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Correlation between antibiotic resistance and virulence of *Pseudomonas aeruginosa* clinical isolates

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Background/aim: Virulent *Pseudomonas aeruginosa* is frequently life-threatening and often challenging to treat, and the emergence of multidrug-resistant isolates presents a critical problem for patients. The aim of the study was concerned with molecular analysis of the virulence factors and antimicrobial resistance profile of multidrug-resistant *P. aeruginosa* (MDRPA).

Materials and methods: Out of 44 MDRPA isolates, 12 isolates representing different resistance profiles and sources of samples were selected for further molecular studies. Polymerase chain reaction (PCR) approaches were applied to identify the genes implicated in antimicrobial resistance or virulence factors in the selected MDRPA isolates.

Results: Multidrug-resistance (*pstS*), β-lactamase (*IMP7*, *IMP10*, *IMP13*, and *IMP25*), and extended spectrum β-lactamase (*blaOXA50*) genes were detected in all of the selected MDRPA isolates. However, only 4 (33%) MDRPA isolates were positive for the presence of the extended spectrum β-lactamase (*blaOXA2*) gene. Furthermore, the hemolytic phospholipase C precursor gene (*plcH*) was detected in all PCR products of the tested MDRPA isolates while the exotoxin A (*toxA*) gene was absent. Other virulence genes were detected with variable percentage in tested isolates.

Conclusion: The statistical analysis revealed a significantly positive correlation (r = 0.779, P = 0.002) between virulence factors and antimicrobial resistance marker profiles of the tested MDRPA isolates.

Key words: Multidrug-resistant *Pseudomonas aeruginosa*, antibiotic, virulence factors, genes, polymerase chain reaction

1. Introduction

The increasing frequency of multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) strains is concerning, as efficacious antimicrobial options are severely limited. Risk factors for MDRPA infection include prolonged hospitalization, exposure to antimicrobial therapy, and immunocompromised states such as human immunodeficiency virus infection. The emergence of MDRPA isolates during therapy was reported in 27%–72% of patients with initially susceptible *P. aeruginosa* isolates. Patients with severe MDRPA infections should be treated with combination therapy, consisting of an antipseudomonal β-lactam with an aminoglycoside or fluoroquinolone, rather than aminoglycoside and fluoroquinolone combinations, to provide adequate therapy and improve patient outcomes (1).

P. aeruginosa produces a number of virulence factors, which, after colonization, can cause extensive tissue damage, bloodstream invasion, and dissemination (2). Pathogenesis is based on multiple virulence factors: endotoxin, exotoxins, and enzymes. Its endotoxin, like that of other gram-negative bacteria, causes the symptoms of sepsis and septic shock (3). Most strains of *P. aeruginosa* produce 2 exotoxins, exotoxin A and exoenzyme S; a variety of cytotoxic substances including phospholipases, pyocyanin, and proteases; and an alginate-like exopolysaccharide that is responsible for the mucoid phenotype. The importance of these putative virulence factors depends upon the site and nature of infection. Proteases play a key role in corneal ulceration, are important in burn infection, and are associated with chronic pulmonary colonization (4). Chitinase produced by *P. aeruginosa* has been investigated as a virulence factor associated with plant diseases (5). Folders et al. (6) reported that ChiC chitinase was produced by clinical isolates of *P. aeruginosa*. These virulence factors help the bacteria

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adhere to and invade their host by damaging the host's immune responses and forming a barrier to antibiotics. Cell-associated and secreted virulence factors are encoded on plasmids or chromosomal genes (7).

The present study is concerned with molecular analysis of the virulence factors and antimicrobial resistance profiles of MDRPA. Polymerase chain reaction (PCR) approaches were applied to plasmid DNA to identify genes implicated in antimicrobial resistance or virulence factors of the selected isolates. Finally, the obtained data were subjected to statistical analysis in order to study the correlation between antimicrobial resistance and virulence factors exhibited by the tested isolates.

2. Material and methods

2.1. Bacterial isolates

A total of 104 antibiotic-resistant *Pseudomonas aeruginosa* clinical isolates were recovered and identified as previously explained by Khalil et al. (8). In brief, these isolates were screened for their susceptibility to 25 antimicrobial agents using the disk agar diffusion method (a modified Kirby–Bauer method) on Mueller-Hinton agar media following the zone diameter criteria recommended by the Clinical Laboratory Standards Institute (CLSI) (9). MDRPA isolates were selected on the basis described by Rossolini and Mantengoli (10), who defined MDRPA as resistance of the isolates to at least 3 of 6 drugs, including amikacin, gentamycin, ciprofloxacin, piperacillin, ceftazidime, and imipenem.

2.2. Isolation of plasmid DNA

The selected MDRPA isolates were subjected to the alkaline lyses method described by Sambrook et al. (11) for total plasmid DNA isolation. The collected plasmid DNA pellets were subjected to gel electrophoresis. O'GeneRuler DNA Ladder Mix (100 bp or 1 kbp) was also applied, and the gel was run at 80 V for 30 min and then photographed.

2.3. PCR detection of antibiotic resistance-associated genes

The PCR technique was applied to plasmid DNA extract of the tested MDRPA isolates in order to identify the genes implicated in their antimicrobial resistance mechanisms. The tested genes were encoded multidrug-resistance (*pstS*), β-lactamase (*IMP7*, *IMP10*, *IMP13*, and *IMP25*) and extended spectrum β-lactamase (*blaOXA50* and *blaOXA2*) genes. Genes were amplified using the specific primers listed in Table 1 (12). DNA was amplified with the following protocol: initial denaturation at 94 °C for 4 min, followed by 30 cycles of 1 min each at 94 °C, 1 min at 55–60 °C, and 1 min at 72 °C, with a final extension step at 72 °C for 10 min. PCR products were subjected to agarose gel electrophoresis in the presence of the O'GeneRuler DNA Ladder.

2.4. PCR analysis of the virulence genes of tested MDRPA isolates

The presence of several virulence-associated genes encoding GDP mannose 6-dehydrogenase (alginate) (*algD*) (13), type IV fimbrial biogenesis protein PilB (*pilB*) (14), neuraminidase (*nan1*) (14), elastase LasB (*LasB*) (13), hemolytic phospholipase C precursor (*plcH*) (13),

Table 1. Primers used for amplification of resistance-associated genes.

Target gene	Nucleotide sequence of primers	Amplicon size (bp)
IMP7	3′-AAGGCAGTATCTCCTCTCATTTTC-5′ 5′-ACTCTATGTTCAGGTAGCCAAACC-3′	243
IMP10	3′-AATGCTGAGGCTTACCTAATTGAC-5′ 5′-CCAAGCTTCTATATTTGCGTCAC-3′	388
IMP13	3′-AGACGCCTATCTAATTGACACTCC-5′ 5′-CCACTAGGTTATCTTGAGTGTGACC-3′	311
IMP25	3′-GCAGTATTTCCTCACATTTCCATAG-5′ 5′-TCACCCAAATTACCTAGACCGTAG-3′	295
pstS	3′-CTTGAAGGGACTCGACAAGG-5′ 5′-TTCAGGTCCGCGTAGTGAAT-3′	606
blaOXA50	3′-GAAAGGCACCTTCGTCCTCTAC-5′ 5′-CAGAAAGTGGGTCTGTTCCATC-3′	400
blaOXA2	3′-ATACACTTTTTGCACTTGATGCAG-5′ 5′-TGAAAAGATCATCCATTCTGTTTG-3′	510

nonhemolytic phospholipase C precursor (*plcN*) (15), exoenzyme S (*exoS*) (13), exoenzyme U (*exoU*) (14), and exotoxin A (*toxA*) (15) in tested MDRPA isolates was assessed by PCR amplification. The virulence genes were amplified using plasmid DNA extract with the specific primers listed in Table 2.

The DNA was amplified using the following protocol: initial denaturation (94 °C for 5 min), followed by 25–30 cycles of denaturation (94 °C for 35–45 s), annealing (53–62 °C from 45 s to 1 min), and extension (72 °C from 45 s to 1 min), with a single final extension of 7 min at 72 °C. PCR products were subjected to agarose gel electrophoresis in the presence of the O'GeneRuler DNA Ladder. Amplified genes were identified on the basis of fragment sizes shown in Table 2.

2.5. Statistical analysis

Statistical presentation and analysis of the present study, including linear correlation coefficients and analysis of variance (ANOVA) tests, was conducted by SPSS 17. In all cases, a P-value was considered indicative of significance if it was equal to or less than 0.05. The obtained data were statistically presented as mean ± standard deviation.

3. Results

Out of 44 MDRPA isolates, 12 isolates representing different resistance profiles and sources of samples were selected for further molecular studies. Plasmid DNA analysis showed that all tested isolates harbored 2 plasmids of molecular sizes 10 and 15 kbp. Table 3 shows the antimicrobial resistance patterns and plasmids profiles of the selected MDRPA isolates.

3.1. PCR detection of some antibiotic resistance genes in tested MDRPA isolates

As observed in Figures 1a–1d, gel electropherograms of the PCR products of all tested MDRPA isolates were positive for IMP genes. This PCR amplification gave an amplicon size of 243 bp, 388 bp, 311 bp, and 295 bp corresponding to the *IMP7*, *IMP10*, *IMP13*, and *IMP25* resistance genes, respectively. PCR amplification to detect the presence of *blaOXA50* and *blaOXA2* genes using a set of *OXA50* and *OXA2* primers gave amplicon sizes of 400 bp and 510 bp, respectively (Figures 1e and 1f). Detected DNA bands indicated that all isolates were positive for the *OXA50* gene. However, only 4 of the 12 MDRPA isolates were positive for the presence of the *OXA2* gene. Interestingly, an amplicon of molecular size 606 bp, corresponding to

Table 2. Primers used for amplification of virulence-associated genes.

Target gene	Nucleotide sequence of primers	Amplicon size (bp)
algD	3′-ATGCGAATCAGCATCTTTGGT-5′ 5′-CTACCAGCAGATGCCCTCGGC-3′	1310
pilB	3′-ATGAACGACAGCATCCAACT-5′ 5′-GGGTGTTGACGCGAAAGTCGAT-3′	826
nan1	3′-ATGAATACTTATTTTGATAT-5′ 5′-CTAAATCCATGCTCTGACCC-3′	1317
lasB	3′-GGAATGAACGAGGCGTTCTC-5′ GGTCCAGTAGTAGCGGTTGG-3′	300
exoS	3′-CTTGAAGGGACTCGACAAGG-5′ 5′-TTCAGGTCCGCGTAGTGAAT-3′	504
exoU	3′-GGGAATACTTTCCGGGAAGTT-5′ 5′-CGATCTCGCTGCTAATGTGTT-3′	428
toxA	3′-CTGCGCGGGTCTATGTGCC-5′ 5′-GATGCTGGACGGGTCGAG-3′	270
plcN	3′-TCCGTTATCGCAACCAGCCCTACG-5′ 5′-TCGCTGTCGAGCAGGTCGAAC-3′	481
plcHI	3′-GAAGCCATGGGCTACTTCAA-5′ 5′-AGAGTGACGAGGAGCGGTAG-3′	307
plcHII	3′-GCACGTGGTCATCCTGATGC-5′ 5′-TCCGTAGGCGTCGACGTAC-3′	608

Table 3. Resistance patterns and plasmid contents of MDRPA isolates.

Isolate code	Resistance patterns*
PA1	AMP, AX, FEP, K, AMC, TIM, ZOX, C, N, PRL, CAZ, CTX, CRO, CFP, ATM, SXT, TE, CN, AK, S, CT
PA4	AMP, AX, FEP, K, AMC, TIM, ZOX, C, N, PRL, CAZ, CTX, CRO, CFP, IPM, MEM, ATM, CIP, SXT, TE, CN, S, TOB, CT
PA6	AMP, AX, FEP, K, AMC, TIM, ZOX, C, N, PRL, CAZ, CTX, CRO, CFP, IPM, ATM, SXT, TE, AK, S, CT
PA17	AMP, AX, FEP, K, AMC, TIM, ZOX, C, N, PRL, CAZ, CTX, CRO, CFP, IPM, MEM, ATM, CIP, TE, CN, AK, TOB
PA18	AMP, AX, FEP, K, AMC, TIM, ZOX, C, N, PRL, CAZ, CTX, CRO, CFP, IPM, MEM, ATM, CIP, SXT, TE, CN, AK, S, TOB, CT
PA27	AMP, AX, FEP, K, AMC, TIM, ZOX, C, N, PRL, CAZ, CTX, CRO, CFP, IPM, MEM, ATM, SXT, TE, S, TOB, CT
PA34	AMP, AX, FEP, K, AMC, TIM, ZOX, C, N, CAZ, CTX, CRO, CFP, IPM, MEM, ATM, SXT, TE, CN, S, CT
PA55	AMP, AX, FEP, K, AMC, TIM, ZOX, C, N, CAZ, CTX, CRO, CFP, MEM, ATM, SXT, TE, CN, AK, S
PA60	AMP, AX, FEP, K, AMC, TIM, ZOX, C, N, CTX, CRO, CFP, IPM, SXT, TE, CN, AK, S
PA63	AMP, AX, FEP, K, AMC, TIM, ZOX, C, N, PRL, CAZ, CTX, CRO, CFP, IPM, ATM, CIP, SXT, TE, CN, S, TOB
PA66	AMP, AX, FEP, K, AMC, TIM, ZOX, C, N, PRL, CAZ, CTX, CRO, CFP, IPM, MEM, ATM, CIP, SXT, TE, CN, S, CT
PA102	AMP, AX, FEP, K, AMC, TIM, ZOX, C, N, CAZ, CTX, CRO, CFP, IPM, ATM, SXT, TE, S, CT

*AMP, ampicillin; AX, amoxicillin; PRL, piperacillin; AMC, amoxicillin/clavulanic acid; TIM, ticarcillin/clavulanic acid; CAZ, ceftazidime; FEP, cefepime; CTX, cefotaxime; CRO, ceftriaxone; ZOX, ceftizoxime; CFP, cefoperazone; IPM, imipenem; MEM, meropenem; ATM, aztreonam; CIP, ciprofloxacin; SXT, co-trimoxazole; TE, tetracycline; C, chloramphenicol; CN, gentamicin; AK, amikacin; N, neomycin; S, streptomycin; TOB, tobramycin; K, kanamycin; CT, colistin sulfate.

pstS (multidrug-resistance gene), was also detected in all 12 representative MDRPA isolates, as shown in Figure 1g.

3.2. Detection of some virulence genes in the selected MDRPA isolates using PCR

Figures 2a–2i illustrate the profiling of the amplification products of different virulence genes in each of the 12 tested MDRPA isolates.

As shown in Figure 2b, *plcH* II was detected in all PCR products of the tested MDRPA isolates, while the *toxA* virulence gene was absent. Other virulence genes, namely *pilB*, *plcH*, *plcN*, and *algD*, were detected in 11 of 12 MDRPA isolates (Figures 2a, 2c, 2h, and 2i, respectively). The *LasB* virulence gene was detected in the amplicons of 9 out of 12 MDRPA isolates (Figure 2e). On the other hand, PCR amplification of the *nan1* gene in the DNA of 12 MDRPA isolates was positive in only 4 isolates: PA4, PA6, PA60, and PA63 (Figure 2d). Another 2 virulence genes, *exoS* and *exoU*, responsible for the production of exoenzymes S and U, respectively, were detected in 10 of the screened MDRPA isolates as shown in Figures 2f and 2g.

3.3. Correlation between virulence factors and antimicrobial resistance marker profiles of the tested MDRPA isolates

Table 4 summarizes the virulence factors and antimicrobial resistance markers of 12 MDRPA isolates. Statistical analysis using Pearson's correlation coefficient between virulence factors and antimicrobial resistance markers was performed. A positive significant correlation was found between virulence factor production and resistance to antimicrobial agents ($r = 0.779$, $P = 0.002$), as shown in Figure 3.

4. Discussion

The development of multidrug-resistance by *P. aeruginosa* isolates requires several different genetic events, including the acquisition of different mutations and/or horizontal transfer of antibiotic resistance genes (12). The implication of plasmids in antibiotic resistance has been previously reported by several studies (16,17). In our study, the PCR technique was applied to plasmid DNA of the tested MDRPA isolates in order to identify the genes implicated in their expressed antimicrobial resistance phenotypes.

β-Lactamases are the most common and most important mechanism of resistance to β-lactam antibiotics as they are capable of hydrolyzing the 4 members of the β-lactam class of antibiotics including penicillins, cephalosporins, monobactams, and carbapenems. These β-lactamases may be plasmid-mediated or chromosomally mediated (18). β-Lactamases can be divided into 4 classes (A, B, C, and D) according to their sequence similarities (19). On the basis of their catalytic mechanisms, 2 groups have been established; the class B enzymes are metallo-β-lactamases

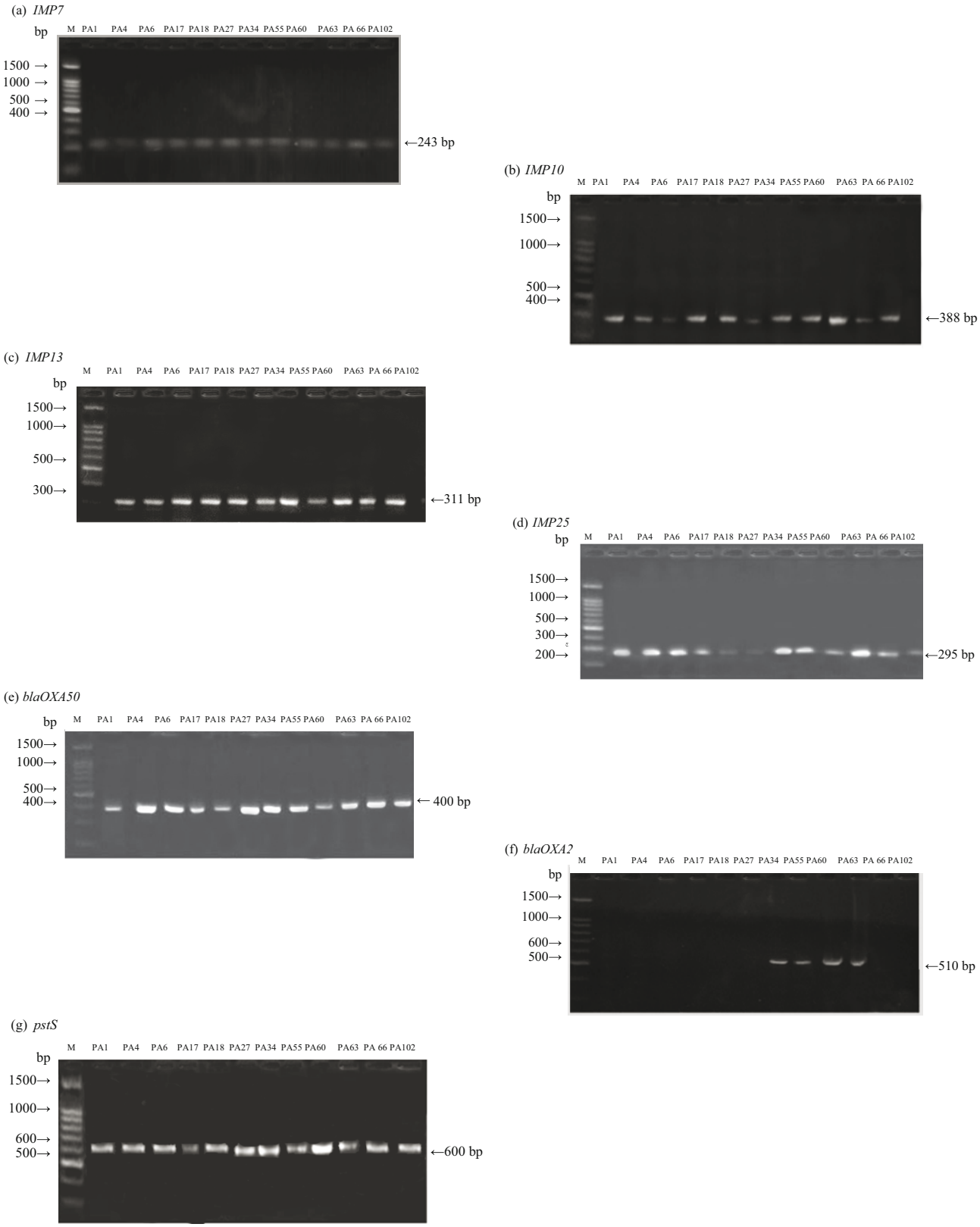

Figure 1. PCR detection of antibiotic-resistant genes of 12 MDRPA isolates: a) *IMP7*, b) *IMP10*, c) *IMP13*, d) *IMP25*, e) *blaOXA50*, f) *blaOXA2*, g) *pstS*. M: Molecular size marker (100 bp DNA ladder). PA: *Pseudomonas aeruginosa* isolate code.

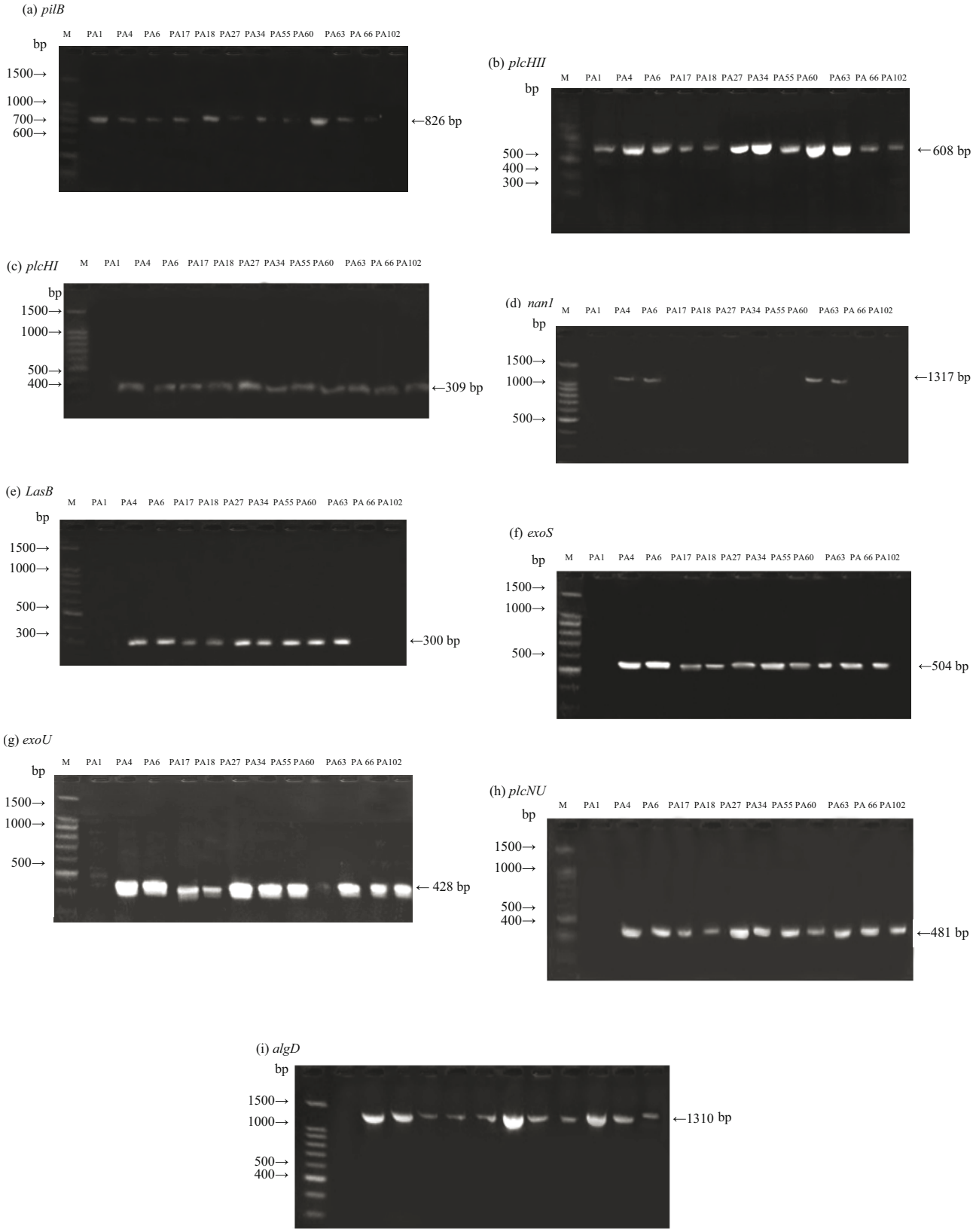

Figure 2. PCR detection of virulence genes of 12 MDRPA isolates: a) *pilB*, b) *plcHII*, c) *plcHI*, d) *nan1*, e) *LasB*, f) *exoS*, g) *exoU*, h) *plcN*, i) *algD*. M: Molecular size marker (100 bp DNA ladder). PA: *Pseudomonas aeruginosa* isolate code.

Table 4. Virulence factors and resistance patterns of 12 MDRPA isolates.

Sample source		Isolate code											
		PA1	PA4	PA6	PA17	PA18	PA27	PA34	PA55	PA60	PA63	PA66	PA102
		Sputum	Burn	Burn	Urine	Urine	Sputum	Burn	Sputum	Sputum	Sputum	Sputum	Blood
Virulence genes	*pilB*	+	+	+	+	+	+	+	+	+	+	+	-
	plcH (II)	+	+	+	+	+	+	+	+	+	+	+	+
	plcH (I)	-	+	+	+	+	+	+	+	+	+	+	+
	nan1	-	+	+	-	-	-	-	-	+	+	-	-
	LasB	-	+	+	+	+	+	+	+	+	+	-	-
	exoS	-	+	+	+	+	+	+	+	+	+	+	-
	ExoU	-	+	+	+	+	+	+	+	-	+	+	+
	plcN	-	+	+	+	+	+	+	+	+	+	+	+
	algD	-	+	+	+	+	+	+	+	+	+	+	+
	toxA	-	-	-	-	-	-	-	-	-	-	-	-
Antimicrobial resistance genes	*IMP7*	+	+	+	+	+	+	+	+	+	+	+	+
	IMP10	+	+	+	+	+	+	+	+	+	+	+	-
	IMP13	+	+	+	+	+	+	+	+	+	+	+	-
	IMP25	+	+	+	+	+	+	+	+	+	+	+	+
	bla*OXA50*	+	+	+	+	+	+	+	+	+	+	+	+
	bla*OXA2*	-	-	-	-	-	-	+	+	+	+	-	-
	pstS	+	+	+	+	+	+	+	+	+	+	+	+

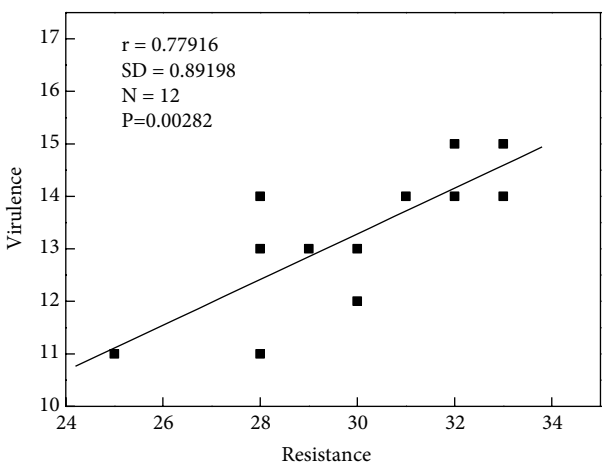

Figure 3. Correlation between antimicrobial resistance and virulence factors production among 12 MDRPA isolates. N: Number of antimicrobial agents. r: Pearson's correlation coefficient.

that require zinc for their activity, and the class A, C, and D β-lactamases contain serine groups in their active site (20). Oxacillinases are Ambler class D β-lactamases with hydrolytic activity against penicillins, extended-spectrum cephalosporins, methicillin, and aztreonam (21). In this study, β-lactamases from class B and class D enzymes, such as IMP and OXA genes, respectively, were identified.

In the present study, all the carbapenem-resistant *P. aeruginosa* isolates were found to harbor the *blaOXA50* gene. In addition, the *blaOXA2* gene was present in 4 isolates. On the other hand, the *blaOXA2* gene was absent in others, probably due to the presence of another type of carbapenem-hydrolyzing enzyme. These genes were found mainly in *P. aeruginosa* isolates from Turkey (22) and France (23). This aspect is important in order to identify and track the spread of MDRPA clones since *blaOXA50* may be a potential clonality marker for *P. aeruginosa* (24).

The other enzyme that encodes the carbapenemases besides oxacillinase is the metallo-β-lactamase (MβL) from class B. Since the first report of acquired MβL in Japan in 1994 (25), genes encoding IMP-type enzymes have spread rapidly among *Pseudomonas* species (26).

MβLs are mostly encoded by integron-borne genes and confer resistance against all β-lactams, except for the monobactams (27). The prevalence of MβL-producing gram-negative bacilli has increased in some hospitals, particularly among clinical isolates of *P. aeruginosa* (28). Since MβL production may confer phenotypic resistance to virtually all clinically available β-lactams, the continued spread of MβL is of major clinical concern (29).

In the present study, PCR amplification of the *blaIMP* gene (*IMP7*, *IMP10*, *IMP13*, and *IMP25*) among the tested carbapenem-resistant isolates using the previously published primers of Crăciunaş et al. (12) was positive in almost all of the tested isolates. The plasmids' location of the MβL genes explains their spread among *P. aeruginosa* strains in specific regions such as Italy and Korea (30). It was determined that the carbapenem resistance in *P. aeruginosa* was due to *IMP7* in Canada (31).

The increasing trend of carbapenem resistance in *P. aeruginosa* worldwide is a concern since it limits drastically the range of therapeutic alternatives. Metallo-β-lactamase, namely *IMP*, has been reported worldwide, especially in Asia and West Europe, and it confers resistance to all β-lactams. Precautionary monitoring of *blaOXA2* in clinical isolates of *P. aeruginosa* should be carried out. Hence, an understanding of carbapenem resistance mechanisms might be crucial for the development of novel therapeutic strategies.

Interestingly, the *pstS* gene was detected in all tested isolates; this gene plays an important role in multidrug-resistance. *pstS* proteins are the cell-bound phosphate-binding elements of the ubiquitous bacterial ABC phosphate uptake mechanisms. Primary and tertiary structures, characteristic of *pstS* proteins, are conserved in proteins, which are expressed in secretory operons and induced by phosphate deprivation, in *Pseudomonas* species (32). The presence of this periplasmic phosphate binding protein (*pstS*) confers a highly virulent phenotype of MDR isolates of *P. aeruginosa* (33).

In the present study, the selected isolates were shown to possess genes encoding virulence factors including GDP mannose 6-dehydrogenase (alginate) (*algD*), neuraminidase (*nan1*), elastase LasB (*LasB*), type IV fimbrial biogenesis protein pilB (*pilB*), exoenzyme S (*exoS*), exoenzyme U (*exoU*), exotoxin A (*toxA*), nonhemolytic phospholipase C (*plcN*), and hemolytic phospholipase C (*plcH*). Surprisingly, none of our isolates was positive for the *toxA* gene. The expression of virulence genes in a given infection is of primary importance in the capacity of an individual *P. aeruginosa* isolate to establish and maintain infection (34). Mitov et al. (35) reported that the frequencies of *pilB*, *exoU*, and *nan1* were significantly higher in MDRPA strains than in non-MDRPA strains. The spread of *nan1* in cystic fibrosis *P. aeruginosa* isolates increases when the clinical state of patients worsens, suggesting the possible role of neuraminidase in cystic fibrosis pulmonary disease evolution (14). Secretion of exoU is a marker for highly virulent *P. aeruginosa* isolates obtained from patients with hospital-acquired pneumonia (36).

From the view point of presence or absence of plasmids in MDRPA isolates, Woodford et al. (37) reported that since many plasmids carry antibiotic resistance determinants contained within mobile genetic elements (transposons) that can be readily acquired or deleted, the DNA compositions of plasmids can change rapidly. Furthermore, the strong selective pressure for organisms to express antibiotic resistance may cause such plasmids to spread rapidly among strains, and even among different species, and persist for prolonged periods within an institution (38).

When strains have multiple antibiotic resistances, the choice of therapy is limited and difficult. The tremendous therapeutic advantages afforded by the introduction of new antimicrobial agents will always be threatened by the emergence of increasingly resistant bacteria pathogens (39).

In conclusion, the data of this study showed a high incidence of antibiotic resistance and virulence properties in *Pseudomonas aeruginosa* isolates, whereas the statistical analysis revealed a significantly positive correlation ($r = 0.779$, $P = 0.002$) between virulence factors and antimicrobial resistance marker profiles of the tested MDRPA isolates. To combat this problem, routine drug susceptibility testing and molecular fingerprinting are recommended monitoring routes of infection and changes in drug resistance patterns.

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