# Outbreak of Multidrug-Resistant *Pseudomonas aeruginosa* Isolates from Burn Infections and Detection of Some Virulence Factors

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## الخلاصة

جمعت 320 مسحة من الحروق المختلفة (مسحة لكل مريض محروق) من عدد من مستشفيات بغداد ، للفترة الواقعة ما بين تشرين الأول 2007 – نيسان 2009 ، من مجموع المسحات أظهرت 276مسحة نمواً بكتيرياً، منها 215 عزلة سالبة لصبغة كرام و61 عزلة موجبة لصبغة كرام بينما لم تظهر44مسحة نمواً بكتيرياً. تم الاعتماد على الفحوصات المظهرية ولبايوكيميائية في التشخيص الأولي للعزلات ومن ثم التشخيص لنهائي بعدة التشخيص api20E . أظهرت النتائج من مجموع 215 عزلة سالبة لصبغة كرام كانت هنالك 48 عزلة تعود لبكتريا *Pseudomonas aeruginosa*. أظهرت الدراسة الحالية مقاومة عالية تجاه 16مضاداً حيوياً مختلفاً، آذ بلغت نسبة المقاومة 100% لكل من مضادات الـ Penicillin, Pipracillin, Amoxicillin, Cefalotin Cefixime, Ceftriaxone, Ceftazidiume, Cefepime, فيما بينت الدراسة الحالية ان الـ Polymixin كان العامل الضد ميكروبي الأكثر فعاليه ضد عزلات *P. aeruginosa* بنسبة 33.33% يتبعه مضاد الـ Azithromycin بنسبة 39.58% . أظهرت هذه البكتريا مقاومه عالية لباقي انواع المضادات الحيويه المدروسه. وكانت مقاومة متعددة للمضادات الحيويه بلغت 11-16 مضاد حيوي مختلف وعلى هذا الاساس قسمت العزلات إلى مجموعتين اعتمادا على عدد المضادات التي قاومتها، وكانت مجموعة B هي المتغلبة في هذه الدراسة. تراوحت قيم التركيز المثبط الادنى (MIC) لمضادات Augmentin، Cefixime، Pipracillin و Cefepime عندما كانت 1024-2 ميكروغرام/مل، 1024-32 ميكروغرام/مل، 1024-64 ،ميكروغرام/مل1024-16 ميكروغرام/مل على التوالي. أظهرت عزلات *P. aeruginosa* القدرة على أنتاج عدد من عوامل الضراوة المرتبطة بامراضيتها فضلاً عن مقاومتها المتعددة للمضادات الحيوية ،فقد أبدت 79.16% من العزلات قابلية إنتاجية لإنزيم البيتالاكتاميز،فضلاً عن انتاج الانزيم المحلل للبروتينات وبنسبة 89.58% والإنزيم المحلل للدهون وبنسبة 87.5% ، في حين أظهرت 87.5% من العزلات قدرتها على الالتصاق بالخلايا الطلائية ، بينما كانت 79.16% منتجة لانزيم اللستنيز، و 72.91% منها منتجة لصبغة البايوسيانين. من خلال هذه الدراسة تم ملاحظة أن لهذه البكتريا ذات المقاومة المتعددة للمضادات الحيويه دور واضح في إصابات المستشفيات سيما وحدات العناية المركزة.نتيجة لامتلاكها عدد من عوامل الضراوة المسئولة عن أمراضيتها.

## ABSTRACT

In this study, during November 2007 till April 2008, 320 swaps from different wound infections were collected from different Baghdad Hospitals, (only one smear per patient was taken). Numbers of swaps showed bacterial growth were 276. From which (215) isolates belong to gram negative bacteria, 61isolates referred to gram positive bacteria, while 44 swaps didn't showed any bacterial growth. Primary identification depending on morphological and biochemical tests were performed followed by using api20E to confirm isolation. The results showed that from the total 215 gram negative isolate only 48 swaps were colonized with *Pseudomonas aeruginosa.* The present study revealed that there were high rate of resistance against 16 different antibiotics were used, 100% of *P. aeruginosa* showed resistance to Penicillin, Pipracillin, Amoxicillin, Cefalotin Cefixime, Ceftriaxone, Ceftazidiume and Cefepime ,the Polymixin was the most in vitro active antimicrobial agent against *P. aeruginosa* (33.33%), followed by

Azithromycin (39.58%). On the other hand multidrug resistance of *P. aeruginosa* was ranging between 11-16 different antibiotics, so that *P.aeruginosa* classified in to 2 groups according to number of resisted antibiotics; group B was the predominant group in this study. Result of sensitivity test agree, with minimum inhibitory concentration (MIC) ratio for, Augmentin، Cefixime، Pipracillin and Cefepime when they were 2-1024µg/ml, 32-1024µg/ml, 64-1024µg/ml, 16-1024µg/ml respectively. *P. aeruginosa* was able to produce number of virulence factors that associated with its pathogencity, 79.16% of isolates were able to produce β-lactamas ,further more able to produce Protease (89,58%),Lipase (87.5%), while 87.5% exhibited ability to adhesion to epithelial cells, 79.16% produce lecithinase and 72.91% were pyocyanin producer. our study demonstrated, High prevalence of nosocomial infections associated with the presence of multidrug resistant *P.aeruginosa*, especially in burn care unit.

# INTRODUCTION

In spite of considerable advances in the treatment of burns, infection continues to pose the greatest danger to burn patients. Approximately 73 per cent of all death within the first five days post-burn has been shown to be directly or indirectly caused by septic processes due to infection with serious pathogen (1).

Nosocomial (hospital-acquired) infections caused by antibiotic-resistant bacteria pose a serious threat to public health (2). Despite interventions aimed at limiting the emergence and spread of antimicrobial-resistant bacteria, including methicillin- resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci*, and multidrug-resistant *Gram-negative bacilli*, recovery of these pathogens continues to rise rapidly (3). Antimicrobial-resistant bacteria are transmitted among patients in hospitals through the contamination of the institutional environment or through human vectors (2).

There are many cases of infection due to different bacteria had been reported but still, the common pathogen isolated from such burn patients is *P. aeruginosa* (4). Multi drug resistant bacteria have frequently been reported as the cause of nosocomial outbreaks of infection in burn units or as colonizers of the wounds of burn patients (5).

The ability of *P. aeruginosa* to invade tissue depends upon it, extracellular enzymes and toxins that break down physical barriers and otherwise contribute to bacterial invasion (6). This study was carried out to determine *P.aeruginosa* isolated from burned patients at different hospital's intensive care unit in Baghdad, then the susceptibility of these isolates to various antibiotics using disk diffusion and minimum

inhibitory concentration methods and performing ß-lactamase production test besides determining some of the major virulence factors produced by these pathogen.

## MATERIAL AND METHOD

**Bacterial isolates and identification procedures.** From November 2007 to April 2008, 48 consecutive *P. aeruginosa* isolates were recovered from 320 patients admitted to the different Baghdad Hospitals (Al-Kindy , Baghdad , Al-Shech zaid ). Only one isolate per patient was included in the study. Bacterial isolates were identified using api20E system (BioMérieux, France) and by conventional biochemical tests: cytochrome oxidase reaction, pigment production, glucose oxidation, arginine dihydrolase activity, and growth at 42°C (7).

**Antimicrobial susceptibility testing.** The susceptibilities of the bacterial isolates to 16 antimicrobial agents including: Penicillin; Pipracillin; Amoxicillin; Augmentin,; Cefalotin; Cefixime; Ceftriaxone; Ceftazidiume; Cefepime; Vancomycin; Erythromycin; Azithromycin; Chloramphenicol; Polymixin; Trimethoprime and Nalidixic acid were determined by the disk diffusion method in accordance with NCCLS guidelines. Briefly, diameter of inhibition zone was measured (mm) and compared with the national committee for clinical laboratory standard (8).

**Minimum Inhibitory Concentration.** The MICs of (4) antimicrobial agents (Augmentin, Cefixime, Pipracillin, Cefepime) were determined by using the twofold dilution method according to (8). Briefly, MICs were determined in Mueller-Hinton broth containing each compound in a twofold serial dilution series. The cells were incubated in the test medium at 37°C for 24 h, and growth was examined visually. The MIC of each compound was defined as the lowest concentration that prevented visible growth.

**ß-lactamase detection**. β-lactam resistant isolates were evaluated for ß-lactamase production by a Standard Iodometric test, If the blue color is lost within 1-3 min, the result was considered to be β-lactams positive .While if changing occurred during (5-10) min , the result will considered as a delay result, the test should be reperform (9).

**Detection of some virulence factors.** To determine production of proteases, lipases and lecithinases *P. aeruginosa* isolates were plated on agar plates containing the appropriate substrates as described below. Production of proteolytic enzymes was determined on media containing 10% skim milk powder (10). After incubation at 30°C for 72 h, plates were flooded with 1 N HCl to observe clearance zones (11). Lipase production was assessed using single-layer agar (11). Single-layer agar

consists of 5% (wt/ vol) clarified butter fat and 1:7,500 Victoria blue B blended into tryptic soy agar. After incubation at 30°C for up to 5 days, plates were observed for the presence of colonies surrounded by dark blue zones. Lecithinase production was determined on egg yolk agar containing 10% egg yolk emulsion. After incubation at 30°C for up to 5 days, plates were observed for the presence of colonies surrounded by brown opaque zones. (12).

**Bacterial adhesion test**. By using Adhesion assay method dependent on epithelial cell suspension obtained from healthy women urine according to (13).

**Pyocyanin production.** By growing the bacteria on nutrient agar then observe pyocyanin production (12)**.**

## RESULT AND DISCUSSION

A total of 320 patients were studied. 48 were colonized with *P. aeruginosa*, at least one isolate from each colonized site of each colonized patient was selected. The clinical samples included burn wound swabs. All of these patients had been treated with various β-lactams, including those with antipseudomonal activity (Piperacillin, Ceftazidime, and Imipenem), and aminoglycosides before the acquisition of *P. aeruginosa* infection or colonization. Other isolates (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and yeast isolates) were recovered from burn wounds of all of these patients during their hospital stays.

Previous studies suggest that the selective pressure from the use of antimicrobial agents is a major determinant for the emergence of resistant strains.

Our result agrees with (14) in which 47 isolates of *P. aeruginosa* were recovered during the 2 months of investigation. In other researches (15) referred *P. aeruginosa* from the patient's endogenous gastrointestinal flora and/or an environmental source is the most common cause of burn wound infections in many centers.

Most of the MDR *P. aeruginosa* isolates were resistant to all antimicrobials tested, except for Polymixin , Chloramphenicol and Azithromycin (Tables 1). Rates of drug resistance were as follow: Penicillin; Pipracillin; Amoxicillin; Cefalotin; Cefixime; Ceftriaxone; Ceftazidium and Cefipime, the percentage was 100% for each .For other antibiotics, Vancomycin, (95.83%); Erythromycin, (95.83%); Augmentin (93.75%) ; Azithromycin, (39.58%); Chloramphenicol, (66.66%); Polymixin, (33.33%); Trimethoprime and Nalidixic acid, (95.83%)for each.

This result agree with in which they showed that *P. aeruginosa* appear resistance to most used antibiotic (16).

The present study revealed that Polymixin was the most *in vitro* active antimicrobial agent against *P. aeruginosa* recovered from clinical burn unit specimens followed by Azithromycin.

In *P. aeruginosa*, secondary β-lactamases with extended substrate specificity can be responsible for acquired resistance to the most powerful antipseudomonal β-lactams, such as expanded-spectrum cephalosporins and carbapenems (17). The secondary ESβLs can degrade Penicillins, expanded-spectrum cephalosporins, and monobactams (but not carbapenems) and are often susceptible to serine-β-lactamase inhibitors (18).

Table -1: Distribution of antimicrobial resistance rates among *P. aeruginosa* isolates isolated in Baghdad hospitals, November 2007 to April 2008

| Antimicrobial agent | Concentration / (μ/l) | No. (%) of resistant isolates from the original 48 |
|---|---|---|
| Penicillin | 10 | 48(100) |
| Pipracillin | 10 | 48(100) |
| Amoxicillin | 100 | 48(100) |
| Augmentin | 30 | 45(93.75) |
| Cephalothin | 30 | 48(100) |
| Cefixime | 30 | 48(100) |
| Ceftriaxone | 30 | 48(100) |
| Ceftazidium | 30 | 48(100) |
| Cefipime | 30 | 48(100) |
| Vancomycin | 30 | 46(95.83) |
| Erythromycin | 30 | 46(95.83) |
| Azithromycin | 30 | 19(39.58) |
| Chloramphenicol | 30 | 32(66.66) |
| Polymixin | 30 | 16(33.33) |
| Trimethoprime | 1.25 | 46(95.83) |
| Nalidixic acid | 30 | 46(95.83) |

The MICs of 4 antimicrobial agents were determined for all the *P. aeruginosa* isolates, it were ranged between: 2-1024 μg/ml for the Augmentin (2 isolates,1isolate,39 isolates and 6 isolates, have MIC value,2,4,512 and 1024 μg/ml respectively) ; for Cefixime were between 32-1024 μg/ml(1 isolate,2isolates and 45 isolates, have MIC value,32,512 and 1024 μg/ml respectively);while for Pipracillin were ranged between 64-1024 μg/ml(3 isolates,7isolates,30 isolates and 8 isolates, have MIC value,64,128,512 and 1024 μg/ml respectively) and for Cefipime were 16-1024 μg/ml(2 isolates, 39 isolates and 7 isolates,

have MIC value,16, 512 and 1024 µg/ml respectively). This result match with (19), they reported that *P. aeruginosa* isolates were resistant to all tested antimicrobials, except for Gentamaicin and Polymixin.

Table-2 present Multidrug resistance of *P. aeruginosa* and it ranged between 11-16 different antibiotics : only 2 isolates showed low MDR against 11 antibiotics , while all isolates ranging between 11 to 16 MDR, finally 2 isolates give resistance to all used antibiotics , so that considered high MDR *P. aeruginosa*.

Nowadays, outbreaks with MDR *P. aeruginosa* strains have become rather frequent (20), and the persistence of an MDR *P. aeruginosa* clone in a burn unit has been reported (14). A serious problem is the emergence of multidrug-resistant (MDR) *P. aeruginosa* strains resistant to β-lactams, aminoglycosides, and quinolones (21). Although intrinsically sensitive to ß-lactams (e.g., Ceftazidime and Imipenem), aminoglycosides (e.g., Amikacin and Tobramycin), and fluoroquinolones (e.g., Ciprofloxacin and Ofloxacin), *P. aeruginosa* resistant to these antibiotics has emerged and is widespread (19).

Table -2: Multidrug resistance of *P. aeruginosa* isolates

| Number of isolates | % of resistant isolates | Number of resistant antibiotics |
|---|---|---|
| 11 | 4.5 | 2 |
| 13 | 40.9 | 18 |
| 14 | 40.9 | 18 |
| 15 | 15.9 | 8 |
| 16 | 6.8 | 2 |

From Table-3 MDR *P. aeruginosa* classified in to 2 groups according to number of antibiotics resisted by the isolates, group A represent 20 MDR isolates that gave 11-13 antibiotic resistance, while group B showed the isolates that resist 14-16 antibiotic to words *P. aeruginosa*. From this result we may predict that group B was the predominant group in this study. Bielecki *etal* (22) Reported that *P. aeruginosa* is prevalent in burn wound infections and it is generally multi-drug resistant.

Table -3: Groping of *P. aeruginosa* according to resistant to antibiotics

| Groups | Number of antibiotics resistant by *P. aeruginosa* | % of resistant isolates |
|---|---|---|
| A | 11-13 | 20 |
| B | 14-16 | 28 |

In our intensive care burn unit, an outbreak of infection due to a *P. aeruginosa* strain was initially suspected on the basis of the

identification of an unusual antibiotype, resistance to all antimicrobial agents routinely tested for activity against *Pseudomonas* species.

Table -4: Virulence factors produce by *P. aeruginosa* isolates isolated from Baghdad hospitals

| Virulence factors | No. (%) of positive isolates |
|---|---|
| β-lactamase | 38(79.16) |
| Protease | 43(89,58) |
| Lipase | 42(87.5) |
| Lecithinase | 38(79.16) |
| Bacterial adhesion | 42(87.5) |
| Pyocyanin production | 35(72.91) |

For β-lactamase the enzyme was detected using Iodometric method which is a simple and convenient technique for susceptibility testing of *P. aeruginosa* in clinical laboratories. Our results revealed the continuing need for quality control of antimicrobial susceptibility tests by concurrently testing known susceptible and resistant strains of *P. aeruginosa* and the utility of attempting to confirm apparent ampicillin resistance of *P. aeruginosa* by demonstration of beta-lactamase activity. 38(79.16%) isolates of *P. aeruginosa* are able to produce these enzyme, Table-4 and this be confirm by sensitivity test, except some isolates not produce enzyme but it is resistant to β-lactam antibiotics. The presence of a chromosomally determined inducible beta lactamase has been reported as characteristic of the genera Pseudomonas(23) .β lactamase may be not always the major mechanism for β-lactam antibiotic resistance , and the resistance may be due to change in target , permeability and efflux pump (24).

Table -4 showed ability of MDR *P. aeruginosa* to produce number of virulence factor that associated with it's pathogencity beside multiple antibiotic resistance. The percentage of production were as following, Protease **(**89,58%**)** ,Lipase (87.5%) and Bacterial adhesion(87.5%) produced in high ratio among MDR *P. aeruginosa* . *P. aeruginosa* extracellular proteases have been associated with virulence (25). Many researches referred the ability of pili from *P. aeruginosa* K (PAK) to act as an adhesion to human epithelial cells (26). *P. aeruginosa* produces three other virulence factor: lecithinase(79.16%). They appear to act synergistically to break down lipids and lecithin and blue pigment, pyocyanin (72.91%), impairs the normal function of human nasal cilia, disrupts the respiratory epithelium, and exerts a proinflammatory effect on phagocytes (25).

The risk of invasive burn wound infection is influenced by the extent and depth of the burn injury, various host factors, and the quantity and

virulence of the microbial flora colonizing the wound (27). Common burn wound pathogens such as *P.aeruginosa* and *Staphylococcus aureus* produce a number of virulence factors that are important in the pathogenesis of invasive infection. *Pseudomonas aeruginosa* produces a number of cell-associated (adhesins, alginate, pili, flagella, and lipopolysaccharide) and extracellular (elastase, exoenzyme S, exotoxin A, hemolysins, iron-binding proteins, leukocidins, and proteases) virulence factors that mediate a number of processes, including adhesion, nutrient acquisition, immune system evasion, leukocyte killing, tissue destruction, and bloodstream invasion (28).

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