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Original Article

Virulence genes and antibiotic resistance of *Pseudomonas aeruginosa* isolated from patients in the Northwestern of Morocco

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Abstract

Introduction: *Pseudomonas aeruginosa* is an ubiquitous bacterium causes various community-acquired and nosocomial infections. In this investigation, we aimed to screen the antibiotic susceptibility patterns and the prevalence of virulence factor genes in a set of *Pseudomonas aeruginosa* isolated from nosocomial and community-acquired infections in the Northwestern of Morocco.

Methodology: A total of 155 of *Pseudomonas aeruginosa* strains were collected (January 2015 - December 2016) from nosocomial and community-acquired infections at hospital centers and clinical laboratories in the Northwestern of Morocco. Antimicrobial susceptibility test was performed by the standard disk diffusion method. In addition, PCR assays were used for screening five virulence encoding genes (*lasB*, *algD*, *plcH*, *exoA,* and *exoS*).

Results: Our results revealed that high level of antimicrobial resistance was detected towards aztreonam (27.1%) followed by meropenem (14.2%). The resistance to imipenem was significantly higher in strains isolated from nosocomial infections (12.7%) than strains isolated from community-acquired infections (1.5%). The results highlighted that *lasB* (98.7%) and *exoS* (98.7%) were the most frequent virulence genes.

Conclusions: This survey provides data about phenotypic and genotypic properties of *Pseudomonas aeruginosa* emerged in the Northwestern of Morocco. It could be helpful for the health workers to improve infection control measures and to establish a surveillance system.

Key words: *Pseudomonas aeruginosa*; nosocomial infections; community-acquired infections; antimicrobial resistance; virulence genes; Morocco.

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an ubiquitous gram-negative bacillus implicated in various community-acquired and nosocomial infections [1]. Through its ability to resist to different physical conditions, this bacterium can survive in community and hospital settings [2]. *P. aeruginosa* is responsible for several nosocomial infections like pneumonia, urinary tract infections, surgical site infections, and some of community-acquired infections such as otitis externa, ulcerative keratitis, and soft tissue infections [1].

The pathogenesis of *P. aeruginosa* is linked to the production of an arsenal of virulence factors classified into cell-associated and secreted [3]. One of these virulence factors that play a main role in tissue lysis and bacterial invasion is exotoxin A (*exoA*). The hemolysin phospholipase H (*plcH*) act to destroy lipids and lecithin contributing to tissue invasion. *P. aeruginosa* also produces exoenzyme S (*exoS*), it is cytotoxin responsible for damage to many types of host cells and elastase B (*lasB*) that play an important role during the acute infection [4,5]. Some strains produce alginate that forms the matrix of the biofilm which protect bacteria from the host defense during the chronic infection. The GDP-mannose 6-dehydrogenase (*algD*) is one of three proteins that are implicated in the production of alginate [6,7].

The treatment of nosocomial and community infections due to *P. aeruginosa* has become a serious concern because of its intrinsic resistance and its capacity to acquire new mechanisms of resistance to many antibiotic groups such as fluoroquinolones, β-lactams, and aminoglycosides [8]. Hence, a better understanding of the resistance patterns, and virulence of this bacterium is indispensable [9].

Information regarding the antimicrobial susceptibility patterns of isolates is needed if we are to decrease the risk of the spread of resistant *P. aeruginosa* strains [10]. Ongoing microbiological surveillance has

been recommended by the World Health Organization (WHO), to improve infection control measures especially in the healthcare setting and to guide the physicians in the choice of empirical or directed treatment [11].

Recent findings have shown that *P. aeruginosa* strains do not necessarily have similar virulence factor genes. Moreover, various studies suggested there was an association between the presence of some virulence genes and certain antibiotic resistance patterns [12,13]. To our knowledge, very few studies have evaluated the genotypic and phenotypic characteristics of *P. aeruginosa* strains in Morocco. However, no survey has been reported exploring the emergence of *P. aeruginosa* in the Northwestern of Morocco.

Therefore, the purpose of the current study was to screen the antimicrobial resistance profile of *P. aeruginosa* isolated from CAI and NI in the Northwestern of Morocco, as well as to determine the prevalence of some virulence factor genes according to the strains origin. Moreover, a statistical analysis was used in order to evaluate whether a significant association exists between the presence of virulence factor genes and antibiotic susceptibility patterns. Such a study could help to better understand the properties of *P. aeruginosa* strains emerged in the region.

Methodology

Bacterial isolates

This study was carried out during the period of January 2015 to December 2016, strains were obtained from laboratory of microbiology, Mohamed V provincial hospital of Tangier (250 beds), Laboratory of Microbiology, Provincial Hospital of Tetouan (524 beds), five Medical Analysis Laboratories from Tangier-Tetouan region situated in the Northwestern of Morocco. A total of 155 non-duplicate isolates of *P. aeruginosa* were recovered from various samples, out of which 87 were from Nosocomial infections (NI) and 68 from Community-acquired infections (CAI) according to patient records. The strains were stored in nutrient agar stab cultures, in a cooler bag, for transporting to Institut Pasteur du Maroc. The isolates were inoculated on Cetrimide agar (Oxoid Ltd, Basingstoke, United Kingdom) and incubated at 42°C for 18 to 24 hours. Identification of *P. aeruginosa* was performed by conventional techniques [14].

Antimicrobial susceptibility testing

The susceptibility testing was determined by the disc diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations. The antibiotic disks (Oxoid Ltd, Basingstoke, United Kingdom) used were: ciprofloxacin (5 µg), ticarcillin (75µg), amikacin (30 µg), ceftazidime (30 µg), aztreonam (30 µg), imipenem (10µg), meropenem (10µg), and doripenem (10 µg). Inhibition zone diameters were evaluated according to EUCAST guidelines (2017) [15]. The minimum inhibitory concentrations (MICs) of imipenem were determined for all strains displayed resistance or intermediate resistance to imipenem, by using the broth microdilution method as described elsewhere [16]. The results were interpreted based on the EUCAST breakpoints (2017) [15]. The multidrug-resistant (MDR) phenotypes defined as a resistance to at least three or more classes of antimicrobial agents [17]. *P. aeruginosa* ATCC 27853 was employed as a control strain.

Virulence factors PCRs

Preparation of template DNA

Genomic DNA extraction of *P. aeruginosa* was performed using boiling-freezing method. Few colonies of overnight culture were picked and resuspended in 400 µL of sterile distilled water, a homogenous

Table 1. Primers used for PCR amplification of virulence factor genes.

Target gene	Primers	Nucleotide sequence of primers (5'– 3')	Amplicon size (bp)	Annealing temperature (°C)	References
algD	*algD*-F *algD*-R	ATG CGA ATC AGC ATC TTT GGT CTA CCA GCA GAT GCC CTC GGC	1310	55	[18]
lasB	*lasB*-F *lasB*-R	GGA ATG AAC GAA GCG TTC TC GGT CCA GTA GTA GCG GTT GG	300	55	[18]
plcH	*plcH*-F plcH-R	GAA GCC ATG GGC TAC TTC AA AGA GTG ACG AGG AGC GGT AG	307	55	[18]
exoS	*exoS*-F *exoS*-R	GCG AGG TCA GCA GAG TAT CG TTC GGC GTC ACT GTG GAT GC	118	58	[19]
exoA	eta1B eta2	AAC CAG CTC AGC CAC ATG TC CGC TGG CCC ATT CGC TCC AGC GCT	207	65	[19]

F: forward primer; R: reverse primer.

suspension was made by vortexing for 60 seconds. The tubes were placed in boiling water bath at 94°C for 20 minutes and immediately frozen at -20°C overnight. A volume of10 µL of RNase A (10 mg/mL Solution, Amresco, Solon, United States of America) was added to remove RNA, then the tubes were incubated for 60 minutes at 37°C and stored at -20°C until further use.

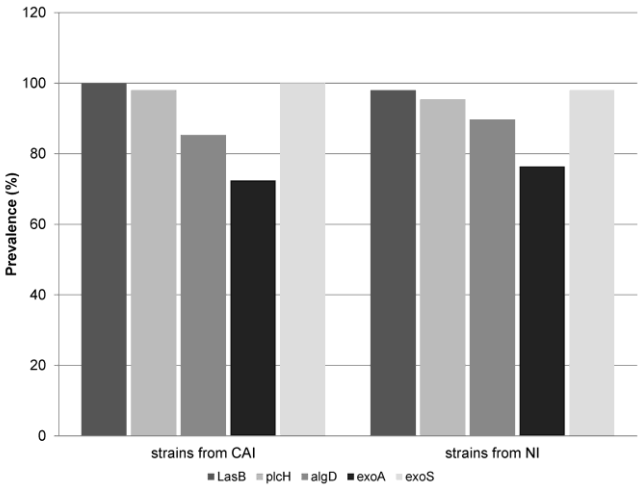
Detection of virulence genes

The virulence genes (*lasB*, *algD*, *plcH*, *exoA,* and *exoS*) in this study were selected on the basis of previous studies that pointed their importance in the pathogenesis. The virulence genes were amplified with specific primers listed in Table 1. PCR amplifications were performed in a final volume of 25 µL. The PCR reaction mixtures consisted of1 X MyTaq Reaction Buffer (Bioline, London, United Kingdom), 1.25 µM of each primer, 1.5U of MyTaq DNA Polymerase (Bioline, London, United Kingdom) and 8µL of template DNA, the volumes of the reaction mixtures were increased to 25 µL using sterile distilled water. PCR conditions have been described previously [18,19]. Positive control strain was *P. aeruginosa* ATCC 27853 for the five virulence genes. A reaction mixture containing sterile water was included as a negative control. PCR products were separated in a 2% agarose gel for 60 minutes at 100V, stained with ethidium bromide (Promega, Madison, USA), and detected by UV transillumination. Amplified genes were identified on the basis of fragment size.

Statistical analysis

Chi-square test of independence (χ^2) was used to compare the antibiotic resistance patterns and the prevalence of virulence factor genes among *P. aeruginosa* strains isolated from NI and CAI. A value of $P < 0.05$ was considered statistically significant.

Figure 1. Prevalence of virulence factor genes among P. aeruginosa strains isolated from NI and CAI.



NI: Nosocomial infections. CAI: Community-acquired infections.

Results

Antimicrobial susceptibility testing

Susceptibility testing has revealed that of all the strains (n = 155), 112 (72.3%) isolates were susceptible to all the antibiotics tested, 43 (27.7%) were resistant to at least one antibiotic, and MDR phenotype was not detected.

No significant difference was found between the strains origin and antimicrobial resistance against the antibiotics tested, with an exception of imipenem (P-value = 0.013), for which the resistance was significantly higher in strains isolated from NI (12.7%) than strains isolated from CAI (1.5%). The MICs of imipenem ranged from 4 to 16 mg/L.

Antimicrobial susceptibility rates are summarized in Table 2. In addition, high level of resistance was detected towards aztreonam (27.1%) followed by meropenem (14.2%), doripenem (11.6%), and ciprofloxacin (11%). Furthermore, a total of 154

Table 2. Comparison of antimicrobial susceptibility between P. aeruginosa strains isolated from NI and CAI.

Antibiotics	Strains from CAI n = 68		Strains from NI n = 87		Total n = 155	
	Susceptibility (%)					
	S	R[a]	S	R[a]	S	R[a]
ticarcillin	83.8	16.2	92	8	88.4	11.6
aztreonam	75	25	71.3	28.7	72.9	27.1
ceftazidime	92.6	7.4	95.4	4.6	94.2	5.8
imipenem[b]	98,5	1.5	87.3	12.7	92.3	7.7
meropenem	89.7	10.3	82.8	17.2	85.8	14.2
doripenem	92.6	7.4	85	15	88.4	11.6
ciprofloxacin	83.8	16.2	93.1	6.9	89	11
amikacin	98,5	1.5	100	0	99.4	0.6

NI: Nosocomial infection; CAI: Community-acquired infection; n: Number; S: Susceptible; R: Resistant; [a]: include intermediate strains; [b]: P-value =0.013.

Table 3. Distribution of virulence factor genes in P. aeruginosa strains isolated from NI and CAI.

	Number of virulence factor genes				
	Five	**Four**	**Three**	**Two**	**One**
Strains from CAI n = 68	44 (64.7%)	20 (29.4%)	4 (5.9%)	0	0
Strains from NI n = 87	53 (60.9%)	28 (32.2%)	6 (6.9%)	0	0
Total n = 155	97 (62.6%)	48 (31%)	10 (6.5%)	0	0

NI: Nosocomial infections; CAI: Community-acquired infections; n: Number.

(99.4%) of *P. aeruginosa* isolates were susceptible to amikacin.

Virulence factors PCRs

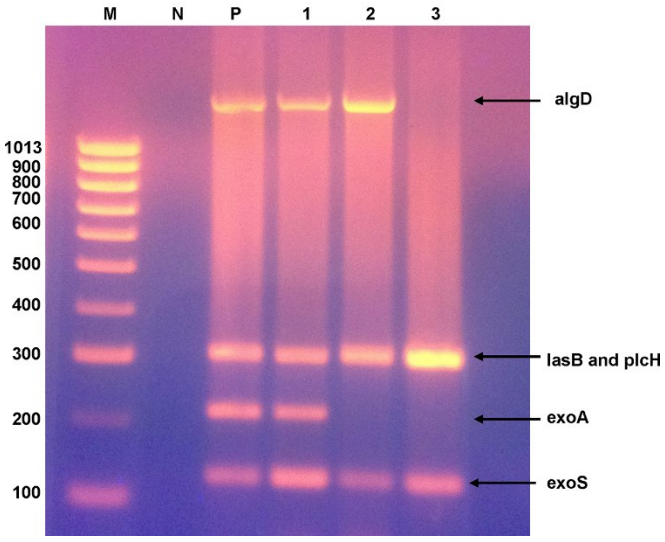
PCR analysis was used for the screening of five virulence encoding genes (*lasB*, *algD*, *plcH*, *exoA,* and *exoS*). The results highlighted that *lasB* (98.7%) and *exoS* (98.7%) were the most frequent virulence genes in *P. aeruginosa* strains, followed by *plcH* (96.1%), and *algD* (87.7%), while the least commonly detected virulence factor gene was *exoA* (74.2%).

No significant association was identified between the prevalence of virulence factor genes and the strains origin (P-value > 0.05). The prevalence of virulence factor genes between *P. aeruginosa* strains isolated from NI and CAI was shown in Figure 1. Moreover, the distribution of virulence factor genes between isolates showed that a total of 97 (62.6%) of *P. aeruginosa* strains carried the five virulence genes (Figure 2).

However, all isolates carried at least three virulence genes. No significant difference was detected by comparing the prevalence of virulence factor genes among *P. aeruginosa* strains isolated from NI and CAI, all P-value were >0.05 (Table 3).

A total of 23 (53.5%) of resistant *P. aeruginosa* isolates carried five virulence genes. No significant association was found between the presence of virulence genes and antibiotic resistance patterns of *P. aeruginosa* strains, all P-value were > 0.05 (Table 4).

Figure 2. PCR products of virulence genes in P. aeruginosa isolates.



M:molecular weight marker (N: negative control. P: Positive control for the five virulence genes (*P. aeruginosa* ATCC 27853). Lane 1: positive isolate for *algD* (1310bp), *lasB* (300bp), *plcH* (307bp), *exoA* (207bp), and *exoS* (118bp) genes. Lane 2: positive isolate for *algD*, *lasB*, *plcH*, and *exoS* genes. Lane 3: positive isolate for *lasB*, *plcH,* and *exoS* genes.

Table 4. Distribution of virulence factor genes among resistant and susceptible P. aeruginosa isolates.

	Susceptible isolates n = 112	**Resistant isolates n = 43**	**Total n = 155**
Virulence factor genes			
lasB	111(99%)	42(97.6%)	153(98.7%)
plcH	109(97%)	40(93%)	149(96.1%)
aLgD	99(88.4%)	37(86%)	136(87.7%)
exoA	85(75.9%)	30(69.7%)	115(74.2%)
exoS	110(98%)	43(100%)	153(98.7%)
Number of virulence factor genes			
1	0	0	0
2	0	0	0
3	7(6.2%)	3(7%)	10(6.5%)
4	31(27.7%)	17(39.5%)	48(31%)
5	74(66.1%)	23(53.5%)	97(62.6%)

n: Number.

Discussion

The present study is to our knowledge the first report about the profile of antimicrobial resistance to the commonly used antibiotics and the prevalence of several virulence factor genes among *P. aeruginosa* strains isolated from CAI and NI, in the Northwestern of Morocco.

According to antimicrobial susceptibility testing, a total of 154 of *P. aeruginosa* isolates were susceptible to amikacin (99.4%). Our results are consistent with work carried out previously by Mohammadzadeh *et al*. (2017). A similar study by Sarwat *et al*. (2015) revealed that (46.6%) of strains were resistant to ticarcillin, this result is in disagreement with the present study [20,21].

It was found that the rates of antibiotic resistance of *P. aeruginosa* were (27.1%) to aztreonam, (11%) to ciprofloxacin, and (5.8%) to ceftazidime, whereas Badamchi *et al*. (2017) reported that (27.1%) of *P. aeruginosa* were resistant to ciprofloxacin and (25.9%) to ceftazidime. Several surveys from developed and developing countries confirmed the direct link between the irrational antibiotics use and the emergence of resistant strains. To reduce this problem, it is very important to implement infection control measures such as good hand hygiene and judicious use of antimicrobial agents [22,23].

In addition, our results displayed decreasing susceptibility of *P. aeruginosa* isolates towards carbapenems (imipenem, meropenem, and doripenem). These findings are in accordance with the previous results of a study done by Gierhart *et al*. (2015) in which all strains exhibited significant decreases in susceptibility to doripenem and imipenem. However, our finding are dissimilar to Kireçci *et al*. (2014) showed that most of the isolates were susceptible to imipenem and meropenem [24,25]. Our study revealed that the resistance to imipenem was significantly higher in strains isolated from NI than strains isolated from CAI, because this antimicrobial agent is used mostly for treatment NI.

WHO recently put *P. aeruginosa* carbapenem-resistant on the list of antibiotic-resistant "priority pathogens", in order to lead and encourage research to discover new and effective antibiotics [26].

Our results reported that the level of resistance of *P. aeruginosa* isolated from CAI was not alarming, but it could increase over time.

In the community, the antimicrobial resistance was related to self-medication with antibiotics, which is common practice in Morocco. Therefore, it is necessary to raise awareness of the general public about the appropriate use of antimicrobial drugs and to improve the policy of sale of antibiotics in pharmacy stores so as to avoid the dissemination of *P. aeruginosa* MDR strains [27].

PCR assays were used for screening five virulence encoding genes (*lasB*, *algD*, *plcH*, *exoA,* and *exoS*) and results suggest that all strains isolated from NI and CAI harbored at least three of the virulence genes tested. As well, the results showed that *lasB* (98.7%) and *exoS* (98.7%) were the most frequent virulence genes in *P. aeruginosa* strains. These results are in line with Bradbury *et al*.(2010) who revealed that *lasB* and *exoS* genes were detected in (100%)of isolates [28].

In this survey the prevalence of *plcH* and *algD* genes was (96.1%) and (87.7%) respectively. These finding are similar to Lanotte *et al*. (2004) found that all strains were harbored *algD* and *plcH* genes [18].

Regarding this investigation, the least commonly detected virulence factor gene was *exoA* (74.2%). A similar finding by Wolska *et al*. (2009) reported that *exoA* gene was presented in (88.7%) of isolates [29].

The absence of some virulence factor genes in some strains may be due to the fact that the infection with this bacterium can be followed by genome reduction which can enhance its ability to persist in the host [30].

In this current research, the *P. aeruginosa* strains isolated from NI and CAI at hospital centers and clinical laboratories in Northwestern Morocco yielded very similar findings and there were no significant differences between strains with respect to the prevalence and the distribution of the virulence genes tested. These results are compatible to Pirnay *et al*. (2009) who found that the clinical *P. aeruginosa* strains were genotypically indistinguishable from environmental strains, so the isolates were highly conserved [31].

No significant difference was found between the presence of virulence factor genes and antibiotic susceptibility patterns. However, previous studies showed that a number of virulence factor genes could affect both virulence and antibiotic resistance. For example, Garey *et al*. (2008) reported that *P. aeruginosa* strains possessing the exoenzyme U gene were significantly more resistant to several antibiotics such as fluoroquinolones and carbapenems [13]. There is also, the transcriptional regulator *PsrA*, it plays a role in biofilm formation and intrinsic and adaptive antibiotic resistance. Another virulence factor is the sensor kinase *CbrA* which participates in acquired resistance and regulates biofilm development and swarming [32].

The *P. aeruginosa* strains isolated from CAI is rarely studied. Therefore, the main advantage of our

investigation was the collection of a set of strains isolated from NI and CAI in order to determine the antibiotic susceptibility patterns and the prevalence of virulence factor genes, and although most previous studies have focused separately on virulence or resistance, our report assessed the potential relationship between the distribution of virulence factor genes and antibiotic resistance profiles in *P. aeruginosa* strains. This survey provides data about phenotypic and genotypic characteristics of *P. aeruginosa* emerged in this region which could be useful for the health workers to improve infection control measures and to establish a surveillance system.

However, a number of potential limitations need to be considered. Firstly, the small number of strains obtained. Secondly, a retrospective study wasn't carried out because we couldn't collect patients' data like demographic characteristics, comorbidities, and other clinical information. Finally, our study didn't investigate the antimicrobial resistance mechanisms and their potential interplay with virulence factor genes, but, we will examine it in further studies.

Conclusion

In summary, the study provides an insight into the phenotypic and genotypic characteristics of *P. aeruginosa* emerged in the Northwestern of Morocco. Our finding highlighted a moderate rate of resistance to antibiotics. In order to reduce the risk of spread of highly resistant strains, we suggest the need to establish a periodic surveillance system, to enhance infection prevention and control measures in healthcare setting, and to increase awareness among physicians and the general public about the rational use of antibiotics. Future work should target mechanisms of resistance and diversity genetic of *P. aeruginosa* strains to draw more far-reaching conclusions.

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