses [3].

Bacterial infection after an injury can be a result of considerable breaches of skin barrier. Burn hospitals often contain multidrug-resistant *P. aeruginosa* which can be an infection source and it has been found to contaminate the floors, bed rails, and nurses' hands [4]. Bacterial flora can be also transmitted into a hospital by the patient and can infect the same patient following an injury [5].

As opposed to multidrug resistance, Hsueh *et al*. (1998) reported a single multidrug-resistant *P. aeruginosa* strain through several years, and stated that it was transmitted asymptomatically by some patients during antibiotic treatment course which were used against *Pseudomonas* and non-*Pseudomonas* infections [6]. Various *P. aeruginosa* virulence factors participate in the pathogenesis of burn wound infection and some researchers have noted their roles in rodents [7].

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A magnificent role has been discovered for *P. aeruginosa* pili and flagella. [8]. Other virulence factors have a role in burn wound infection such as phospholipase C [9] and lipopolysaccharide (LPS) [10].

LPS are the most essential constituents of bacterial outer membranes released after bacterial growth or death as it serves an effective permeability barrier against antibiotics and host defenses [11]. The present study aims to isolate and identify clinical isolates of *P. aeruginosa* from wound and burn infections. Furthermore, lipopolysaccharide will be extracted by hot EDTA method and partial purification by gel filtration chromatography using the Sephadex G-200.

MATERIALS and METHODS

Collection of bacterial samples

The samples were collected from patients who suffered from burns and wounds attending Baghdad Teaching Hospital, Baghdad, Iraq. These samples were taken by sterile cotton swabs and placed in sterile tubes containing normal saline and transported directly to laboratory of Biology Department, College of Science, University of Baghdad [12]. The study was carried out by following approval from the ethics committee of University of Baghdad, Baghdad, Iraq.

Isolation of *P. aeruginosa*

The collected material was inoculated onto both MacConkey and blood agar then incubated at 37°C for 24 h. The large flat colonies with zones of Beta-haemolysis and a grape like odor on the blood media and the pale colonies on MacConkey agar were re-cultured onto Cetramide agar. On these media, colonies were yellow-blue/green pigmented. Therefore, it is an assumption that they might belong to *Pseudomonas* species. The colonies were then further sub-cultured onto nutrient agar to check for purity. Pure colonies were used for the biochemical tests for identification of *P. aeruginosa* [13].

Identification of *P. aeruginosa*

Bacterial identification was performed by using biochemical tests. These included Gram stain, growth characteristics, and other test methods [14]. The VITEK 2 DensiCheck instrument, fluorescence system (bioMe´rieux) (ID-GNB card) includes 43 non-enterobacterial Gram-negative taxa. Testing was performed according to the instructions of the manufacturer and the analysis was done using the identification card for Gram-negative bacteria (ID-GNB card) containing 41 fluorescent biochemical tests. Cards are automatically read every 15 min. Data were analyzed using the VITEK 2 software version VT2- R03.1 [15].

Antibiotic susceptibility

Susceptibility of *P. aeruginosa* to the several antibiotics (Ampicillin, amoxicillin/Clavulanic acid, ampicillin/Sulbactam, Piperacillin/ Tazobactam, cefazolin, ceftazidime, ceftriaxone, cefepime, imipenem, gentamicin, tobramycin, ciprofloxacin, levofloxacin, nitrofurantoin,trimethoprim/sulfamethoxazole, tricarcillin, amikacin) was performed using the VITEK 2 DensiCheck instrument (bioMe´rieux) [16].

Extraction and partial purification of LPS of *P. aeruginosa*

After identification, the LPS was extracted from the bacterium by hot EDTA extraction method. Bacterial growth was carried out using twelve flasks (250ml) of brain heart broth and inoculated with 1ml of brain heart broth containing the overnight bacterial growth and incubated for 24 h at 37ºC. The cells were harvested by washing them with phosphate buffer saline and cooling centrifuge. Then they were suspended in PBS containing 0.5% formalin and centrifuged. Cells were dried by acetone. Cell disruption was done according to Johnson and Perry, 1976 using the enzymes Proteinase K, DNase and RNase [17]. The extraction step was done by the addition of EDTA-PBS solution, autoclaved, and left to cool at room temperature. The mixture was centrifuged to obtain the supernatant containing the LPS which was then taken and placed in dialysis tubes. The extract was lyophilized and the dry extract was called water dissolved LPS [18]. Partial purification of LPS was done by gel filtration chromatography using the Sephadex G-200[17]. The sample was eluted [19] and the absorbency was measured at 280 nm for protein [20], 490 nm for carbohydrates [21], 260 nm for nucleic acids [22], and at 525 nm for lipids [23].

RESULTS

Twenty five samples were obtained from burn patients. One isolate was identified as *P. aeruginosa* by the routine biochemical testing and confirmed using the VITEK 2 system. Antibiotic susceptibility testing was also carried out and it showed the isolate was sensitive to piperacillin, ceftazidime, cefepime, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin and levofloxacin while resistant to Amipicillin/Slubactam, Cefazolin, ceftriaxone, tigecycline and trimethoprim/sulfamethoxazole (**Table 1**).

Table 1. Minimum inhibition concentrations (MIC) of different antibiotics for clinical isolates of P. aeruginosa. S, sensitive to antibiotic; R, resistant to antibiotic. The antibiotic MIC was tested by VITEK 2 DensiCheck instrument (bioMe´rieux).

Antibiotic	MIC µg/ml	Interpretation
Piperacillin/Tazobactam	8	S
Ceftazidime	4	S
Cefepime	2	S
Imipenem	1	S
Meropenem	<=0.25	S
Amikacin	<=2	S
Gentamicin	<=1	S
Tobramycin	<=1	S
Ciprofloxacin	<=0.25	S
Levofloxacin	1	s
Amipicillin/Slubactam	>=32	R
Cefazolin	>=64	R
Ceftriaxone	16	R
Tigecycline	>=8	R
Trimethoprim/ Sulfamethoxazole	160	R

Sephadex G-200 gel filtration was used to purify LPS that extracted from *P. aeruginosa*. The peak of carbohydrate and lipids were represent the elution of high purity LPS. The LPS was collected from the tubes. Fraction that composed of high amount of sugar and lipid and low level of DNA and protein was chosen for further experiments.In our laboratory the several experiments will be done to check the role of LPS in stimulating several kind of immune cells that collected from patients suffering from rheumatoid arthritis.

DISCUSSION

It was easy to isolate P. aeruginosa from wound infections as this bacterium is commonly known as nosocomial pathogen. As stated by other workers, the likely reason for this might be factors related to attaining nosocomial pathogens in hospitalized patients, complicating illnesses, administration of antimicrobial agents, or the immunosuppressive consequences of burn trauma [24]. The studied bacteria isolate in current study is known for its intrinsic resistance to a wide spectrum of antimicrobial agents and capability to develop multidrug resistance by chromosomal mutations which can lead to a serious therapeutic dilemma [25-27]. Numerous antimicrobial agents, involving several Beta–lactams are effective against *P. aeruginosa* such as extended-spectrum Penicillins. Ciprofloxacin is the most effective against *P. aeruginosa*, although the aminoglycosides have remained the treatment of choice for these infections [28,29]. Lipopolysaccharide is the major outer membrane constituent of gram negative bacteria which makes up about 75% of the surface [1] and 5-10% of the total dry weight of gram negative bacteria [2]. This structure has three parts: lipid A, core oligosaccharide and polysaccharide labeled as "O" antigen. Since the role of LPS has been detected in many diseases, there have been many trials for its extraction and purification. Methods include trichloroacetic acid extraction at 4 ºC [5], aqueous butanol [6], triton/Mg+2 [30], cold ethanol [7] and extraction in water at 100ºC [8]. Other purification protocols with phenol, chloroform, petroleum-ether [9] have been outlined specifically for rough LPS. Contamination with proteins and nucleic acids are among the disadvantages of some methods for LPS purification which delay decisive application of the end product in molecular and immunological experiments. In this study, we have used the hotEDTA extraction method along with Proteinase K digestion of bacterial proteins and nuclease elimination of nucleic acids. This method is simpler, safer and more efficient in extracting LPS antigens than the hot phenol method as stated by Chandan *et al.*(1994) and it is consistent with our results which showed a good purity of the partially purified LPS [18].

The link between LPS and rheumatoid arthritis (RA) is very impressive that they test medications for rheumatoid arthritis by testing their effect on LPS receptors. LPS is a strong substance that secretes various mediators, such as interleukin-12 (IL-12) and interferon-c (IFN-c), participating in cellular immunity and many studies have showed that LPS plays a role in some diseases along with auto-antibodies or self-antigen-specific T cells. For example, LPS enhances nephritis, autoimmune uveitis, autoimmune myocarditis and autoimmune enterocolitis [31]. LPS induces Semaphorin4A expression along with the expression of Sema4A in monocytes/macrophage which shows its involvement in persistent synovial inflammation in RA [32]. In our laboratory, we are working on the role of LPS in stimulate particular immune cells and role of each one in the severity of RA, this work is going on.

It can be concluded from current study that the LPS of *P. aeruginosa* can be easily purified by hotEDTA with a good amount of LPS.

Conflict of interest

The authors declare that they have no conflict of interests.

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