Acta Biologica Szegediensis

Volume 65(1):105-112, 2021
Acta Biologica Szegediensis
http://abs.bibl.u-szeged.hu/index.php/abs

DOI:10.14232/abs.2021.1.105-112

ARTICLE

Insights on carbapenem-resistant *Pseudomonas aeruginosa*: phenotypic characterization of relevant isolates

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ABSTRACT *Pseudomonas aeruginosa* (*P. aeruginosa*) is ubiquitous in nature, and may be a causative agent in severe, life-threatening infections. In >60% of cases, β-lactam antibiotics are used in the therapy of *P. aeruginosa* infections, therefore the emergence of carbapenem-resistant *P. aeruginosa* (CRPA) is a significant clinical concern. In this study, phenotypic methods were used to characterize fifty-four (n = 54) *P. aeruginosa* isolates, which were included based on their suspected non-susceptibility to meropenem. Minimum inhibitory concentrations (MICs) of meropenem, ceftazidime, cefepime, ciprofloxacin, gentamicin, were determined using E-tests, while colistin MICs were determined using broth microdilution. The isolates were subjected to the modified Hodge test (MHT), the modified carbapenem-inactivation method (mCIM) and the imipenem/EDTA combined disk test (CDT). AmpC and efflux pump overexpression was studied using agar plates containing cloxacillin and phenylalanine-arginine β-naphthylamide (PAβN), respectively. Assessment of biofilm-formation was carried out using the crystal violet tube-adherence method. 38.9% of the strains showed meropenem MICs in the resistant range (>8 mg/L). Efflux-pump overexpression and AmpC-hyperproduction was seen in 44.4% and 35.2% of isolates, respectively. 88.8% of the isolates were characterized as strong biofilm-producers. On the other hand, the presence of carbapenemases was suspected in a minority (16.7%) of tested isolates. As safe and effective therapeutic options in carbapenem-resistant Gram-negative infections are severely limited, characterization of these isolates using phenotypic and molecular-based methods is important to provide insights into the epidemiological features of these pathogens.
Acta Biol Szeged 65(1):105-112 (2021)

KEY WORDS
antibiotic
biofilm
carbapenem
carbapenem-resistance
efflux pump
phenotypic assay
Pseudomonas

ARTICLE INFORMATION
Submitted
11 May 2021.
Accepted
28 July 2021.
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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a non-fastidious, motile, oxidase-positive non-fermenting Gram-negative bacterial pathogen (belonging to the rRNA group I. among non-fermenters, based on the genomic classification of Palleroni) (Palleroni 1993; Palleroni 2010). *P. aeruginosa* is ubiquitous in nature (in soil, on plants, and in aquatic environments, in addition to being transmitted by birds and smaller mammals as reservoirs) and it is also frequently found in healthcare-associated environments (e.g., persisting in water taps and other inanimate surfaces, irrigation fluids, respiratory tubes, surgical theaters and on medical equipment, spreading via aerosol-formation) as a colonizer on patients (8-20% of infections are preceded by colonization of the relevant anatomical regions) (Blanc et al. 2007; Gumey et al. 2020; Hall et al. 2016). This pathogen may be a causative agent in severe, life-threatening infections (especially in immunocompromised individuals or in patients treated in intensive care units), including pneumonia (Szabó et al. 2005), bacteremia/sepsis (Behzadi et al. 2021), skin and soft tissue infections associated with burns and surgeries (Maenni et al. 2017), otitis externa, keratitis (Cannas et al. 2015) and urinary tract infections (Gajdács et al. 2020). In addition, *P. aeruginosa* may be an important colonizer in the airways of patients with cystic fibrosis and chronic obstructive pulmonary disease (COPD), leading to acute exacerbations and decreased quality of life (Clark et al. 2015; Shariff and Beri 2017).

P. aeruginosa is characterized by having a large (5.5-

7 Mb) genome a with pronounced genomic plasticity, including many regulatory genes, affecting motility, efflux proteins, metabolic pathways and the expression of virulence factors and antibiotic resistance determinants (Algammal et al. 2020; Behzadi and Behzadi 2011; Behzadi et al. 2021). The heterogeneity of the *P. aeruginosa* genome may be further increased through horizontal gene transfer, by the introduction of various mobile genetic elements (Suenaga et al. 2017). A plethora of virulence-determinants have been described in *P. aeruginosa*, including secreted virulence factors such as pigments, exotoxins, proteases and other enzymes (e.g., lipases, alkaline protease, elastase A, DNase), secretion systems (type I-VI) and an exopolysaccharide biofilm; in addition, bacterial cell wall-associated structural components, including lipopolysaccharide (LPS), flagella (for swarming, swimming, and twitching motility), pili, adhesins and lectins (Barrak et al. 2020; Behzadi et al. 2021; Hogardt and Heesemann 2013; Jain et al. 2012).

In the era of multidrug resistant (MDR) pathogens, the therapy of *P. aeruginosa* infections is becoming increasingly difficult (Bassetti et al. 2018; Füzi et al. 2017). To start with, this pathogen is intrinsically resistant to aminopenicillin/β-lactamase-inhibitor combinations, I–II. generation cephalosporins and orally administered III. generation cephalosporins, rifampin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole and macrolides (Bassetti et al. 2018; Bonomo and Szabó 2006). Thus, the therapy of pseudomonad infections heavily relies on a select group of antibiotics, including III. and IV. generation parenterally administered cephalosporins, carbapenems, fluoroquinolones, aminoglycosides and colistin. Nevertheless, in >60% of cases, β-lactam antibiotics are used in the therapy of *P. aeruginosa* infections, therefore resistance against these agents (especially carbapenems) is a significant clinical concern (Algammal et al. 2020; Bassetti et al. 2018; Behzadi et al. 2021). The emergence of carbapenem-resistant *P. aeruginosa* (CRPA) may occur through a combination of resistance mechanisms, including decreased membrane permeability and porin loss (e.g., OprD, OprF porin mutants), overexpression of efflux pumps (MexAB-OprM and MexCD-OprJ), changes in penicillin-binding proteins (PBPs) and the production of either chromosomally-encoded or plasmid-mediated β-lactamase enzymes (carbapenemases) capable of hydrolyzing these drugs (Hassuna et al. 2020; Mirzaei et al. 2020). While in some non-fermenters possess chromosomally-mediated β-lactamases are the norm (e.g., L1 and L2 metallo-β-lactamases in *Stenotrophomonas malthophilia*), carbapenemases encoded on mobile genetic present a serious public health issue, as they are capable of widespread dissemination (Gajdács and Urbán 2019; Poole 2011); additionally, these genetic elements often include a wide range of other resistance determinants. The accumulation of intrinsic and plasmid-mediated resistance in *P. aeruginosa* may lead to the emergence of MDR, extensively-drug resistant (XDR) and pandrug-resistant (PDR) isolates (Gajdács 2019; Ranjan et al. 2020).

The characterization of carbapenem-resistant Gram-negative isolates using phenotypic and molecular-based methods is important to provide insights into the epidemiological features of these pathogens, both locally and internationally (Eszik et al. 2016; Mirzaei et al. 2020; Nordmann and Poirel 2019). The aim of our present laboratory-based study was to characterize a selection of carbapenem non-susceptible *P. aeruginosa* isolates using various phenotypic methods.

Materials and methods

Bacterial strains

A total of fifty-four (n = 54) *P. aeruginosa* isolates were included in this study, which were kindly provided by various Hungarian and Italian hospitals, originating from different clinical materials. Inclusion of these strains was based on the non-susceptibility criteria to meropenem (MER) used in routine clinical microbiology, defined by EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines v.9.0 (meropenem disk diameter 23-18 mm: intermediate, <19 mm: resistant) (https://www.eucast.org/clinical_breakpoints/). Identification of the isolates was carried out based on classical phenotypic and biochemical panel-based methods (Leber 2016). All isolates included in the study were re-identified as *P. aeruginosa* before further assays. For shorter time periods (<1 month), the bacterial strains were maintained on blood agar with continuous passage. For longer periods, the strains were kept in a -80 °C freezer, in a 1:4 mixture of 85% glycerol and liquid Luria-Bertani medium. During our experiments *P. aeruginosa* ATCC 27853 was used as a control strain.

Minimum inhibitory concentrations (MICs) of meropenem and ancillary antibiotics

MICs of MER, ceftazidime (CEF), cefepime (CFP), gentamicin (GEN) and ciprofloxacin (CIP) were determined by E-tests (Liofilchem, Roseto degli Abruzzi, Italy) on Mueller-Hinton agar plates (Oxoid, Basingstoke, UK). MIC determination for colistin (COL) was carried out using the broth microdilution method in cation-adjusted Mueller-Hinton broth (MERLIN Diagnostika, Berlin, Germany) (Gajdács et al. 2020). The interpretation of the results was based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints v.9.0 (https://www.eucast.org/clinical_breakpoints/).

Phenotypic detection of AmpC overexpression

Overexpression of AmpC β-lactamase enzymes was detected by an agar plate method, where the agar base was supplemented with cloxacillin (250 μg/mL), as cloxacillin inhibits the effects of AmpC β-lactamases (Khalili et al. 2019). A two-fold decrease in CEF MICs in the presence of cloxacillin, compared to MICs without cloxacillin, was considered as positivity for AmpC overexpression (Akhi et al. 2018).

Phenotypic detection of carbapenemase and metallo-β-lactamase (MBL) production

To establish carbapenemase-production in the isolates included in the study, the isolates were subjected to the modified Hodge test (MHT) and the modified carbapenem-inactivation method (mCIM), optimized for *P. aeruginosa*, as previously described (Chou et al. 2020; Pitout et al. 2008; Rao et al. 2019). In both assays, MER disks (10 μg; Oxoid, Basingstoke, UK) were utilized and *Escherichia coli* ATCC 25922 was used as an indicator organism.

Metallo-β-lactamase (MBL) production was tested using the imipenem/EDTA combined disk test (CDT), as described previously (Makharita et al. 2020). In preparation to this assay, imipenem/EDTA disks were prepared by adding 750 μg of a sterile 0.5 M EDTA solution to a 10 μg imipenem disk, then disks were dried in a 37 °C incubator. The assay was considered positive if the inhibition zone diameter (≥17 mm) of the imipenem/EDTA disk increased compared to the imipenem disk alone (Makharita et al. 2020).

Phenotypic detection of efflux pump overexpression

The effect of phenylalanine-arginine β-naphthylamide (PAβN; a compound with well-known efflux pump inhibitory activity) on the MICs of MER was detected using the agar dilution method described previously (Khalili et al. 2019). During the experiments, the concentration of PAβN was 40 μg/mL in the agar base. A two-fold decrease in MER MICs in the presence of PAβN, compared to the MIC values without the inhibitor, was considered as positivity for efflux pump overexpression (Khalili et al. 2019; Gajdács 2020).

Detection of biofilm-production by the tube-adherence method

Assessment of biofilm-formation was carried out in the tube-adherence method described previously (Dumaru et al. 2019; Senobar Tahaei et al. 2021). In short, glass tubes containing 1 mL of sterile trypticase soy broth (bioMérieux, Marcy-l'Étoile, France) were inoculated with 1 μL of the overnight culture of a respective bacterial strains. Respective tubes were then incubated statically for 24 h at 37 °C. Verification of planktonic growth was observed visually. After the incubation period, the supernatant was then discarded, the adhered cells were rinsed three times with phosphate buffer saline (PBS; Sigma-Aldrich; Budapest, Hungary) and the tubes were patted dry on a paper towel. The contents of the tubes were treated with a 1 mL solution of 0.1% crystal violet (CV; Sigma-Aldrich; Budapest, Hungary) to stain the adhered biomass; the tubes were incubated for 3 h at room temperature with the staining solution. The CV solution was then discarded, and the tubes were again rinsed three times with PBS and the tubes were patted dry on a paper towel. Biofilm-formation was observed visually; based on the appearance of visible biofilm lining at the bottom and on wall of the glass tubes, the strains were classified as non-biofilm producers (-), weak biofilm producers (-/+) and strong biofilm producers (+) (Dumaru et al. 2019). All experiments were evaluated by two independent researchers.

Statistical analysis

Descriptive statistical analysis (including means and percentages to characterize data) was performed using Microsoft Excel 2013 (Microsoft Corp.; Redmond, WA, USA).

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki and national and institutional ethical standards. Ethical approval for the study protocol was obtained from the Human Institutional and Regional Biomedical Research Ethics Committee, University of Szeged (registration number: 140/2021-SZTE [5019]).

Results

MICs of the tested antibiotics, phenotypic detection of AmpC overexpression

The MICs of the tested antibiotics, including MIC_{50}, MIC_{90} values, MIC ranges and the percentage of resistant isolates are presented in Table 1. The highest levels of resistance were observed for CIP (n = 42, 77.8%), followed by CEF (n = 36, 66.7%) and CFP (n = 33, 61.1%). All tested isolates were susceptible to COL, with MIC values ranging between 0.064 and 2 mg/L. Based on EUCAST breakpoints, n = 21 (38.9%) of isolates showed MICs above the resistance breakpoint for MER (8 mg/L), with MICs ranging between 0.5 and 64 mg/L. Among the tested *P. aeruginosa* isolates, overexpressions of AmpC-enzymes were observed in n = 19 (35.2%), where a two-fold decrease in the CEF MICs was seen in the presence of cloxacillin.

Table 1. MIC values of meropenem and ancillary antibiotics on the tested bacterial strains.

	Resistant strains (n, %)	MIC range (mg/L)	MIC50 (mg/L)	MIC90 (mg/L)
Meropenem (MER)	21 (38.9%)	0.5-64	4	16
Ceftazidime (CEF)	36 (66.7%)	0.5-256	8	64
Cefepime (CFP)	33 (61.1%)	0.5-256	8	64
Gentamicin (GEN)	28 (51.9%)	0.25-128	2	16
Ciprofloxacin (CIP)	42 (77.8%)	0.128-16	1	4
Colistin (COL)	0 (0%)	0.064-2	0.128	0.5

Phenotypic detection of carbapenemase, MBL production and efflux pump overexpression

To detect the presence of carbapenemases, two distinct phenotypic methods were utilized: n = 11 (20.4%) isolates presented with positive results in the modified Hodge test (MHT), while this number was n = 9 (16.7%) during the modified carbapenem-inactivation (mCIM) test. If we consider the results of the antibiotic susceptibility testing (MER MIC > 8 mg/L) as a reference in our study, the agreement between the results of the MIC determination and the results of the MHT and mCIM tests were 52.3% and 42.9%, respectively. MBL-production was noted in n = 3 (5.6%) of isolates, using the imipenem/EDTA combined disk test (CDT). Efflux pump-overexpression (based on the PAβN screening agar) was detected in n = 24 (44.4%) of isolates. In the case of n = 3 isolates, efflux pump-overexpression and MHT/mCIM-positivity were detected, while for n = 2 isolates, AmpC-hyperproduction and MHT/mCIM-positivity was seen; for n=6 isolates, high MER MICs were seen with efflux pump-overexpression and AmpC-hyperproduction and negative MHT/mCIM tests.

Biofilm production in the tested isolates

Majority (n = 48, 88.8%) of the tested isolates were strong (+) biofilm producers in the CV tube-based assay, while n = 4 (7.4%) were weak biofilm producers (-/+) and two (3.7%) did not form biofilm (-).

Discussion

Antimicrobial resistance has emerged as a significant public health issue in the 21st century, mainly stemming from the imprudent use of these agents in both community-based and inpatient settings, which has exacerbated the prevalence of resistant bacterial isolates (Aslam et al. 2020; Gajdács et al. 2018; Hemlata and Tiwari 2017); it has been suggested that infections caused by MDR bacteria may be one of the major causes of mortality by 2050 (Gajdács et al. 2021; Shallcross et al. 2015). Drug-resistant *P. aeruginosa* is a significant nosocomial pathogen with the potential to cause serious, difficult-to-treat infections (Kadri et al. 2018). For this reason, it is unsurprising that *P. aeruginosa* has been included among the so-called „ESKAPE" pathogens (designated by the Infectious Diseases Society of America; IDSA), listing the MDR bacteria that are of particular concern for healthcare (Pachori et al. 2016; Rice 2008). From a clinical standpoint, infections caused by CRPA present an important therapeutic problem, as even with the introduction of some novel antimicrobials (e.g., β-lactam-β-lactamase combinations, neoglycosides), there are limited number of safe and effective therapeutic alternatives remaining (Hu et al. 2019). Based on the data of the US Centers for Disease Control (CDC), the prevalence of CRPA was around ~12%, corresponding to ~51 000 nosocomial infections per year (Nordmann and Poirel 2019); while based on the data of the European Antimicrobial Resistance Surveillance Network (EARS-Net) for 2017, the population-weighted mean prevalence of CRPA in invasive isolates was 17.4% (lowest in Iceland [0%], highest in Romania 63.4%]) (ECDC 2019).

Carbapenem resistance in *Pseudomonas* more commonly occurs due to alternations in the membrane permeability (i.e. porin deficiency) and the overexpression of efflux pumps, while the production of carbapenemases is observed less frequently, especially when compared to the members of *Enterobacteriaceae* and *Acinetobacter* spp. (Nordmann and Poirel 2019). Initially, metallo-β-lactamases were characteristic for *Pseudomonas* spp.; in fact, both IMP (active-on-imipenem) and VIM-type (Verona intergon-encoded MBL) enzymes were first described in pseudomonads (Lauretti et al. 1999; Watanabe et al. 1991). Since then, the presence of many other carbapenemases (including the *Klebsiella pneumoniae* carbapenemase [KPC], and the New Delhi metallo-beta-lactamase [NDM]) were reported in CRPA isolates (Halat and Moubareck 2020; Nordmann and Poirel 2019). In our present study, a collection of *P. aeruginosa* isolates – suspected of being CRPA – were included and their characterization was carried out using various phenotypic assays. Among the isolates, 38.9% of the strains showed MER MICs in the resistant range, while apart from COL (which showed 100% susceptibility), resistance rates were high (>50%) against the other tested antibiotics as well. Similarly to

other reports in the literature, carbapenemase-detection methods were positive in a lower number of isolates, i.e. 20.4% for MHT and 16.7% for mCIM, whereas the presence MBL was suggested in only 5.6%, respectively. On the other hand, the prevalence of efflux pump overexpression was seen in a much higher rate, in 44.4% of isolates. Another common characteristic of clinical *P. aeruginosa* isolates is the production of a protective biofilm, which was verified *in vitro* in 88.8% of our isolates.

Between 2003 and 2005, the first outbreaks and the spread of MBL-producing *Pseudomonas* sp. was reported in Hungary, corresponding to two distinct groups of isolates based on serotyping (O11 and O12, respectively) with the carriage of VIM-4 (Libisch et al. 2006). Most of these isolates originated from either urine or tracheal aspirate samples and most of them had high MICs corresponding to imipenem (>256 mg/L) and MER (>32 mg/L) (Libisch et al. 2006). The first detection of a PER-1 and VIM-2-producing *P. aeruginosa* isolate was reported in 2008, which was associated with a Hungarian patient who was initially hospitalized in Egypt (Szabó et al. 2008); in this clinical case, three distinct *P. aeruginosa* strains were detected, one resistant to antipseudomonal cephalosporins (with MICs>256 mg/L), one resistant to imipenem only (MIC>32 mg/L) and one presenting with high-level resistance against imipenem (MIC>256 mg/L) and MER (MIC>32 mg/L) (Szabó et al. 2008). In an effort to provide the most effective therapy for patients affected by invasive *P. aeruginosa* infections, a Monte Carlo simulation was performed using the susceptibility data from Hungarian *P. aeruginosa* isolates: the results suggest that due to the worsening rates of resistance, as increasing doses, frequencies or infusion times, in addition to combination antimicrobial therapy may be relevant in the empiric therapy of *P. aeruginosa* in Hungary (Ludwig et al. 2006). Based on the surveillance data of the Hungarian National Public Health Centre, bloodstream infections were among the more common nosocomial infections caused by *P. aeruginosa* in recent years (Epi-Net 2021). A study group in Hungary has published the occurrence of MDR *P. aeruginosa* found in environmental sites contaminated by hydrocarbons between the period of 2002-2007; carbapenem-resistance was noted in 33% of isolates (Kaszab et al. 2010). The same study group has also recently reported ceftriaxone and imipenem resistance in 25.0% of tested environmental *P. aeruginosa* isolates; in addition, five out of the 44 isolates originating from sources as groundwater, soil or compost showed close genetic relatedness to clinically relevant pulse-field types based on pulse-field gel electrophoresis (PFGE) (Kaszab et al. 2019). In a laboratory-based study, n = 250 carbapenem-resistant *P. aeruginosa* were surveyed for their susceptibilities against ceftazidime-avibactam (C/A) and ceftolozane–tazobactam (C/T), in addition to a phenotypic-genotypic study for carbapenemase-production: prevalence of resistance to C/A and C/T was 33.6% and 32.4%, respectively; isolates producing positive CIM-tests were VIM (80%) or NDM (11%) producers (O'Neall et al. 2020). This study has concluded that in case of a negative CIM-test for a relevant carbapenem non-susceptible *P. aeruginosa* isolate, either C/A or C/T may be an effective therapeutic choice (O'Neall et al. 2020). A study involving n = 57 carbapenem-resistant, but cephalosporin susceptible (Car-R/Ceph-S) *P. aeruginosa* isolates originating from urinary tract infections has highlighted the role of efflux pump overexpression and overproduction of AmpC β-lactamases in the development of the carbapenem-resistant phenotype; the study has also highlighted that – although rare – these isolates may constitute a viable target for colistin-sparing strategies (Gajdács 2020). A similar study was performed in Turkey, where n=243 isolates were assessed by phenotypic and molecular methods: in this report, carbapenemase-producing isolates were not detected, while overexpression of MexAB-OprM (60.9%) and MexXY-OprM (68.8%) efflux pumps and decreased permeability due to OprD-related porin deficiency (68.8%) were the principal mechanisms of resistance (Khalili et al. 2019).

Due to the disadvantageous developments in antibiotic resistance trends globally (and the increasing prevalence of CRPA), it has been suggested that alternative antimicrobial therapeutic strategies should be explored more extensively (Hauser et al. 2016; Usai et al. 2019). These strategies may include physico-chemical modalities, such as sonobactericide (ultrasound), lasers and the use of photosensitizers for photodynamic therapy (Lattwein et al. 2020; Stájer et al. 2020); in contrast, novel pharmacological modalities include bacteriophages, probiotics, antimicrobial peptides, antibodies and antivirulence compounds, many of which were already included in some human clinical trials (Ghosh et al. 2019; Kumar et al. 2021). Compounds of natural origin have long been proposed as a potential source of novel antimicrobials for the treatment of drug resistant infections (either as monotherapy or as adjuvant), including MDR *Pseudomonas* (Ding et al. 2021; Amorese et al. 2018). These may include extracts of various plant materials, new bioactive compounds or phytopharmaceuticals and essential oils (Le NT et al. 2020); these compounds are often characterized by good safety and tolerability *in vivo*, in addition to having established indications in ethnopharmacology/traditional medicine (Le et al. 2020; Mazzarello et al. 2020). It may be assumed that – in addition to novel antibiotics – the introduction of such alternative treatments to the clinical practice may have a pronounced role in the treatment of difficult-to-treat infections in the 21st century.

Acknowledgements

M.G. was supported by the János Bolyai Research Scholarship (BO/00144/20/5) of the Hungarian Academy of Sciences. The research was supported by the ÚNKP-20-5-SZTE-330 New National Excellence Program of the Ministry for Innovation and Technology from the source of the National Research, Development and Innovation Fund. Support from Ministry of Human Capacities, Hungary grant 20391-3/2018/FEKUSTRAT is acknowledged. M.G. would also like to acknowledge the support of ESCMID's "30 under 30" Award.

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