

Molecular characterization of *Pseudomonas aeruginosa* isolates from various clinical specimens in Khartoum/Sudan: Antimicrobial resistance and virulence genes

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Abstract

Background: *Pseudomonas aeruginosa* is a pathogenic organism responsible for frequent wound and nosocomial infections worldwide. Its infections are difficult to control since the organism is known to rapidly develop antibiotic resistance and becomes multidrug-resistant (MDR) during treatment of patients.

Aim of the study: This study was intended to investigate the occurrence of certain important types of (ESBL) and (MBL) enzymes in association with important specific virulence factors associated with *P. aeruginosa* clinical isolates from Khartoum, Sudan.

Methods: This study investigated 70 *P. aeruginosa* isolates which were collected from patients admitted to four major hospitals in Khartoum (Fedail, Ribat, Ibn Sina and Soba hospitals). These isolates were recovered from 40 wound swabs (57.1%), 27 urine samples (38.6%), and 3 pleural fluid samples (4.3%) of patients. Higher numbers of isolates were recovered from males 42 (60%) than in females 28 (40%). All *P. aeruginosa* isolates were first confirmed by conventional biochemical and second using molecular PCR tests. PCR methods were also used for detecting the presence of the virulence genes *ToxA*, *AlgD*, *LasB*, *exoS*, *exoU*, *CTX*, *GES-1*, and genes of *VIM*, *IMP*, *KPC*, *CTX*, *VEB-1* and *SHV-1*.

Results: Antimicrobial susceptibility testing of *P. aeruginosa* isolates showed a high resistance to azetronam 49 (70%), followed by ceftazidime 32 (45.7%), 16 ciprofloxacin (22.9%), gentamicin 13 (18.6%),

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piperacillin-tazobactam 11 (15.7%), amikacin 9 (12.9%), and imipenem 6 (8.6%) showed the least resistance. All isolates were positive for *algD* and *lasB* (100%), followed by *toxA* (90%), *exoS* (34.3%), *exoU* (24.3%), respectively. The rates of detected ESBL genes *blaTEM*, *blaCTX-m*, *blaSHV-1*, *GES-1*, were 3.3%, 6.6%, 10%, 3.3%, 10%, respectively, but all isolates were negative for *bla-KPC* and *bla-VIM* and *IMP*. The percentages of pigment production were 61.4% for *pyocyanin*, 37.1% for *pyoverdin* and 1.4% for *pyorubin*.

Conclusion: The study demonstrated high rates of antimicrobial resistance markers to most commonly used antibiotics in treatment of *P. aeruginosa* infections. The majority of the isolates from urine and wound samples carried at least three potential virulence factor genes of *algD*, *lasB* and *toxA* and without any significant relation to their antimicrobial resistance markers.

Keywords

Pseudomonas Aeruginosa; Antimicrobial Resistance; Virulence Factors; Khartoum; Sudan.

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen. The organism is frequently causes nosocomial infection, wound sepsis, pneumonia and urinary tract infections (UTIs) in hospitalized patients, especially in intensive care units (ICUs) [1-5].

P. aeruginosa needs very limited nutritional requirements. It can survive in distilled water [6]. The organism survives at wide range of temperature (4-42 °C), and it is also tolerant to disinfectants and high concentrations of salts [7]. *P. aeruginosa* is capable of producing several different pigments, which are pyocyanin (blue), pyoverdin (yellow and fluorescent), pyorubin (red), and pyomelanin (brown) [8].

Multidrug-resistant *P. aeruginosa* is considered to be a serious problem in all types of infections because the organism has intrinsic resistance to several drug classes and it is able to acquire resistance

to most normally effective anti-Pseudomonas drugs within a short period [9].

There are many virulence factors that may contribute to pathogenic potential of *P. aeruginosa*, such as elastase, alkaline protease hemolysins (phospholipase and lecithinase) and pyocyanin diffusible pigment. In addition, most *P. aeruginosa* strains produce a variety of toxins especially exotoxin encoded by the *toxA* gene, which inhibits protein biosynthesis and is responsible for tissue necrosis. Exoenzyme S is encoded by the *exoS* gene, which is secreted by a type-III secretion system directly into the cytosol of host epithelial cells and subsequently causes their destruction [10]. Other important virulence factor found in *P. aeruginosa* include *algD*, which codes for GDP mannose dehydrogenase, which is transcriptionally activated in mucoid strains, and elastase B, a zinc metallopro-

tease encoded by the (*lasB*) gene, which attacks eukaryotic cells [11-12]. *P. aeruginosa* may also possess flagellae and pili, which helps in motility and adherence to host cells [13].

In recent years, a new variety of β -lactamases have been detected in *P. aeruginosa* clinical isolates. Broad spectrum β -lactam resistance attributed to β -lactamases is generally due to the metallo- β -lactamases (MBLs) such as IMP and VIM. The over expression of chromosomally encoded cephalosporinase, AmpC, is prevalent in *P. aeruginosa*. These MBLs hydrolyze anti-Pseudomonas cephalosporins and carbapenems effectively and their activity is not suppressed by the β -lactamase inhibitors that are currently available for treatment [14-15].

This study aimed to investigate *P. aeruginosa* isolates obtained from various clinical specimens of patients in Khartoum, Sudan, for their antimicrobial resistance profile, the occurrence of most important MBL genes and the common specific virulence factors using PCR molecular and culture tests.

Materials and Methods

This study included a total of 70 different clinical *P. aeruginosa* isolates collected from patients admitted to four hospitals in Khartoum, Sudan, during the period between September to November 2017. All patients were on treatment with various antibiotics.

The data about each patient was obtained and registered as the following: name, gender, age, and type of specimen, and all patients are agreed for any necessary medical process once they are admitted to each hospital.

Culture and isolation

All *P. aeruginosa* isolates were preserved in cryo tubes containing brain heart infusion with 15% glycerol samples and transported from Sudan to Jordan. All isolates were then subcultured on *Pseudomonas* Agar plates and incubated for 24 to 48 hours at 37

°C, and were examined for the presence of pigmentation and grape-like odor of *P. aeruginosa* at the research laboratories of the School of Medicine, The University of Jordan, Amman, Jordan.

Identification and storage

All growth of *P. aeruginosa* isolates was first identified using conventional biochemical tests including oxidase test, develop pigmentation and growth on 42 °C and produce no chemical reaction during culture in Kligler's iron agar tube were stored in cryo tubes at -70°C.

Antimicrobial susceptibility using disc diffusion method

Antimicrobial susceptibility using disc diffusion test was performed according to the guidelines of the Clinical Laboratory and Standards Institute (CLSI, 2016) [16].

Minimum inhibitory concentration (MIC) using E-test

This test was only performed on the *P. aeruginosa* isolates, which were MDR to three or more antibiotic classes. Four classes of antibiotics were tested (ceftazidime, ciprofloxacin, imipenem and amikacin). Results were interpreted according to the CLSI 2016 guidelines [16].

DNA extraction and PCR

The DNA extraction was performed according to the Promega kit (USA). Two PCR assays were performed to confirm the isolates; one is specific for the genus *Pseudomonas*, while the other is specific for *P. aeruginosa*. Two pairs of primers were used for each assay based on 16S ribosomal DNA (rDNA) sequence as shown in **Table 1**.

The bacterial plasmid of freshly cultured *P. aeruginosa* isolates was extracted according to manufacturer instructions using the Zyppy™ Plasmid Miniprep Kit (QIAGEN, Germantown, MD, USA) for detection of ESBLs, MBLs and virulence genes. All

Table 1. 16S rDNA primers sets for differentiation of *P. aeruginosa* from *Pseudomonas* species and their sequences and product size.

Target	Primer	Primer sequence (5' to 3')	Product size(bp)	Reference
<i>Pseudomonas species</i>	PA-GS-F PA-GS-R	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	618	17
<i>P. aeruginosa</i>	PA-SS-F PA-SS-R	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCACCCG-	956	17

Table 2. Primer targets, sequences and their product size for five ESBLs and virulence genes.

Target	Primer	Primer sequence (5' to 3')	Product size (bp)	Reference
blaCTX-M	CTX-M(F) CTX-M(R)	CGCTTTGCGATGTGCAG ACCGGATATCGTTGGT	550	18
blaTEM	TEM-A TEM-B	ATAAAATTCTTGAAGAC TTACCAATGCTTAATCA	1,075	19
blaVEB-1	VEB-FOR VEB-REV	CGACTTCCATTTCCCGATGC GGACTCTGCAACAAATACGC	642	19
blaSHV-1	SHV-FOR SHV-REV	TGGTTATGCGTTATATTCGCC GCTTAGCGTTGCCAGTGCT	867	20
blaGES-1	GES-1(F) GES-1(R)	ATGCGCTTCATTCACGCAC CTATTTGTCCGTGCTCAGG	864	21
blaIMP	IMP-A IMB-B	GAAGGCGTTTATGTTTCATAC GTACGTTTCAAGAGTGATGC	587	22
blaVIM	VIM2004A VIM2004B	GTTTGGTGCATATCGCAAC AATGCGCAGCACCAGGATAG	382	22
blaKPC	KPC (F) KPC (R)	ATGTCACTGTATCGCCGTCT TTACTGCCCGTTGACGCCCA	880	23
algD	algDF algDR	CGTCTGCCGCGAGATCGGCT- GACCTCGACGGTCTTGCGGA	313	24
lasB	lasBF lasBR	GGAATGAACGAAGCGTTCTCCGAC TTGGCGTCGACGAACACCTCG	284	24
toxA	toxAF toxAR	CTGCGCGGGTCTATGTGCC GATGCTGGACGGGTCGAG-3	270	24
exoS	exoS1-F exoS1-R	TCTGAATTCTCCAGGCGGGTGAACATCA TTAGATCTCACCTGGTATCCAAGGCGA	504	25
exoU	exoU-F exoU-R	GGG AAT ACT TTC CGG GAA GTT- CGA TCT CGC TGC TAA TGT GTT-	428	26

primers, sequences and their product size for detection of extended-spectrum- β -lactamases (ESBLs), metallo- β -lactamases (MBLs) and virulence genes were used according to references as shown in **Table 2** [18-26].

Statistic

Data generated from the study were tabulated as Microsoft Excel sheet and uploaded to Statistical Package for Social Sciences (SPSS version 20). Isolates from clinical sources were calculated for the categorical data by Fisher's exact test, to deter-

mine whether there are any statistical differences in comparison to type of antibiotic resistance and virulence factors. The level of significance was set at a p-value of 0.05 to test the hypothesis of no association; Fisher's exact test is used for small sample size.

Results

A total of 70 *P. aeruginosa* isolates were included in this study, and these were recovered from 40 wound swabs (57.1%), 27 urine samples (38.6%), and 3 pleural fluid samples (4.3%) of patients. Higher rate of isolates were recovered from males 42(60%) than in females 28(40%). Antimicrobial susceptibility testing showed a high to low resistance rates to aztreonam 49 (70%), followed by ceftazidime 32 (45.7%), ciprofloxacin 16 (22.9%), gentamicin 13 (18.6%), piperacillin-tazobactam 11 (15.7%), amikacin 9 (12.9%), and imipenem 6 (8.6%) respectively. All isolates were positive for potential genes of *algD* and *lasB* (100%), followed by *toxA* (90%), *exoS* (34.3), *exoU* (24.3%) respectively. The rates of detected ESBL genes *bla*_{TEM}, *bla*_{CTX-m}, *bla*_{SHV-1}, *GES-1*, were 3.3%, 6.6%, 10%, 3.3%, and 10% respectively. However, all isolates were negative for *bla*-KPC and *bla*-VIM and IMP genes. The percentages of pigment production were 61.4% for pyocyanin, 37.1% for pyoverdine and 1.4% for pyorubin. (Tables 3, 4, 5 & 6)

Discussion

This study included *P. aeruginosa* isolated from different clinical specimens of patients whom were admitted for medical care at four major hospitals in Khartoum, Sudan. The majority of the isolates were recovered from wound swabs (57.1%), followed from urine (38.6%) and the least percentage was (4.3%) from pleural fluid. The study shows that all isolates have β-hemolytic activity and are mostly

Table 3. Antimicrobial susceptibility of 70 *P. aeruginosa* isolates .

Antimicrobial agent	Resistant Average	
	no	%
Aztreonam	49	70
Ceftazidime	33	47
Ciprofloxacin	16	23
Gentamicin	13	19
Piperacillin- tazobactam	11	16
Amikacin	9	13
Imipenem	6	09

Table 4. MICs of 29 MDR isolates .

Domains/ Facets	MIC ₅₀	MIC ₉₀	MIC Range	Breakpoints for susceptible
	µg/ml	µg/ml	µg/ml)	µg/ml*
Amikacin	7.7	13.9	0.24- >256	≤ 16
Ceftazidime	3.1	5.6	0.75- >256	≤ 8
Imipenem	2.3	4.1	0.38- >32	≤ 2
Ciprofloxacin	2.4	4.2	0.19- > 32	≤ 1

Table 5. Incidence of Virulence genes .

Among 70 <i>P. aeruginosa</i> isolates Gene	<i>P. aeruginosa</i> isolates	
	no	%
AlgD	70	100
LasB	70	100
ToxA	63	90
exoS	24	34.3
exoU	17	24.3

Table 6. Shows distribution of EBLs genes, plasmid-mediated virulence genes of KPC and VIM2/IMP.

GENE	<i>P. aeruginosa</i> isolates	
	no	%
<i>bla</i> _{TEM}	1	3.3
<i>bla</i> _{CTX-M}	2	6.6
<i>bla</i> _{SHV-1}	3	10
<i>bla</i> _{GES-1}	1	3.3
<i>bla</i> _{VEB}	3	10
KPC	0	0
VIM2/IMP	0	0

producing pigmentation, either pyocyanin (61.4%) or pyoverdinin (37.1%) and more rarely the pigment pyorubin (1.4%).

Many studies have reported that most important virulence factors of *P. aeruginosa* are production of pigments, including mostly both pyoverdinin and pyocyanin and blood hemolysis, which contribute to its pathogenicity during the infection process [27, 28]. However, there are few studies comparing the incidence of pyocyanin and pyoverdinin production of *P. aeruginosa* isolates according to their clinical sources. It has been demonstrated that pyocyanin has numerous antagonistic effects and damages host tissues *in vitro* and *in vivo*, including pro-inflammatory and free radical effects [29].

Treatment of infections caused by *P. aeruginosa* has become challenging in recent years due to the ability of this pathogen to rapidly develop resistance to multiple classes of antibiotics. Therefore, the investigation of the genetics of antimicrobial resistance of this organism may help to control and treat its infections by selection of useful antibiotics [30].

This study also showed high to low rates of resistance markers to certain commonly used drugs in treatment of *Pseudomonas* infections including aztreonam (70%), ceftazidime (47%), ciprofloxacin (23%), gentamicin (19%), piperacillin-tazobactam (16%), amikacin (13%) and imipenem (9%) (Table 3). A previous study conducted also in Sudan [31], reported that

P. aeruginosa isolates from clinical samples of patients was 49.3%. The study showed that 74 of the isolates were resistant to carbenicillin [31]. While a new study conducted in Sudan found a multidrug-resistant (MDR) *P. aeruginosa* isolate from urine of patient with a urinary tract infection was highly resistant to many antibiotics including ciprofloxacin, gentamicin, ceftazidime, piperacillin, and meropenem [32].

The most important mechanisms of resistance to carbapenems are production of carbapenemase enzymes, which have the ability to hydrolyze

penicillins, cephalosporins, monobactams and carbapenems. Carbapenem-resistant *Klebsiella* (*KPC* β -lactamases) hydrolyze most β -lactams, including carbapenems. [33, 34]. Therefore, early detection of this gene will help to avoid incorrect treatment, controlling hospital infections, and preventing the increase spread of resistant strains in hospital and community [35].

The study didn't find any *bla*-*VIM/IMP* and *KPC* genes in *P. aeruginosa* isolates. Other mechanisms might be responsible for the resistance other than the *KPC* genes. It is interesting to note here that similar results to ours have been recently reported by a Jordanian study [2].

The study has demonstrated that all *P. aeruginosa* isolates carried potential *AlgD* and *lasB* genes (100%), followed by other toxic genes (Table 6). All MDR isolates were highly associated with these virulence genes compared to less resistant isolates. However, no significant associations were found between antibiotic resistance markers and putative virulence genes neither among MDR nor all *P. aeruginosa* isolates from urine or wound samples *P. aeruginosa* excreted many virulence factors and forms biofilms that contribute to its infection process. Virulence of *P. aeruginosa* is multifactorial, involving both secreted and cell-associated bacterial products such as elastase, alkaline protease, protease IV, exotoxin A and exoenzymeS. However, most studies consider that exotoxin A, exoenzymeS and exoenzymeU are major virulence factors produced by most clinical *P. aeruginosa* strains, and the most toxic effects of these factors are causing host tissue damage [2, 36].

Conclusion

This study demonstrated high rates of antimicrobial resistance markers to most commonly used antibiotics in the treatment of *P. aeruginosa* infections. There was no significant relationship discovered between antimicrobial resistance markers and

potential virulence genes among all *P. aeruginosa* isolates including MDR isolates from either urine or wound samples.

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