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Original Article

Association of Biofilm Inducer with *bla*VIM, *blaIMP,* and *bla*NDM in *Pseudomonas aeruginosa* Isolates

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is a ubiquitous opportunistic organism that is hard to treat. This study aimed to investigate the association of *bla*VIM, *blaIMP,* and *bla*NDM prevalence with Cyclic di-GMP (c-di-GMP) in *P. aeruginosa*. To this end, 27 clinical isolates of *P. aeruginosa* were obtained from different hospitals in Baghdad, Iraq. The phenotypic detection of carbapenem and biofilm assays was performed by the M63 minimal medium, supplemented with glucose, magnesium sulfate. The polymerase chain reaction was utilized to detect carbapenem genes. The results showed that the isolates were highly resistant to Imipenem (37%) and Meropenem (63%). Imipenem (37%) and Meropenem (63%) demonstrated a moderate sensitivity against *P. aeruginosa*. The *P. aeruginosa* No.5 showed high resistance to carbapenem by *bla*VIM$^+$, *blaIMP*$^+$, and *bla*NDM$^+$, followed by a robust biofilm confirmed with c-di-GMP levels and the twitching motility ability. Upon these findings, the use of antibiotics should be restricted to severe bacterial infections to avoid the rapid emergence of new resistant isolates, which leads to the hard treatment of infection with *P. aeruginosa*. It is highly recommended that these findings be notified for infectious control. Future studies can investigate the link between transferable resistant genes and c-di-GMP values.

Keywords: Cyclic di-GMP, *bla*VIM, *blaIMP*, *bla*NDM, *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an important principled nosocomial pathogen. This organism is highly resistant to antimicrobials and causes difficulty in treating infections (1). Due to the small number of efficient antimicrobials as the intrinsic resistance, resistance is acquired through mutations and plasmids (2). The most important factor of resistance would be transferable resistance agents, such as Metallo-ß-lactamases (MβL) (3). It is well documented that carbapenem is pivotal to the treatment of MβL-resistant bacteria (4).

As a case of resistance genes, *bla*VIM, *blaIMP*, and *bla*NDM genes could be a plasmid or chromosomal region of class 1 integron used to encode *bla*VIM gene beside aminoglycoside-modifying genes. The global dissemination of MβL genes is considered a clinically important issue caused by integron. The emergence of isolates resistant to multiple antimicrobial agents is due to the ability of *P. aeruginosa* to merge variable resistance genes, which leads to strictly lowering the perspective treatment options of infections (5, 6).

The *P. aeruginosa* in a biofilm could tolerate 1,000-fold antibiotic concentrations when compared to the same bacteria in an individual, free-living, planktonic state (7). As a result, within these concentrations, it may not clear biofilm infections, allowing the bacterial population to persist, recover, and spread (8). The

biofilm formation, polysaccharide, and pellicle in *P. aeruginosa* are regulated by Cyclic di-GMP (c-di-GMP) (9). The c-di-GMP is a second messenger that promotes biofilm formation and contribution to pathogenic infection, as well as multidrug resistance in several Gram-negative pathogens. Therefore, the elevation of c-di-GMP in *P. aeruginosa* leads to the initial bacterial infection. On the other hand, low concentrations of c-di-GMP decrease the development of biofilm and infection (10). This study, therefore, attempted to investigate the association of bla_{VIM}, bla_{IMP}, and bla_{NDM} with the level of c-di-GMP in clinical isolates of *P. aeruginosa* that form strong biofilm.

2. Materials and Methods

2.1. Collection and Identification

Twenty-seven clinical isolates of *P. aeruginosa* were collected and diagnosed in this study. Bacteria were isolated from different infected patients at three major teaching hospitals in Baghdad (Al-Kindey, Al-Kadhymiaand, and Ibn-Albalady). Five isolates were obtained from individuals suffering from urinary tract infections, seven isolates from sputum patients suffering from respiratory tract infections, five isolates from burn infections, five isolates from wound infections, and five isolates from bacteremia. The criteria described previously were applied for bacterial identification (11).

2.2. Antibiotic Susceptibility Tests

An antimicrobial susceptibility test was conducted on all isolates using the disc diffusion method by the CLSI (12). The study used two types of carbapenem discs: Imipenem and Meropenem. After 18 h, the inhibition zone's diameter was measured, followed by its comparison with *P. aeruginosa* ATCC 27853 as the control strains.

2.3. Molecular Examination

The polymerase chain reaction (PCR) was used to detect three common carbapenems by a bacterial genomic DNA extraction kit (Bioneer, Daejeon, Korea). The oligonucleotide primer sequences used to detect the three genes were as follows: bla_{VIM} (390 bp, amplified size), by VIM-F 5′- GAT GGT GTT TGG TCG CAT A-3′ and VIM-R 5′- CGA ATG CGC AGC ACCAG-3′, bla_{IMP} (740bp, amplified size) by IMP-F 5′- TGA GCA AGT TAT CTG TAT TC -3′ and IMP-F 5′- TTA GTT GCT TGG TTT TGA TG -3′, as well as bla_{NDM} (621 bp, amplified size) by NDM-F5′- GGT TTG GCG ATC TGG TTT TC-3′ and NDM-R 5′- CGG AAT GGC TCA TCA CGA TC-3′, according to Wu, Lin (13), as well as Christensen, Simpson (14). Both positive and negative controls were used in the PCR.

2.4. Biofilm Quantification Assay

Biofilm formation assay was quantified as previously described by Musafer, Kuchma (15). The M63 minimal medium supplemented with glucose, Magnesium sulfate, and CAA was used to perform all biofilm assays.

2.5. Measurement of C-di-GMP

The c-di-GMP measurement was performed, according to the method previously described by Musafer (16). Within 18 h, the *P. aeruginosa* culture growth medium (1L of LB) was centrifuged at 250 rpm. Afterward, formaldehyde was added to prevent the degradation of c-di-GMP, and then, it was centrifuged for 10 min at 8,000 rpm at 4˚C. The nucleotide extraction was performed according to the method described by Amikam, Steinberger (17).

2.6. Statistical Analysis

Error bars±standard deviations of averages were measured for three independent experiments. Statistical analysis was performed using correlation coefficient (r) and LSD0.05 using the Microsoft EXCEL application (2016).

3. Results and Discussion

The sensitivity test for Imipenem and Meropenem antibiotics showed that the inhibition zone value of the resistant isolates was greater than the breakpoint, as defined by CLSI (12) while it would be susceptible if it was less than the breakpoint. The results in table 1 clearly showed that 27 isolates of *P. aeruginosa* were

tested for carbapenem susceptibility, including Imipenem and Meropenem discs, which had a different range of resistance. Table 1 showed that No.6, No.19, and No.21 of *P. aeruginosa* isolates were resistant to Imipenem antibiotic whereas *P. aeruginosa* No.5, No.7, No.12, No.13, and No.19 were resistant to Meropenem antibiotic. The remarkable point is that *P. aeruginosa* No.19 showed resistance to both Imipenem and Meropenem. As Riera, Cabot (18) indicated in their study, the resistance of clinical isolates may contribute to the impermeability resistance, modification enzyme coded by plasmid or chromosome, the mutation in efflux pump that uptakes antibiotic, new carbapenem resistance mechanism, and target side changing for the antibiotic action. Resistance to Imipenem and Meropenem acquired by MβL genes are located on mobile genetic elements, such as plasmids or transposons, thereby enabling widespread dissemination (19).

Table 1. The carbapenem sensitivity test results *P. aeruginosa* isolates

Clinical Isolates	Clinical Source	Imipenem			Meropenem		
		S	**I**	**R**	**S**	**I**	**R**
		23	**20-22**	**19**	**23**	**20-22**	**19**
PAO1 Wild type	Wild type	-	-	-	-	-	-
P. aeruginosa#1	Sputum	-	21	-	25	-	-
P. aeruginosa#2		-	22	-	30	-	-
P. aeruginosa#3		-	22	-	26	-	-
P. aeruginosa#4		-	22	-	30	-	-
P. aeruginosa#5		-	20	-	-	-	No I.Z
P. aeruginosa#6	Urine	-	-	19	-	20	-
P. aeruginosa#7		24	-	-	-	-	15
P. aeruginosa#8		25	-	-	25	-	-
P. aeruginosa#9		23	-	-	25	-	-
P. aeruginosa#10		-	21	-	23	-	-
P. aeruginosa#11		-	22	-	30	-	-
P. aeruginosa#12		23	-	-	-	-	18
P. aeruginosa#13		26	-	-	-	-	18
P. aeruginosa#14		25		-	30	-	-
P. aeruginosa#15			21	-	32	-	-
P. aeruginosa#16		25	-	-	34	-	-
P. aeruginosa#17			22	-		20	-
P. aeruginosa#18		34	-	-	24	-	-
P. aeruginosa#19	Blood	-	-	14	-	-	No I.Z
P. aeruginosa#20		-	22	-	30	-	-
P. aeruginosa#21	Burn	-	-	18	-	21	--
P. aeruginosa#22		-	22	-	30	-	-
P. aeruginosa#23	Wound	-	20	-	-	21	-
P. aeruginosa#24		-	20	-	-	22	-
P. aeruginosa#25		-	20	-	25	-	-
P. aeruginosa#26		23	-	-	30	-	-
P. aeruginosa#27		24	-	-	30	-	-
		37%	51%	11%	62.9%	18.5%	18.5%

R, resistant; **I**, intermediate; **S**, sensitive; **I.Z**, Inhibition zone

As shown in table 2, there are two main findings. First, all carbapenem-resistant isolates showed a positive prevalence of bla_{VIM}. Second, *P. aeruginosa* No.5, No.7, No.12, No.13, and No.19 were bla_{VIM}, bla_{IMP}, and bla_{NDM} positive, resistant to Meropenem, and sensitive to Imipenem. On the other hand, *P. aeruginosa* No.21 and No.1 were negative for bla_{IMP} and bla_{NDM}. The findings have been compared to the *P. aeruginosa* (wild type) standard strain for *P. aeruginosa*. These results were in agreement with previous studies mentioning that resistance to Meropenem was related to Metallo-carbapenems encoded by carbapenem genes, and resistance to Imipenem antibiotic alone was related to porins gene (20).

The present study found that the biofilm producer in *P. aeruginosa* No.5, No.7, No.*12*, No.13, and No.19 had all carbapenem genes bla_{VIM}^{+}, bla_{IMP}^{+}, and bla_{NDM}^{+} positive, compared to *P. aeruginosa* No.6, No.11, No.26, and No.18 with negative carbapenem genes. The correlation coefficient (r) was estimated at 0.96, meaning there is a strong positive correlation between the biofilm formation values and the presence of bla_{VIM}^{+}, bla_{IMP}^{+}, and bla_{NDM}^{+} genes, as shown in table 3. These findings were in line with the findings of a study by Drenkard and Ausubel (21), pinpointing that the antibiotic-resistant variants of phenotypic *P. aeruginosa* could promote biofilm formation in the lungs of cystic fibrosis patients and in *in vitro*. The horizontal gene transmission within bacteria turns into a biofilm producer and multidrug resistance mechanisms against beta-lactam, aminoglycosides, and fluoroquinolones antibiotics (22).

Table 2. Carbapenem resistance pattern and the existence of related genes

Isolates	Carbapenem pattern		bla_{VIM}	bla_{IMP}	bla_{NDM}
PAO1	IPM-S	MEM-S	-	-	-
P. aeruginosa#5	IPM-I	MEM-R	+	+	+
P. aeruginosa#7	IPM-S	MEM-R	+	+	+
P. aeruginosa#12	IPM-S	MEM-R	+	+	+
P. aeruginosa#13	IPM-S	MEM-R	+	+	+
P. aeruginosa#19	IPM-R	MEM-R	+	+	+
P. aeruginosa#21	IPM-R	MEM-I	+	-	-
P. aeruginosa#1	IPM-I	MEM-S	+	-	-
Percentage	57%	86%	100%	71%	71%

PAO1: *Pseudomonas aeruginosa*, IPM-S: Imipenem-Sensitive, IPM-I: Imipenem-Intermediate, IPM-R: Imipenem-Resistance, MEM-S: Meropenem-Sensitive, MEM-I: Meropenem-Intermediate, MEM-R: Meropenem-Resistance, *P. aeruginosa*: *Pseudomonas aeruginosa*

Table 3. Biofilm formation and carbapenem genes of *Pseudomonas aeruginosa* isolates

Isolates No.	Biofilm values ($A_{490} \pm SD^{b}$)	Carbapenem gene patterns
PAO1	0.11±0.0169	bla_{VIM}^{-}, bla_{IMP}^{-}, bla_{NDM}^{-}
P. aeruginosa#5	0.92±0.0124	bla_{VIM}^{+}, bla_{IMP}^{+}, bla_{NDM}^{+}
P. aeruginosa#7	0.92±0.0141	bla_{VIM}^{+}, bla_{IMP}^{+}, bla_{NDM}^{+}
P. aeruginosa#12	0.85±0.0249	bla_{VIM}^{+}, bla_{IMP}^{+}, bla_{NDM}^{+}
P. aeruginosa#13	0.85±0.0205	bla_{VIM}^{+}, bla_{IMP}^{+}, bla_{NDM}^{+}
P. aeruginosa#19	0.80±0.0169	bla_{VIM}^{+}, bla_{IMP}^{+}, bla_{NDM}^{+}
P. aeruginosa#21	0.53±0.0163	bla_{VIM}^{+}, bla_{IMP}^{-}, bla_{NDM}^{-}
P. aeruginosa#1	0.54±0.0124	bla_{VIM}^{+}, bla_{IMP}^{-}, bla_{NDM}^{-}
P. aeruginosa#6	0.35±0.0163	bla_{VIM}^{-}, bla_{IMP}^{-}, bla_{NDM}^{-}
P. aeruginosa#11	0.36±0.0235	bla_{VIM}^{-}, bla_{IMP}^{-}, bla_{NDM}^{-}
P. aeruginosa#26	0.37±0.0216	bla_{VIM}^{-}, bla_{IMP}^{-}, bla_{NDM}^{-}
P. aeruginosa#18	0.24±0.0518	bla_{VIM}^{-}, bla_{IMP}^{-}, bla_{NDM}^{-}

P. aeruginosa: Pseudomonas aeruginosa
PAO1: *Pseudomonas aeruginosa*

Concentrations of the cellular c-di-GMP of *P. aeruginosa* were measured as follows: *P. aeruginosa* No.5, No.11, and No.26 were measured by high performance liquid chromatography (HPLC), as stated previously by Musafer (16). Figure 1 shows c-di-GMP levels (0.02 pmol/mg) in standard *P. aeruginosa*. The lowest c-di-GMP levels (0.007pmol/mg and 0.008 pmol/mg) in *P. aeruginosa* were found in No.5 and No.26, respectively, whereas the highest level was 0.03 pmol/mg in *P. aeruginosa* No. 5. The finding confirmed that the high biofilm (bla_{VIM}^{+}, bla_{IMP}^{+}, and bla_{NDM}^{+}) exists in *P. aeruginosa* No.5. In addition, the experiment on twitching motility confirmed the results of high biofilm since twitching is an important step in biofilm formation (15). Based on the findings, this study showed significant levels of c-di-GMP in isolates with a high biofilm producer and the three carbapenem genes in *P. aeruginosa* clinical isolates. These results are in agreement with the findings of Musafer (16) on c-di-GMP.

Imipenem (37%) and Meropenem (63%) demonstrated a moderate sensitivity to *P. aeruginosa*, as a clinically important antibiotic. The *P. aeruginosa* No.5 showed high resistance to carbapenem by bla_{VIM}^{+}, bla_{IMP}^{+}, and bla_{NDM}^{+}, followed by a robust biofilm that confirmed c-di-GMP levels and the twitching motility ability (Figure 2). Upon these findings, using antibiotics should be restricted to severe bacterial infections to avoid the rapid emergence of new resistant isolates, which leads to the hard treatment of infection with *P. aeruginosa*. It is highly recommended that these findings be notified by infectious control departments. Future studies can investigate the link between transferable resistance genes and c-di-GMP values.

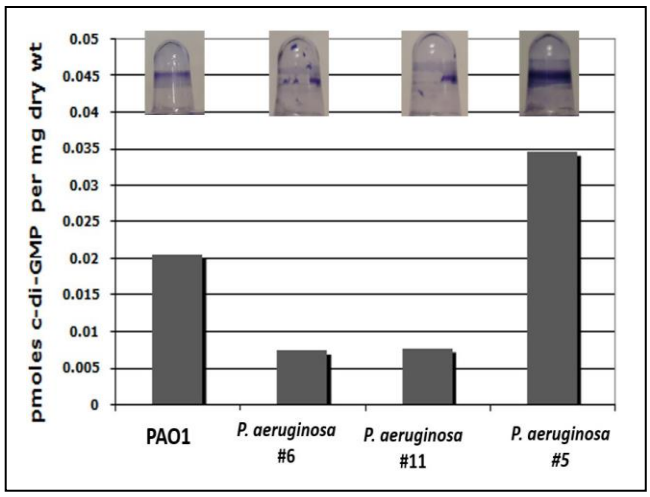

Figure 1. Cellular c-di-GMP's quantification and concentrations by HPLC from 30 mg of cells. A significant difference was considered when $P<0.05$

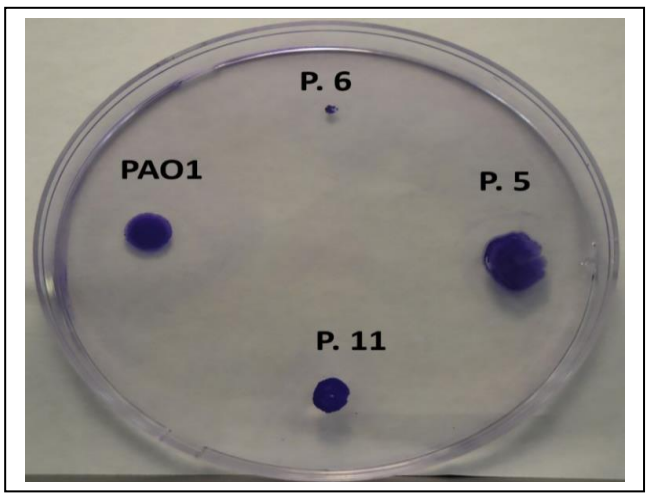

Figure 2. Explanation of the ability of *Pseudomonas aeruginosa* with a strong biofilm of twitching motility

Authors' Contribution

Study concept and design: H. K. M. and F. N. J.
Acquisition of data: H. K. M.
Analysis and interpretation of data: M. B. A. A.
Drafting of the manuscript: M. B. A. A.
Critical revision of the manuscript for important intellectual content: H. K. M. and F. N. J.
Statistical analysis: M. B. A. A.
Administrative, technical, and material support: H. K. M.

Ethics

The study protocol was approved by the medical ethics board of the Al-Mustansiriyah University, Ministry of Higher Education and Research, Baghdad, Iraq. Written informed consents were provided by all the subjects participated in the study

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Obritsch MD, Fish DN, MacLaren R, Jung R. National surveillance of antimicrobial resistance in Pseudomonas aeruginosa isolates obtained from intensive care unit patients from 1993 to 2002. Antimicrob Agents Chemother. 2004;48(12):4606-10.
2. Khan W, Bernier SP, Kuchma SL, Hammond JH, Hasan F, O'Toole GA. Aminoglycoside resistance of Pseudomonas aeruginosa biofilms modulated by extracellular polysaccharide. Int Microbiol. 2010;13(4):207.
3. Gutiérrez O, Juan C, Cercenado E, Navarro F, Bouza E, Coll P, et al. Molecular epidemiology and mechanisms of carbapenem resistance in Pseudomonas aeruginosa isolates from Spanish hospitals. Antimicrob Agents Chemother. 2007;51(12):4329-35.
4. Jesudason MV, Kandathil A, Balaji V. Comparison of two methods to detect carbapenemase & metallo-beta-lactamase production in clinical isolates. Indian J Med Res. 2005;121(6):780.
5. Henrichfreise B, Wiegand I, Sherwood KJ, Wiedemann B. Detection of VIM-2 metallo-β-lactamase in Pseudomonas aeruginosa from Germany. Antimicrob Agents Chemother. 2005;49(4):1668-9.
6. Viedma E, Juan C, Villa J, Barrado L, Orellana MÁ, Sanz F, et al. VIM-2–producing multidrug-resistant Pseudomonas aeruginosa ST175 clone, Spain. Emerg Infect Dis. 2012;18(8):1235.
7. Høiby N. Prospects for the prevention and control of pseudomonal infection in children with cystic fibrosis. Paediatr Drugs. 2000;2(6):451-63.
8. Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg E. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature. 2000;407(6805):762-4.
9. Sakuragi Y, Kolter R. Quorum-sensing regulation of the biofilm matrix genes (pel) of Pseudomonas aeruginosa. J Bacteriol. 2007;189(14):5383-6.
10. Cole SJ, Lee VT. Cyclic di-GMP signaling contributes to Pseudomonas aeruginosa-mediated catheter-associated urinary tract infection. J Bacteriol. 2015;198(1):91-7.
11. Atlas RM, Brown AE, Parks LC. Laboratory manual of experimental microbiology: Mosby; 1995.
12. CLSI. Performance standard for antimicrobial susceptibility testing. 2016.
13. Wu M-C, Lin T-L, Hsieh P-F, Yang H-C, Wang J-T. Isolation of genes involved in biofilm formation of a Klebsiella pneumoniae strain causing pyogenic liver abscess. PloS One. 2011;6(8): 23500.
14. Christensen GD, Simpson WA, Younger J, Baddour L, Barrett F, Melton D, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol. 1985;22(6):996-1006.
15. Musafer HK, Kuchma SL, Naimie AA, Schwartzman JD, Al-Mathkhury HJF, O'Toole GA. Investigating the link between imipenem resistance and biofilm formation by Pseudomonas aeruginosa. Microb Ecol. 2014;68(1):111-20.
16. Musafer H. Anti-Biofilm Strategy: Meropenem Modulates Biofilm Formation in Acinetobacter lwoffii. Pakistan J Biotechnol. 2018;15(4):879-82.
17. Amikam D, Steinberger O, Shkolnik T, Ben-Ishai Z. The novel cyclic dinucleotide 3′-5′ cyclic diguanylic acid binds to p21 ras and enhances DNA synthesis but not cell replication in the Molt 4 cell line. Biochem J. 1995;311(3):921-7.
18. Riera E, Cabot G, Mulet X, García-Castillo M, del Campo R, Juan C, et al. Pseudomonas aeruginosa carbapenem resistance mechanisms in Spain: impact on the activity of imipenem, meropenem and doripenem. J Antimicrob Chemother. 2011;66(9):2022-7.
19. Enwuru N, Enwuru C, Ogbonnia S, Adepoju-Bello A. Metallo-β-lactamase production by Escherichia coli and Klebsiella species isolated from hospital and community subjects in Lagos, Nigeria. Nat Sci. 2011;9(11):1-5.
20. Sardelic S, Pallecchi L, Punda-Polic V, Rossolini GM. Carbapenem-Resistant Pseudomonas aeruginosa–Carrying VIM-2 Metallo-β-Lactamase Determinants, Croatia. Emerg Infect Dis. 2003;9(8):1022.
21. Drenkard E, Ausubel FM. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature. 2002;416(6882):740-3.
22. Potera C. Forging a link between biofilms and disease. American Association for the Advancement of Science; 1999.