ACTA MICROBIOLOGICA BULGARICA Volume 39 / 4 (2023)

Incidence and Characterization of Carbapenem Resistance Mediated by Acquired Beta-Lactamases in *Pseudomonas aeruginosa* Isolates from a Bulgarian Cancer Hospital

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Abstract

Detection of clinical isolates of *Pseudomonas aeruginosa* producing metallo-β-lactamases (MBL) and extended-spectrum β-lactamases (ESBL) is essential to adequately treat patients and control the spread of resistance. We investigated the frequency of carbapenem resistance mediated by acquired β-lactamases in *P. aeruginosa* isolates from a tertiary cancer hospital in Bulgaria. The prevalence of *P. aeruginosa* infections was relatively low - approximately 2% of all hospital-acquired infections, while the carbapenem resistance rate was estimated at 25% in 2023. Carbapenem resistance due to VIM and NDM MBL types was observed in 8/54 (14.81%) isolates. PER, VEB, or GES ESBLs were detected in a total of 8/54 (14.81%) isolates including 3 isolates co-harbouring NDM and GES enzymes. The overall frequency of 24.07% (13/54) of acquired carbapenemase and ESBL genes in our study reflects the multicomponent nature of carbapenem resistance in *P. aeruginosa* and highlights the need for reliable screening.

Keywords: *Pseudomonas aeruginosa*, metallo-β-lactamase (MBL), extended-spectrum β-lactamase (ESBL).

Резюме

Откриването на клинични изолати *Pseudomonas aeruginosa*, продуценти на метало-β-лактамази (MBL) и β-лактамази с разширен спектър (ESBL) е от съществено значение за адекватното лечение на пациенти и контрола върху разпространението на резистентност. Изследвана е честотата на резистентност към карбапенеми, медиирана от придобити β-лактамази, в изолати *P. aeruginosa* от университетска онкологична болница в България. Честотата на *P. aeruginosa* инфекциите през 2023 г е сравнително ниска - приблизително 2% сред всички придобити в болницата инфекции, докато честотата на карбапенемната резистентност е 25%. Карбапенемна резистентност, дължаща се на VIM и NDM MBL типове е наблюдавана при 8/54 (14.81%) изолата. ESBL от типа PER, VEB и GES бяха открити при 8/54 (14.81%) изолата, включително 3 изолата с ко-експресия на ензими от типа NDM и GES. Общата честота на придобитите карбапенемазни и ESBL гени, установена в нашето проучване, 13/54 (24.07 %), отразява комплексния характер на карбапенемната резистентност при *P. aeruginosa* и подчертава необходимостта от надежден скрининг.

Introduction

Pseudomonas aeruginosa is a clinically important opportunistic pathogen with intrinsic resistance to many antibiotics, restricting the therapeutic options to a limited number of antimicrobial agents. This intrinsic multidrug resistance is a result of synergy between chromosomal AmpC β-lactamase, broad specificity efflux pumps, and decreased outer membrane influx (Reynolds and Kollef, 2021). In recent years, this species has developed resistance to all beta-lactams, including carbapenems. Although in *P. aeruginosa* carbapenem resistance mostly arises from mutations leading to loss or inactivation of OprD outer membrane porin or upregulation of efflux pumps, production of carbap-

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Acta Microbiol. Bulg. 2023;39(04). https://doi.org/10.59393/amb23390408

enemases is increasingly reported as well (Reyes *et al.*, 2023). MBL carbapenemases have been reported globally (mainly VIM and IMP), but OXA types and serine carbapenemases have also been detected, including KPC and GES variants. In addition, ESBLs derivatives of OXA-2 and -10, and class A ESBLs of VEB and PER types were the most prevalent ESBLs in carbapenem-resistant *P. aeruginosa* (CR-PA) (Zafer *et al.*, 2014).

CR-PA in Bulgaria has commonly been associated with VIM-type enzymes (Schneider *et al.*, 2008). Recently, the co-occurrence of NDM-1 and GES-5 (Kostyanev *et al.*, 2020), and OXA-50 has also been documented (Petrova *et al.*, 2019).

Detection of MBL- and ESBL-producing *P. aeruginosa* is essential for adequate therapy and infection control. The aim of the present study was to assess the incidence and to characterize carbapenem resistance mediated by acquired β-lactamases in *P. aeruginosa* isolates from a tertiary cancer hospital in Bulgaria.

Materials and Methods

From January 2013 to May 2023, a total of 22198 non-repetitive, clinically relevant isolates were recovered from patients admitted to a 252-bed cancer hospital in Sofia, Bulgaria. Of those, 420 isolates (1.9%) were identified as *P. aeruginosa* using GNI cards on the VITEK 2 system (bioMerieux Vitek Inc., Hazelwood, MO). As carbapenem resistance mechanisms in *P. aeruginosa* can affect multiple β-lactams, all isolates displaying resistance to at least one carbapenem were selected during routine susceptibility testing and subsequently stored at -80°C for further study. Identification of CR-PA isolates was confirmed via MALDI Biotyper with MALDI Reference 2022 Library v4.0.0 (Bruker Daltonik GmbH & Co. KG, Bremen, Germany).

Antimicrobial susceptibility testing (AST) was performed by disk diffusion with disks supplied by Becton Dickinson (Sparks, MD). In addition, minimal inhibitory concentrations (MICs) of selected antimicrobial agents were determined with the MICRONAUT-S Pseudomonas MIC plates according to the manufacturer's protocols (Bruker Daltonics GmbH & Co. KG, Bremen, Germany). MICs for imipenem/relebactam and meropenem/vaborbactam were determined using a gradient test with MIC strips (Liofilchem, Italy). Susceptibility test results were interpreted following EUCAST recommendations (EUCAST v13.0, 2023).

Total genomic DNA was extracted from overnight cultures on Mueller-Hinton agar. A few colonies were resuspended in 10% Chelex 100 in TE buffer (Bio-Rad, CA, USA). The suspension was heated at 96°C for 10 minutes, followed by 2 minutes on ice and centrifugation at 17,000×g for 10 minutes. The resulting supernatant containing the extracted DNA was collected for further analysis.

Multiplex touchdown PCR was performed for detection of carbapenemase-encoding genes using previously published primers for bla_{SIM}, bla_{SPM}, $bla_{OXA\text{-}48\text{-}like}$, bla_{GES}, bla_{KPC}, bla_{IMP}, bla_{VIM} and bla_{NDM} (Poirel *et al.*, 2006; Mendes *et al.*, 2007; Cole *et al.*, 2009; Gröbner *et al.*, 2009; Goudarzi *et al*., 2019). The PCR reaction was carried out in a final volume of 20 µL containing 2 µL of 10x Complete PCR buffer, 0.25 mM dNTP mix, 0.05 U/µL DFS Hot Taq DNA Polymerase (Metabion), 2% DMSO, 32 mM Tetrapropylammonium chloride (Sigma) and 10-30 ng DNA template. The PCR protocol consisted of an initial denaturation step at 95°C for 4 minutes, followed by touchdown PCR. Cycle denaturation was at 95°C for 20s, 65°C annealing for 35s (with -1°C/cycle for the first 10 cycles) and 70°C elongation for 40s. Following the touchdown cycles, an additional 21 cycles were carried out with annealing at 54°C for 25s and final elongation at 72°C for 3 minutes. Multiplex PCR for identification of bla_{VEB} and bla_{PER} was conducted using the same PCR conditions with previously described primers (Lee *et al.*, 2005). PCR for $bla_{OXA\text{-}2\text{-}like}$ and $bla_{OXA\text{-}10\text{-}like}$ genes was carried out as previously published (Mlynarcik *et al.*, 2019; 2020). High-resolution capillary electrophoresis was used with protocol 0M800 (3 kV for 800 s) for precise size estimation and fragment analysis (QIAxcel, QIAGEN, Hilden, Germany).

Results

For the ten-year survey period (January 2013 to May 2023), a total of 420 *P. aeruginosa* isolates were obtained from patients admitted to the cancer hospital. They represented about 2% (420/22198) of the total number of clinical isolates (Table 1).

The applied screening criteria – resistant to at least one carbapenem, resulted in 54 CR-PA isolates. They represented 13% (54/420) of the total number of *P. aeruginosa* isolates. Multiplex PCR revealed the presence of carbapenemase and ESBL genes in 13/54 (24.07%) CR-PA isolates dispersed through the survey period (Table 2).

Class B MBL genes, either bla_{VIM} or bla_{NDM}, were detected in CR-PA from 8 patients (8/54, 14.81% in total). CR-PA isolates from 5 patients carried bla_{VIM} as the only β-lactamase detected. bla_{NDM} co-existing with bla_{GES} was found in 3 isolates. Similarly, three CR-PA isolates carried a sin-

gle bla_{PER} ESBL gene. In isolate PA110, bla_{PER} was found together with both $bla_{OXA-2-like}$ and $bla_{OXA-10-like}$ genes, while in PA4685 bla_{PER} was co-existent with $bla_{OXA-10-like}$. Lastly, bla_{VEB} ESBL genes were found in CR-PA isolates from 2 patients, accompanied by $bla_{OXA-10-like}$ in one of them (PA1031).

According to AST results, all 13 β-lactamase-producing CR-PA isolates exhibited susceptibility to colistin and cefiderocol. Resistance was observed against piperacillin, ceftolozane/tazobact-

Table 1. Incidence of carbapenem-resistant *Pseudomonas aeruginosa* isolates from cancer patients (2013 - May 2023)

Year	Total isolates n	PA isolates n	% of total isolates	CR-PA isolates	
				n	% of PA isolates
2013	1189	46	3.87	6	13.04
2014	1031	36	3.49	5	13.89
2015	1501	33	2.20	0	0.00
2016	1908	43	2.25	4	9.30
2017	2010	51	2.54	2	3.92
2018	2408	32	1.33	3	9.38
2019	2131	27	1.27	3	11.11
2020	1528	70	4.58	17	24.29
2021	1404	42	2.99	6	14.29
2022	1268	20	1.58	3	15.00
2023	681	20	2.94	5	25.00
Total	22198	420	1.89	54	12.86

Abbreviations: PA, *Pseudomonas aeruginosa*; CR-PA, carbapenem-resistant *P. aeruginosa*; CR-PA isolate is defined as resistant to at least one carbapenem (meropenem, imipenem, doripenem).

Table 2. Characteristics of clinical *Pseudomonas aeruginosa* strains with acquired beta-lactamase-mediated carbapenem resistance isolated from cancer patients (2013 - May 2023)

Isolate	Specimen type	Date of isolation	Patient location	Minimal inhibitory concentrations (μg/mL)[a]										Beta lactamase genes
				COL	PIT	CTA	CAZ	CAA	IMP	IMR	MEM	MEV	ATM	
PA6000	Tracheal aspirate	04/10/2013	ICU	2	>64	>8	64	>16	>8	>32	>128	>256	16	bla_{VIM}
PA5257	Pleural fluid	23/10/2014	Outpatient	≤1	16	>8	32	>16	>8	>32	32	24	4	bla_{VIM}
PA2015	Urine	19/05/2016	Gynecology	≤1	16	>8	16	16	>8	>32	16	16	4	bla_{VIM}
PA2267	Urine	08/06/2016	Urology	≤1	32	>8	64	>16	>8	>32	128	64	4	bla_{VIM}
PA1140	Blood	08/03/2018	ICU	≤1	>64	>8	>128	16	>8	8	32	12	>16	bla_{PER}
PA1602	Urine	17/04/2018	Outpatient	2	16	>8	16	16	>8	>32	>128	>256	8	bla_{VIM}
PA4685	Urine	04/11/2019	Outpatient	≤1	>64	>8	>128	16	>8	8	32	12	>16	$bla_{PER;}$ bla_{OXA}
PA110	Urine	13/01/2020	Urology	≤1	>64	>8	>128	>16	>8	4	32	16	>16	$bla_{PER;}$ bla_{OXA}
PA1793	Urine	08/06/2020	Radio-therapy	2	32	>8	>128	>16	>8	>32	>128	>256	16	$bla_{NDM;}$ bla_{GES}
PA2295	Tracheal aspirate	14/07/2020	ICU	2	64	>8	>128	>16	>8	4	16	16	>16	bla_{VEB}
PA131	Urine	19/01/2022	Urology	2	32	>8	>128	>16	>8	>32	>128	>256	16	$bla_{NDM;}$ bla_{GES}
PA1031	Urine	20/03/2023	Urology	2	64	>8	>128	16	>8	4	64	24	>16	$bla_{VEB;}$ bla_{OXA}
PA1596	Abdominal fluid	09/05/2023	ICU	4	32	>8	>128	>16	>8	>32	>128	>256	16	$bla_{NDM;}$ bla_{GES}

[a]COL, colistin; PIT, piperacillin/tazobactam; CTA, ceftolozane/tazobactam; CAZ, ceftazidime; CAA, ceftazidime/avibactam; IMP, imipenem; IMR, imipenem/relebactam; MEM, meropenem; MEV, meropenem/vaborbactam; ATM, aztreonam. MIC values in bold are those corresponding to a resistance categorization.

am, cefepime, ceftazidime, ceftazidime/avibactam, doripenem, imipenem, imipenem/relebactam, meropenem and meropenem/vaborbactam in all of them (Table 2). Three isolates (PA5257, PA2015, PA1602) with bla_{VIM} MBL were categorized as susceptible, to increased exposure to piperacillin/tazobactam. All isolates with bla_{VIM} or bla_{NDM} MBL genes were categorized as susceptible, to increased exposure to aztreonam. Finally, all the 13 CR-PA were resistant to ciprofloxacin, levofloxacin, amikacin, and tobramycin, except for the bla_{GES} and bla_{NDM}-positive isolates that were tobramycin-susceptible.

Discussion

P. aeruginosa is a common cause of nosocomial infections, such as pneumonia, surgical site infections, urinary tract infections, and bacteremia. It is estimated that *P. aeruginosa* has a prevalence of 7.1%–7.3% among all healthcare-associated infections (Reynolds and Kollef, 2021). In our study, the prevalence of *P. aeruginosa* infections was relatively low – about 2% among all hospital infections. This can be explained by the rapid initiation of antimicrobial therapy as well as infection control procedures. The resistance rate of *P. aeruginosa* to carbapenems reached 25% in 2023 consistent with the 2021 EARS-Net data for Bulgaria (retrieved from the ECDC Surveillance Atlas of Infectious Diseases).

Carbapenem resistance in this study was attributed to carbapenemases of VIM and NDM types in 14.81% (8/54) of the isolates. Additionally, 14.81% (8/54) of the CR-PA isolates carried class A ESBLs, including PER, and VEB enzymes. Notably, the recently emerged bla_{NDM} and bla_{GES} tandem were demonstrated in three isolates (Kostyanev *et al.*, 2020). The presence of acquired carbapenemase and ESBL genes, found in 24.07% (13/54) of the isolates, underscores the intricate nature of carbapenem resistance in *P. aeruginosa,* frequently leading to restricted therapeutic options and emphasizes the importance of reliable screening.

Conclusion

The prevalence of *P. aeruginosa* infections in this study was relatively low, accounting for approximately 2% of all hospital infections. However, worrying trends were observed, as the CR-PA rate reached 25% in 2023. A notable frequency of MBL-producing and ESBL-producing isolates among CR-PA was found, limiting the number of effective antimicrobials to only colistin and cefiderocol. These findings underscore the importance of adopting proactive measures and innovative strategies to effectively address the rise in carbapenem resistance and control of MBL- and ESBL-producing *P. aeruginosa* in healthcare settings.

Acknowledgements

This study was supported by The Bulgarian National Science Fund under Grant КП-06-Н23/5.

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