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Original Article

Occurrence of VIM-4 metallo-β-lactamase-producing *Pseudomonas aeruginosa* in an Algerian hospital

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Abstract

Introduction: *Pseudomonas aeruginosa* is one of the most common nosocomial pathogens, known with a wide resistance to antimicrobials. Carbapenemases producing *Pseudomonas aeruginosa* is a growing global public health concern as this pathogen is easily transmissible among patients. Metallo-Beta-lactamases is the most important class of these carbapenemases with their broad-spectrum resistance profile. This study was conducted to investigate the prevalence of MBL-producing *P. aeruginosa* collected in an Algerian hospital.

Methodology: All Metallo-β-lactamase (MBL)-producing *P. aeruginosa* isolates recovered from patients during a 2 years period (2015-2016) were studied using a combination of phenotypic and molecular typing methods (susceptibility testing, molecular characterization of carbapenemase-encoding genes, multi-locus sequence typing and pulsed-field gel electrophoresis).

Results: A total of twenty-six MBL producing *P. aeruginosa* of 188 isolates were investigated. The burns unit ranked in the first position of the majority of identified cases with 73.07%. About 73.07% of total MBL isolates were mainly isolated from pus samples. The studied isolates were subjected to the molecular typing, in which 4 different Dra1-PFGE patterns and 3 sequences type were assigned (ST244, ST381, and ST1076), and all isolates were revealed positive for VIM-4.

Conclusions: We report the third description of *bla*$_{VIM-4}$ in Algeria indicating the emergence and spread of carbapenemase-encoding genes among *P. aeruginosa* in the hospital environment.

Key words: *Pseudomonas aeruginosa*; antibiotic resistance; MBL; VIM-4; PFGE; sequence type.

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Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen that is considered as a major cause of many infections [1]. Its high genetic plasticity enables it to colonize a wide variety of environments and infect almost all anatomical sites [2]. It is responsible for severe hospital-acquired infections especially for the patients compromised by underlying disease, age or immune deficiency (burns, cystic fibrosis, meningitis, abscess, urinary tract infections, catheter-associated infections, and ocular infections) [3]. The intrinsic and acquired resistance of *P. aeruginosa* against many different types of antibiotics, in particular,

β-lactams complicates the treatment of these infections**,** often associated with high mortality rates.

P. aeruginosa has become increasingly resistant to broad-spectrum cephalosporins leading to the use of the last lines agents such as carbapenems [4]. As a result of the intensive use of these molecules, *P. aeruginosa* jeopardizes therapy and developed a resistance that threatened health care systems [5].

An important mechanism of carbapenem resistance is the production of carbapenemases often seen in the clinical isolates of *P. aeruginosa* [6]. The most important carbapenemases are zinc-dependent Metallo-beta-lactamases with a resist pattern to almost all β-lactams. The known MBLs of *Pseudomonas aeruginosa* are IMP (active on IMiPenem), SPM (Sao Paulo Metallo-β-lactamase), AIM (Australia IMipenemase), GIM (German IMipenemase), VIM

(Verona Integron-encoded Metallo-β-lactamase), and more recently NDM-1 (New Delhi Metallo-β-lactamase) and FIM-1 (Florence IMipenemase) where the VIM type enzymes were prevalent and most frequently detected worldwide in *P. aeruginosa* [7].

Metallo-*β*-lactamase-producing *P. aeruginosa* has been extensively reported from different countries in the world [8]. However, data on the prevalent carbapenems resistant phenotypes are limited and poorly investigated in Algeria [9-13]. This work is one among the fewest studies conducted on hospitals in Algeria aiming to project the light on the state of the antibioctic resistance, its level and the main emergent mechanisms. Thus, using a combination of molecular typing methods, a study was conducted to better understand the prevalence and current distribution of MBLs producing clinical isolates of *P. aeruginosa* recovered from the university hospital of a medium-sized city (Batna, North-Eastern Algeria).

Methodology

Bacterial isolates

During the period from January 2015 to December 2016, 188 non-redundant clinical isolates of *P. aeruginosa* were recovered from different pathological samples (pus, blood, urine, cerebrospinal fluid, pleural fluid, throat swab, ascites and others) obtained from hospitalized patients and outpatients consulting the University Hospital Center of Batna (Northeastern Algeria) (Table 1); a structure of 635 beds.

The isolates were presumptively identified by routine tests; colony morphology, pigmentation, isolation on cetrimide agar, oxidase test, and API 20NE System (bioMérieux SA, Marcy-l'Etoile, France). For further analysis, MBL-producing isolates were identified using matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF-MS) with Microflex LT control software (Bruker Daltonik GmbH, Bremen, Germany).

Antibiotic susceptibility and MBL phenotypic determination

Antibiotic susceptibility testing was performed according to the recommendations of the Clinical Laboratory Standards Institute [14] using the disk diffusion method on Mueller Hinton agar. Fourteen antimicrobial agents were tested including; ticarcillin (75µg), piperacillin (100µg), ticarcillin/clavulanic acid (75/10µg), ceftazidime (30µg), imipenem (10µg), aztreonam (30µg), amikacin (30µg), tobramycin (10µg), gentamicin (10µg), netilmicin (30µg), ciprofloxacin (5µg), levofloxacin (5µg) fosfomycin (50µg+ 50µgG6P) and rifampicin (30µg). The antibiotic disks and the used media were purchased from (HiMedia, Mumbai, India). The plates were inoculated with a 1/100 dilution of 0.5 McFarland suspension and incubated at 37ºC for 24 hours and the diameters of zones of inhibition were compared to reference values [14] to determine the susceptibility or resistant pattern of the isolates. *P. aeruginosa* ATCC 27853 was used as a wild-type for quality control. The production of Metallo-β-lactamases was screened using the Modified Hodge test (MHT) and the double disk synergy test (DDST) according to the CLSI guidelines [14].

Molecular typing

Twenty-six *Pseudomonas* isolates were selected for their resistance to all beta-lactams except the aztreonam (MBL strains) and subjected to molecular analysis.

Metallo-β-lactamases molecular identification

Carbapenem-hydrolyzing enzyme-encoding genes were determined by polymerase chain reaction (PCR) for the simultaneous detection of bla_{IMP}, bla_{VIM} and bla_{NDM} using primers and conditions as described by Ellington *et al*. [15] and Poirel *et al*. [16]. All amplified products were then sequenced.

Pulsed Field Gel Electrophoresis Typing (PFGE)

Pulsed-field gel electrophoresis (PFGE) analysis was performed as described by Talon *et al*. [17] to study the epidemiological relatedness of these isolates. Genomic DNA was digested with *Dra1*(Roche Diagnostics, Meylan, France) at 37°C according to the manufacturer's instructions and the restriction fragments were separated in 1.2% agarose gels. The PFGE was carried out at 6V/cm for 24 hours using the CHEF DR III apparatus (Bio-Rad, Hercules, USA) and gels were stained with ethidium bromide. The relatedness of each PFGE fingerprint was determined using the unweighted pair group method and GelCompar software (Applied Maths, Kortrijk, Belgium) for cluster analysis and to establish a DNA similarity matrix *Staphylococcus aureus* NCTC 8325 was included as a reference to compare the gels. The banding patterns were interpreted based on the criteria proposed by Tenover *et al*. [18].

Multi-Locus Sequence Typing (MLST)

The epidemiological relatedness of *P. aeruginosa* was studied by multilocus sequence typing (MLST) as described [19]. The following seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA,* and *trpE*)

were used for the characterization. Alleles and sequence type (ST) assignment were performed at the *P. aeruginosa* MLST website (https://pubmlst.org/paeruginosa/).

Data analysis

Frequencies of *P. aeruginosa* isolates recovered from different wards were calculated as the percentage of a number of isolates to the total of surveyed patients hospitalized in different units. Pearson's Chi-squared test (χ^2) was performed to test the statistical significance of differences between groups and the statistical significance was defined as $P < 0.05$.

Results

Epidemiological survey

During the study period, 188 *P. aeruginosa* were isolated, including (63.3%) male and (36.7%) female

Table 1. Distribution of *Pseudomonas aeruginosa* among the hospitalized patients.

Variable	Admitted patients (n/%)	*P*-value	Patients associated with MBL, n (%)	*P*-value
Gender				
Males	119 (63.3)	**< 0.001**	19 (9.5)	**0.01**
Females	69 (36.7)		7 (3.5)	
Age				
Mean ± SD	37.6 ± 17.86		22.94 ± 18.76	
0-10	51 (27.13)	**< 0.001**	9 (34.6)	**NS**
10-20	26 (13.83)		5 (19.2)	
20-40	61 (32.45)		5 (19.2)	
40-60	32 (17.02)		7 (26.9)	
60-90	18 (9.57)		0	
Hospital ward				
Burns	68 (36.17)	**< 0.001**	19 (73.07%)	**< 0.001**
Neurosurgery	27 (14.36)		2 (7.69)	
ICU	24 (12.76)		0	
Hematology	13 (6.91)		2 (7.69%)	
Internal medicine	10 (5.31)		0	
Traumatology	10 (5.31)		1 (3.84)	
External	10 (5.31)		0	
Nursery	7 (3.72)		1 (3.84)	
Endocrinology	4 (2.13)		0	
Nephrology	3 (1.6)		0	
Surgery	3 (1.6)		0	
Cardiology	3 (1.6)		0	
Pediatrics	3 (1.6)		1 (3.8)	
Emergency	2 (1.06)		0	
Forensic medicine	2 (1.06)		0	
Orthopedics	1 (0.53)		0	
Specimen				
Pus	114 (60.64)	**< 0.001**	19 (73.07)	**< 0.001**
Blood	17 (9.04)		1 (3.84)	
Cerebrospinal fluid	16 (8.51)		1 (3.84)	
Tracheal protected	14 (7.45)		0	
Urine	9 (4.8)		1 (3.84)	
Catheter	4 (2.13)		1 (3.84)	
Pleural fluid	4 (2.13)		0	
Throat swab	3 (1.6)		1 (3.84)	
Urinary catheter	3 (1.6)		2 (7.69)	
Bladder catheter	2 (1.06)		0	
Ascites	1 (0.53)		0	
Adenitis	1 (0.53)		0	
Skin wound	1 (0.53)		0	
Other	1 (0.53)		0	
Total	**188 (100)**		**26 (13.82)**	

NS. Not significant.

with a sex ratio of 1.72 and a mean age of 37.6 ± 17.86 years (Table 1). All patients were hospitalized at the time of *Pseudomonas* isolation, with the exception of 10 cases identified during outpatient visits. As shown in Table 1, burns, neurosurgery and intensive care unit (ICU) grouped the majority identified cases with 36.17%, 14.36%, and 12.76% respectively. In addition, hematology, internal medicine, traumatology, and external medicine shared frequencies ranged from 5.31% to 6.91% of total confirmed cases. *P. aeruginosa* strains are mainly isolated from pus sample (60.64%). Further, blood samples, cerebrospinal fluid and tracheal protected represent 7.47% to 9.04% of the total isolated sources of *P. aeruginosa*.

Antimicrobial susceptibility

The results of the antimicrobial susceptibility rates of the total clinical isolates are presented in (Table 2). Various resistance levels to β-lactam tested drugs were noted including ticarcillin (32%), ticarcillin/clavulanic acid (35.11%), piperacillin (26.06%) and imipenem (20.75%). The aminoglycoside resistance rates ranged from (31%) amikacin, (32%) netilmicin, (26.06%) tobramycin and (26%) for gentamicin. Whereas ciprofloxacin, levofloxacin, and fosfomycin were categorized as the effective molecules on the tested strains.

As shown in Table 1, twenty-six isolates (13.82%) of *P. aeruginosa* presented a positive double disk synergy test and a positive modified Hodge test. These isolates were mainly recovered from pus samples (73.07%, $P< 0.001$) of hospitalized burned patients for the antibiotic susceptibility test, MBLs isolates were found to be highly resistant (100%) to ticarcillin, ticarcillin/clavulanic acid, piperacillin, imipenem, ceftazidime, and tobramycin (Table 2). No resistance was detected against aztreonam, levofloxacin, cirpfloxacin and fosfomycin. In this study, we focused on the production of MBL as carbapenem-resistance pattern; other mechanisms of resistance to carbapenems are not the subject of this paper.

Molecular typing

PCR analysis of the selected isolates revealed positive results for a $bla_{\text{VIM-4}}$ gene in all MBL strains (100%, 26/26) (Figure 1). In addition, bla_{IMP} and bla_{NDM} were not detected in any of the tested strains. The DNA fingerprint patterns of the studied strains revealed four different clusters. Cluster 1, grouped most of the isolates (15 strains) showing a significant resistant homogeneity. As shown in Figure 1, three different sequence types were noted (ST244, ST381, and ST1076) in which the most frequent ST was ST244 with 18 isolates in total.

Discussion

Our results showed that *P. aeruginosa* strains were mainly isolated in burns unit (pus samples) which is due to the fact that skin damage (traumatic or surgical wounds and burns) is a factor contributing to *P. aeruginosa* infections [20]. Patients hosted in high-risk units with reduced immune defenses appear to be susceptible to pathogens infections as *P. aeruginosa*ones, also selection pressure from the prescription of broad-spectrum antibiotics enhancing bacterial infection. Further, and according to Oberholzer *et al*. [21] sex is considered as a risk factor and the incidence of infection increases significantly in

Table 2. Antibiotic susceptibility patterns of *Pseudomonas aeruginosa* isolates.

Antibiotics	Antibiotic susceptibility (%)					
	Total *P.aeruginosa* (N = 188)			MBL *P. aeruginosa* (N = 26)		
	Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive
Ticarcillin (TIC)	32	0	68	100	0	0
Ticarcillin-clavulanic acid (TCC)	35.11	0	64.89	100	0	0
Piperacillin (PIP)	26.06	0	73.94	100	0	0
Imipinem (IMP)	20.75	1.06	78.19	100	0	0
Ceftazidime (CAZ)	15.00	0.53	84.47	100	0	0
Aztreonam (ATM)	0	1.06	98.94	0	0	100
Amikacin (AK)	31	3.91	65.09	96.15	0	3.85
Gentamicin (GEN)	26	3.19	70.81	96.15	0	3.85
Netilmicin (NET)	32.00	0.53	67.47	96.15	0	3.85
Tobramycin (TOB)	26	2.66	71.34	100	0	0
Rifampicin (RIF)	90	9.04	0.96	92.31	3.85	3.85
Levofloxacin (LEV)	2	1	97	0	0	100
Fosfomycin (FOS)	2.66	0	97.34	0	0	100
Ciprofloxacin (CIP)	0	0	100	0	0	100

men (*P. aeruginosa* infections are more common in men especially with burn wounds).

P. aeruginosa is a major pathogen of nosocomial infections and known for its wide range of antimicrobial resistance, the increased antimicrobial exposure lead to the selection of resistant organisms [22].The results of the antimicrobial susceptibility patterns of the all isolated *P. aeruginosa* showed various resistance levels to β-lactam tested drugs and aminoglycoside (with no resistance to aztreonam, levofloxacin, ciprofloxacin and fosomycin of MBL strains). Our data stand in contrast with those reported by Meradji *et al*. [12] where high rates of resistance to ticarcillin, piperacillin and ciprofloxacin were noted.

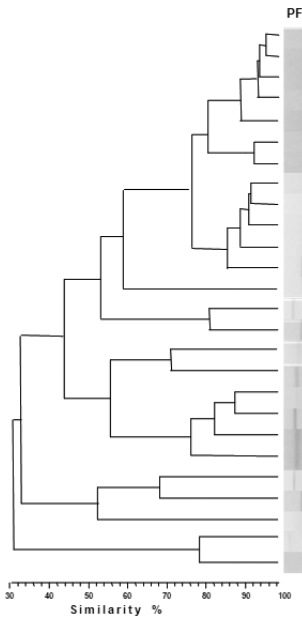
The emergence and spread of clinical carbapenemase-producing *P. aeruginosa* (MBLs) has become a serious concern and health care problem around the world [23]. The MBL prevalence is 13.82% which is similar to that in Côte d'Ivoire (12.5%) [24], but lower than that in Egypt 68.7% [25]. The presence of VIM-type enzymes was responsible of carbapenem resistance in our MBL-producing isolates. The *bla*$_{\text{VIM-4}}$ carrying *P. aeruginosa* isolates were detected in 6 different wards of the hospital mainly in burns, neurosurgery, hematology, traumatology, pediatrics and nursery and associated with three different clones (ST244, ST381, and ST1076), indicating the gene spread and the clonal dissemination which affect the prevalence and the distribution of carbapenemase-encoding genes in the hospital environment.This situation is in general restricted to specific patient and locations, where patients are often exposed to broad-spectrum antimicrobial agents and with an increased risk of cross-transmission of resistant germs [26].

The *bla*$_{\text{VIM-4}}$ genes have been reported in several countries around the world, including Greece [27], Sweden [28], Poland [29], Hungary [30], Canada [31], Egypt [32] and other European countries [33], mainly detected among related *P. aeruginosa* clones which are encoded by an integron-borne gene cassette with high dissemination potential.

Few reports of VIM class MBL isolates in Algeria were depicted, the first one was the *bla* $_{VIM\text{-}19}$described in five *Enterobacteriaceae* clinical isolates [9], then the *bla*$_{\text{VIM-2}}$ has been noted in *P.aeruginosa* clinical strains [10,11] and the *bla*$_{\text{VIM-4}}$ gene that has been recently reported among clinical isolates of *P. aeruginosa* [13, 34], by this study we report the third detection of VIM-4 gene carrying *P. aeruginosa* in Algeria indicating its widespread in the hospital environment.

Pseudomonas aeruginosa is well known by its epidemic population with several sequence types as ST111.ST175, ST235, ST 244 and ST 395 that are commonly associated with outbreaks [35]. MBLs are commonly associated with epidemic high-risk clones [36]. The clone belonging to ST244 was the most frequent in our study and corresponded to the second most prevalent Mediterranean *P. aeruginosa* clone [37] and further was detected in Asia [38]. Similarly, the ST1076 clone was also reported among clinical isolates

Figure 1. Dra1 pulsed-field gel electrophoresis (PFGE) profiles and resistance patterns of 26 VIM-4 positive *P. aeruginosa* isolates. CAZ, ceftazidime; GEN, gentamicin; TCC, ticarcillin-clavulanic acid; TIC, ticarcillin; TOB, tobramycin; NET, netilmicin; RIF, rifampicin; AK, amikacin; PIP, piperacillin; IMP, imipenem; M, male; F, female.



PFGE profile	Isolate	Cluster		Sex/age	Unit	Sample	Resistance pattern	VIM-4	MLST
	PaB165			M/34yr	Traumatology	Pus	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB274			M/03yr	Burns	Pus	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB300			F/20yr	Burns	Pus	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB196			M/55yr	Burns	Pus	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB257			M/23yr	Neurosurgery	Catheter	TIC, TCC, PIP, IMP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB403			F/21yr	Burns	Pus	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB149			F/24yr	Burns	Pus	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB357	A	I	F/36yr	Hematology	Blood	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB374			M/15yr	Burns	Pus	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB545			M/59yr	Hematology	Urinary catheter	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB358			F/45yr	Burns	Pus	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB290			F/06yr	Burns	Pus	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB261	B		M/07yr	Burns	Pus	TIC, TCC, PIP, CAZ, GEN, NET, RIF, TOB	+	ST244
	PaB262	C		M/05yr	Burns	Pus	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB368bis			M/20yr	Burns	Pus	TIC, TCC, IMP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB159	A		M/44yr	Burns	Pus	TIC, TCC, IMP, CAZ, AK, GEN, NET, TOB	+	ST381
	PaB216			M/54yr	Burns	Pus	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST381
	PaB275d			M/1mon	Neurosurgery	CS fluid	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST381
	PaB1315	B	II	F/2.5yr	Burns	Pus	TIC, TCC, PIP, IMP, CAZ, AK, GEN, NET, RIF, TOB	+	ST381
	PaB1320			M/23yr	Burns	Pus	TIC, TCC, PIP, IMP, CAZ, AK, GEN, NET, RIF, TOB	+	ST381
	PaB2097			M/11yr	Pediatrics	Urine	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST381
	PaB506	A	III	F/07yr	Burns	Pus	TIC, TCC, PIP, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB156			F/55yr	Burns	Pus	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB301	B		M/20yr	Burns	Urinary catheter	TIC, TCC, PIP, CAZ, AK, GEN, NET, RIF, TOB	+	ST244
	PaB143		IV	M/06yr	Burns	Pus	TIC, TCC, IMP, CAZ, GEN, NET, TOB	+	ST1076
	PaB64			M/03yr	Nursery	Throat swab	TIC, TCC, PIP, IMP, CAZ, AK, GEN, TOB	+	ST1076

of *P. aeruginosa* in Algeria [11,13] with imipenem-susceptible and VIM-4 producing *P.aeruginosa*, also Sefraoui *et al*. [10] detected the ST 381 in imipenem-suscpetible strains of *P.aeruginosa*. In contrast, a study conducted by Mathlouthi *et al*. [39] showed that the ST911 and ST235 were the most frequent clones in Libya. Treepong *et al*. [35] reported the clonal spread of ST 235 with antibiotic resistance genes in isolates of *P. aeruginosa* across countries and continents. We report the first detection of ST 244 and ST 381 in VIM-4 producing *Pseudomonas aeruginosa* in Algeria.

Conclusion

In conclusion, these results revealed a particular concern that the carbapenemase-producing *Pseudomonas aeruginosa* (VIM-4) have been detected and dramatically spread in Algerian hospitals, with a dominance of the clone ST244 mainly isolated from burns unit. These findings suggest that the emergence of these resistant strains limit the therapeutic options and threaten public health. Thus, suggesting that hospitals need to develop better strategies to prevent and control infections by the implementation of strict hygiene protocols to control cross-transmission between patients including antibiotic use policies, isolation of patients with MBL strains and regular surveillance studies. The understanding of the antibiotic resistance in the Algerian hospitals needs more in-depth studies focusing on all resistant strains and their transmission across the hospitals and the creation of a consistent epidemiological data network connecting all hospitals in the country.

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