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ORIGINAL RESEARCH

Virulence Factors Of Carbapenem-Resistant *Pseudomonas aeruginosa* In Hospital-Acquired Infections In Mansoura, Egypt

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Purpose: The problem of carbapenem-resistant *Pseudomonas aeruginosa* in health-care settings is growing worse. This study was conducted to investigate the rate of carbapenemase genes, antibiotic resistance, and virulence factors in carbapenem-resistant *P. aeruginosa* associated with hospital-acquired infections.

Patients and methods: Isolates of *P. aeruginosa* were collected from patients with hospital-acquired infections at Mansoura University Hospital in Mansoura. Carbapenem susceptibility was done by broth dilution. The presence of carbapenemase genes and quorum-sensing genes was assessed by PCR. Production of protease, pyocyanin, twitching motility, hemolytic activity and biofilm formation was evaluated.

Results: Out of 80 *P. aeruginosa* isolates, 34 (42.5%) were resistant to carbapenem. Among carbapenem-resistant *P. aeruginosa* isolates, 21 (61.8%) were carbapenemase producers. The most prevalent gene detected was *blaVIM*. The frequency of protease, pyocyanin, twitching motility, hemolytic activity and biofilm formation was 76.2%, 58.8%, 83.8%, 93.8% and 77.5%, respectively. Biofilm formation was significantly associated with carbapenem-resistant *P. aeruginosa*. On the other hand, pyocyanin production was significantly lower in carbapenem-resistant isolates. No correlation existed between carbapenem resistance and any other studied virulence factors or quorum-sensing genes.

Conclusion: Association of carbapenem-resistant *P. aeruginosa* with other antibiotic resistance or the presence of virulence factors in hospital-acquired infection may represent a warning that enhances the need for a stringent surveillance program.

Keywords: *Pseudomonas aeruginosa*, carbapenem, virulence factor, resistance

Introduction

Hospital-acquired multidrug-resistant (MDR) *Pseudomonas aeruginosa* infections are increasing worldwide and have become a global issue.[1] Though carbapenems represent the most effective antibiotics in the treatment of MDR *P. aeruginosa* infections, carbapenem resistance has been increasingly described all over the world.[2] Various mechanisms are involved in carbapenem resistance such as carbapenemase production, intrinsic RND efflux pump systems and lack of outer membrane porin (OprD).[3] Carbapenemase genes represent a serious issue, as the resistance can be transmitted horizontally to other species.[5] Metallo-β-lactamase (MBL) represents the principal carbapenemases formed by *P. aeruginosa*.[4]

Pathogenesis of *P. aeruginosa* is multifactorial, and many virulence factors are produced that include secreted factors such as alkaline protease, elastase, exotoxin A,

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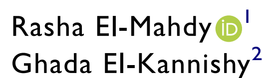

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pyoverdine, pyocyanin, rhamnolipid structural component lipopolysaccharide, pili, flagella and biofilm formation.[6] Production of various virulence factors was regulated by two quorum-sensing (QS) systems las and rhl.[7]

The association between virulence and resistance in *P. aeruginosa* is still poorly understood.[8] The aim of this study was to determine the occurrence of antibiotic resistance, carbapenemase genes and virulence factors in carbapenem-resistant *P. aeruginosa* associated with hospital-acquired infections at Mansoura University Hospitals.

Materials And Methods

Study Setting

The study was conducted in Mansoura University Hospital, Mansoura, Egypt. This study was approved by the Mansoura University's Faculty of Medicine Institutional Review Board—Code number: R.17.10.09.

Isolates

A total nonduplicate 80 isolates of *P. aeruginosa* isolated from clinical samples from September 2018 to April 2019, recovered from patients with hospital-acquired infections at Mansoura University Hospital.

Isolates of *P. aeruginosa* were identified as non-lactose fermenting colonies on MacConkey's medium, Gram's stain, positive oxidase reaction, citrate utilization test, triple sugar iron, growth at 42°C and growth on cetrimide agar.[9] Identification of isolates was confirmed by API 20E (BioMérieux, Marcy l'Étoile, France).

Antimicrobial Susceptibility Testing

The following antibiotics were tested: polymyxin B (300 units), colistin (10 µg), ceftazidime (30 µg), cefepime (30 µg), piperacillin (100µg), piperacillin/tazobactam (100/10 µg), aztreonam (30 µg), tobramycin (10 µg), gentamicin (10 µg), amikacin (30 µg), levofloxacin (10 µg), ciprofloxacin (5 µg), (Oxoid, UK) by disk diffusion method according to the Clinical and Laboratory Standards Institute protocol.[10]

Minimum inhibitory concentrations (MICs) of imipenem and meropenem (manufacturers: GlaxoSmithKlein, AstraZeneca Pharma, Cairo, Egypt) were tested by broth microdilution method according to Clinical Laboratory Standards Institute guidelines. *P. aeruginosa* isolates defined as carbapenem resistant when imipenem or/and meropenem MIC ≥8mg/L determined.[11]

Resistance phenotypes were defined as MDR *P. aeruginosa* for isolate that is non-susceptible to at least one agent in ≥3 antimicrobial categories; XDR isolate is non-susceptible to at least one agent in all but ≤2categories; pandrug resistant means non-susceptible to all antimicrobial categories.[12]

Phenotypic Detection Of Carbapenemases

Carbapenem-resistant isolates were screened for carbapenemase production by modified Hodge test (MHT)[13] and a combined disk diffusion method.[14]

Carbapenemases And Quorum-Sensing Genes

PCR for the following carbapenemase *genes blaIMP; blaVIM; blaNDM; blaKPC; blaOXA-48* and quorum-sensing genes *lasR and rhlR was done as* previously described.[15,16] Genes used in this study are listed in Table 1.

Hemolytic Activity

Five microliters of each strain were streaked on agar base supplemented with 5% sheep erythrocytes and then incubated overnight at 22°C and 37°C. Plates were examined for the presence of β-hemolysis around the colonies.[17]

Twitching Motility

Each strain was stabbed with a sterile toothpick to the bottom 1% Luria–Bertani agar plate and then incubated overnight at 37°C. Twitching motility was evaluated by the presence of a hazy zone surrounding the point of inoculation at the agar–plate interface. Another way to visualize motility was by removal of the agar and addition of crystal violet for 5 mins and then rinsing with tap water.[18]

Pyocyanin Assay

Culture grew in Pseudomonas broth (20 g peptone, 1.4 g $MgCl_2$ and 10 g K_2SO_4 per liter of distilled water). Pyocyanin was extracted as previously described.[19] Concentrations were determined by multiplying the optical density at 520 nm (OD520) by 17.072. Assays were performed three times. *P. aeruginosa* PAO1 was used as a positive control strain for pyocyanin assay.

Protease Assay

Strains of *P. aeruginosa* were streaked on skim milk agar plates (10% w/v skimmed milk) and then incubated at 37°C for 24 hrs. The presence of a clear zone surrounding the colonies indicates a positive test.[17]

Biofilm

Biofilm formation was evaluated by the semiquantitative determination. Briefly,

125 µL of diluted overnight growth of isolates was inoculated in sterile flat-bottomed 96-well microtiter plates

Table 1 Specific Primers Used In This Study

Gene	Sequence (5′–3′)	Product Size (bp)	Reference
blaIMP	GGAATAGAGTGGCTTAAYTCTC GGTTTAAYAAAACAACCACC	232	15
blaVIM	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390	15
blaOXA-48	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	438	15
blaNDM	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	621	15
blaKPC	CGTCTAGTTCTGCTGTCTTG CTTGTCATCCTTGTTAGGCG	798 232	15
lasR	AAGTGGAAAATTGGAGTGGAG GTAGTTGCCGACGACGATGAAG	130	16
rhlR	TGCATTTTATCGATCAGGGC CACTTCCTTTTCCAGGACG	133	16

and incubated at 37°C for 24 hrs. Each well was washed three times with 300 µL distilled water, dried in an inverted position at room temperature. 125 µL of 0.1% crystal violet was added for 10–15 mins then rinsed three times with distilled water. 2 mL of 95% ethanol was added. Uninoculated medium was used as control. Spectrophotometer was used to measure the absorbance at 600 nm.[20]

Statistical Analysis

Data were statistically analyzed using the Statistical Package for Social Sciences (SPSS) version 16 (SPSS Inc, Chicago, IL, USA). Qualitative data are described as numbers and percentages. The Chi-square test or Fisher's exact test was used for comparison between groups, as appropriate. Results with $p<0.05$ were considered significant.

Results

Clinical Isolates

In our study, 80 isolates of *P. aeruginosa* were isolated from different clinical samples, including wound (n = 33), burn (n = 30), respiratory tract (n = 6), urine (n = 6) and blood (n = 5).

Susceptibility Pattern

All isolates were sensitive to colistin, polymyxin B. The susceptibility rate reached up to 52.5%, 45%, 42.5% and 37.5% toward piperacillin/tazobactam, levofloxacin, ciprofloxacin and aztreonam, respectively. Ceftazidime was the least effective antibiotic, with the susceptibility rate being 15%. Fifty-eight (72.5%) isolates were MDR or extensive drug resistance (XDR).

Of the 80 isolates, 34 (42.5%) were carbapenem resistant. Modified Hodge test (MHT) and combined disc diffusion test were positive in 22 (64.7%) and 18 (52.9%), respectively. The sensitivity of MHT and combined disc diffusion was 81% and 95.2%, and the specificity was 92.3% and 84.6%, respectively.

The *blaVIM, blaKPC* and *blaNDM* genes were detected in 18 (52.9%), 1 (2.9%) and 1 (2.9%) of the carbapenem-resistant *P. aeruginosa* isolates, respectively. Besides, 1 isolate (2.9%) carries two carbapenemase genes *VIM+KPC*. None of the isolates carry the *bla OXA48* gene.

Virulence Factors

The frequency of evaluated virulence factors protease, pyocyanin, twitching motility, hemolytic activity and biofilm formation was 76.2%, 58.8%, 83.8%, 93.8% and 77.5%, respectively. All isolates of *P. aeruginosa* carry quorum-sensing *lasR* and *rhlR* genes. Biofilm formation was significantly associated with carbapenem resistance and MDR and XDR *P. aeruginosa*, while pyocyanin production was significantly correlated to carbapenem sensitive isolates. Antimicrobial resistance and virulence factors in carbapenem-resistant

P. aeruginosa and the carbapenem-susceptible *P. aeruginosa* are presented in Table 2.

Virulence factors produced in MDR and non-MDR strains *P. aeruginosa* are illustrated in Figure 1.

Discussion

Multidrug-resistant *P. aeruginosa* is a rising health problem that limits the options for treatment and prolongs hospitalization.[21] In our study, MDR and XDR *P. aeruginosa* represents 72.5% of isolates of *P. aeruginosa*. Furthermore, more than half of these MDR and XDR isolates were carbapenem resistant. A similar rate was reported in Venezuela in which MDR and XDR *P. aeruginosa* increased to 71.9% in 2016.[22] Also, Rossi Goncalves[23] concluded a high rate of MDR (73.9%). However, a lower rate of MDR was reported in other studies conducted in Egypt.[24,25] This high rate of resistance among our isolates may be due to the fact that they were from hospital-acquired infections.[26]

The rate of imipenem resistance varies widely from 1% to 73.5%.[12,16,23–25,27,28] In our study, the frequency of imipenem resistance in *P. aeruginosa* was 42.5%. Moreover, these isolates were significantly associated with resistance to other antibiotics such as cefepime, piperacillin-tazobactam and gentamicin. The same finding was previously reported.[23,29]

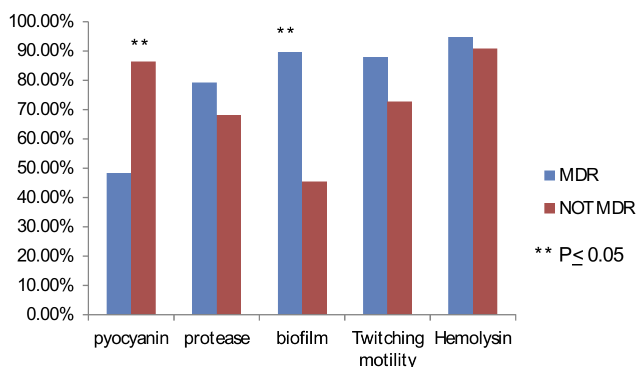

Figure 1 Virulence factors in MDR and non-MDR *P. aeruginosa* strains.

This high rate of imipenem resistance may be due to uncontrolled carbapenem use in hospital infections.[22] Carbapenem resistance among *P. aeruginosa* isolates is attributed mainly to carbapenemase production.[5] In the current study, 65% of carbapenem-resistant isolates carry carbapenemase genes, with *blaVIM* being the most common gene. Numerous studies reported that *blaVIM* gene is the most frequent MBL found in carbapenem-resistant *P. aeruginosa*.[3,30] However, *blaIMP* gene was the most common detected carbapenemase gene in *P. aeruginosa* in a study conducted in Iran.[29] Colistin and Polymyxin B showed a high rate of susceptibility and

Table 2 Comparison Of Antibiotic Resistance And Virulence Factors In Carbapenem-Resistant *P. aeruginosa* And The Carbapenem-Susceptible *P. aeruginosa*

Variable	Carbapenem-Resistant *P. aeruginosa* (34)	Carbapenem-Susceptible *P. aeruginosa* (46)	P value	Odds Ratio	CI
MDR	31(91.2%)	27(58.7)	0.001	7.272	(1.938–27.287)
Ceftazidime	(33) 97.1%	35 (76.1%)	0.009	10.371	(1.268–84.833)
Cefepime	33 (97.1%)	31 (67.4%)	0.001	15.968	(1.989–128.166)
Aztreonam	28 (82.4%)	22 (47.8%)	0.002	5.091	(1.773–14.615)
Piperacillin-tazobactam	21 (61.8%)	17 (37.0%)	0.028	2.756	(1.104–6.879)
Piperacillin	31 (91.2%)	31 (67.4%)	0.012	5	(1.315–19.016)
Gentamicin	29 (85.3)	27(58.7%)	0.010	4.081	(1.337–12.458)
Amikacin	28 (82.4%)	23 (50.0%)	0.003	4.667	(1.626–13.393)
Tobramycin	26(76.5%)	28 (60.9%)	0.141	2.089	(0.777–5.618)
Ciprofloxacin	21 (63.6%)	24 (52.2%)	0.310	1.604	(0.642–4.006)
Levofloxacin	20 (58.8%)	24 (58.8%)	0.555	1.310	(0.535–3.205)
Colistin	0	0	NA	NA	NA
Polymyxin B	0	0	NA	NA	NA
Virulence factors					
Protease	25(73.5%)	36(78.3%)	0.623	0.772	(0.274–2.172)
Biofilm	32(94.1%)	30 (65.2%)	0.002	8.533	(1.807–40.288)
Pyocyanin	0.153±0.053	0.179±0.036	0.012		(0.005742–0.045278)
Hemolysin	32(94.1%)	43 (93.5%)	0.907	1.116	(0.176–7.077)
Twitching motility	30 (88.3)	37 (80.4)	0.35	1.824	(0.511–6.512)

Abbreviation: NA, not applicable.

remain successful options for treatment of infections caused by MBL-producing *P. aeruginosa*.[31] All our isolates were sensitive to colistin and Polymyxin B. In the absence of MBL enzymes, carbapenem resistance may be attributed to increasing production of AmpC chromosome-encoded cephalosporinase, increasing expression of efflux pump permeability and loss of porins.[32]

Virulence genes coexisting with resistance mechanisms to multiple antimicrobial in carbapenem-resistant *P. aeruginosa* have developed an emerging threat.[3] On the other hand, other investigators found no certain associations between antibiotic resistance and virulence genes in MDR *P. aeruginosa*.[30] Bogiel[33] suggested that reduction of virulence in MDR *P. aeruginosa* may be owing to the fact that some genes selectively silence and activate other ones or reduced virulence of MDR strains is the suitable bacterial genome controlling that tolerates for existence in the presence of the antibiotic.

In this study, biofilm formation in carbapenem sensitive and carbapenem-resistant was 65.2% and 94.1%, respectively. Also, Ghanbarzadeh Corehtash[34] demonstrated that the production of biofilm in MDR *P. aeruginosa* isolates was significantly higher than that in non–MDR *P. aeruginosa* isolates. Additionally, many authors described a significant positive correlation between MBL and AmpC β-lactamase production and biofilm formation.[35] In contrast to our results, other studies found no significant difference in the association of biofilm formation and the presence of multidrug resistance.[22,36] Also, no statistical significance was found between MBL and biofilm formation.[31]

Hemolysin and proteases were important virulence factors that were observed in 93.8% and 76.2% of our isolates. No significant difference was detected between carbapenem-resistant and carbapenem-sensitive *P. aeruginosa* for either production of hemolysin or protease or twitching motility. The same finding was formerly reported.[25,37,38] Pyocyanin is a characteristic pigment produced by *P. aeruginosa*.[19] In our isolates, pyocyanin production was significantly reduced in both carbapenem-resistant and MDR isolates. Likewise, other authors revealed that pyocyanin production by *P. aeruginosa* is reduced in MDR strains; furthermore, transduction of MBL genes into non-MDR *P. aeruginosa* strain production of pyocyanin reduced.[36,39] But, others found that imipenem resistance was associated with a lack of alkaline protease production but not associated with a reduction in pyocyanin production.[40]

Several virulence factors investigated in this work such as alkaline protease, pyocyanin and biofilm formation were controlled by (QS) systems.[40] A previous study found that there is an association between QS deficiency and decreased susceptibility to antimicrobials.[40] In our study, all isolates of *P. aeruginosa carry lasR and rhl* genes. Likewise, QS genes were detected in all isolates from wound and respiratory tract secretions from infected patients who underwent cardiovascular surgery.[7] QS deficient strains that fail to create successful infection were related to a decrease in the making of virulence factors.[41] A major limitation of the study is that the gene expression of quorum sensing was not assessed.

Conclusion

Carbapenem resistance in *P. aeruginosa* represents a problem in hospital-acquired infections. The magnitude of this problem may arise if associated with other antibiotic resistance that limits options for treatment or presence of virulence factors that may increase the severity of the infection. Carbapenem-resistant *P. aeruginosa* was significantly associated with other antimicrobial resistance and biofilm formation but shows reduced production of pyocyanin. Surveillance program is needed to trace the origin and limit transport to other patients.

Disclosure

The authors report no conflicts of interest in this work.

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