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***Carbapenemase -producing *Pseudomonas aeruginosa* isolates from Turkey:
First report of *P. aeruginosa* high-risk clones with VIM-5- and IMP-7- type
carbapenemases in a tertiary hospital***

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Abstract

1
2 We investigated the presence of carbapenemases in carbapenem-resistant *Pseudomonas*
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4 *aeruginosa* isolates, which were collected over a 14-month period in a Turkish hospital, with
5
6 in-depth molecular characterization of carbapenemase-producing isolates. Among 45 study
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8 isolates; two isolates were identified as carbapenemase producers by both Carba NP and
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10 Carbapenem Inactivation Method (CIM) tests, and only one of them gave a positive result in
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12 PCR tests for a carbapenemase gene (*bla*_{VIM}). Whole genome sequencing (WGS) of the two
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14 isolates revealed the presence of *bla*_{VIM-5} gene in an ST308 isolate while the other one expressed
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16 IMP-7 in an ST357 isolate, both STs are considered high-risk clones. The two carbapenemase-
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18 producing isolates were multidrug resistant, as they harbored other resistance determinants,
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20 including a variant of the recently described plasmid-encoded fluoroquinolone resistance
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22 determinant *crpP* gene, *crpP-2*. We report for the first time *P. aeruginosa* high-risk clones
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24 carrying VIM-5- and IMP-7- type carbapenemases with multiple resistance determinants in
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Introduction

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2 Multidrug resistant (MDR) gram-negative pathogens, and especially
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4 *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, are emerging
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6 worldwide. The MDR pattern is relatively common with resistance appearing to all major
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8 classes of anti-gram-negative agents (e.g. β -lactams, fluoroquinolones, and aminoglycosides),
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10 and in some cases, resistance to all available drugs [1,2]. This is particularly worrisome in view
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12 of the current dearth of new compounds active against MDR gram-negatives in the pipeline. β -
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14 Lactams, due to their safety, reliable killing properties and clinical efficacy, are among the most
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16 frequently prescribed antibiotics used to treat bacterial infections. However, their utility is being
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18 threatened by the worldwide proliferation of β -lactamases (BL) with broad hydrolytic
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20 capabilities, especially in MDR gram-negative bacteria. Currently, BL-mediated resistance
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22 does not spare even the newest and most powerful β -lactams (i.e. 3rd generation cephalosporins
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24 and carbapenems), whose activity is challenged by extended spectrum β -lactamases (ESBLs),
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26 plasmid encoded cephalosporinases and carbapenemases [2].
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35 In *P. aeruginosa*, carbapenem resistance may arise via hyperproduction of a
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37 chromosomal AmpC β -lactamase, acquisition of carbapenemases, and/or upregulated efflux or
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39 impermeability [3]. Most carbapenem resistance is due to impermeability, which arises via the
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41 loss of the OprD (D2) porin, but acquired transferrable carbapenemases are increasingly
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43 reported, with some of them coming from strains that have caused large outbreaks [3-5]. The
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45 carbapenemases currently found in *P. aeruginosa* belong to Ambler classes A (GES and KPC),
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47 B (IMP, VIM, NDM, SPM, AIM and GIM) and rarely D (OXA-198) [6]. Among acquired
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49 carbapenemases in *P. aeruginosa*, VIM-type and IMP-type enzymes are the most common
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51 types with worldwide distribution, and the VIM and GES types have been the most reported
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53 enzymes in Turkey in a few studies [6-15].
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The aim of the present study is to investigate the presence of carbapenemases both by phenotypic and genotypic methods in a collection of carbapenem-resistant *P. aeruginosa* isolates from a tertiary care hospital in Istanbul. Antimicrobial susceptibilities including ceftolozane/tazobactam, which is known to be highly effective against multidrug resistant *P. aeruginosa* but poorly studied in Turkey, and the presence of high-risk clones were also investigated among study isolates.

Materials and Methods

Bacterial isolates, identification, and susceptibility testing

A collection of 45 non-duplicates clinical *P. aeruginosa* isolates (1 isolate per patient), resistant to at least imipenem or meropenem, from the Şişli Hamidiye Etfal Training and Research Hospital Clinical Microbiology Laboratory between August 2015-October 2016 were included in the study.

P. aeruginosa was identified using standard biochemical tests (API NE, bioMérieux, Marcy l'Etoile, France) in conjunction with matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Germany). Susceptibility testing for amikacin, aztreonam, ceftazidime, cefepime, ciprofloxacin, levofloxacin, gentamicin, piperacillin-tazobactam, imipenem, meropenem, netilmicin, tobramycin, ticarcillin and piperacillin was performed by disk diffusion method. A selection of 12 isolates, which were multidrug resistant including imipenem and ceftazidime has also been tested for ceftolozane/tazobactam susceptibility using E-test (Liofilchem, Italy). Colistin minimal inhibitory concentrations (MICs) were determined by BMD (Sensititre, Thermo Fisher Scientific, Basingstoke, UK) according to the guidelines of the CLSI and EUCAST joint subcommittee [16]. Imipenem and/or meropenem resistance revealed by disk diffusion, was confirmed using Etest (Oxoid M.I.C. Evaluator, Thermo Fisher Scientific). For isolates found

1 as ceftazidime resistant, the presence of an ESBL was investigated by a synergy image detection
2 using the double-disc synergy test (DDST) performed with ceftazidime and
3 ticarcillin/clavulanic acid discs on cloxacillin (200 mg/L)-containing Mueller-Hinton (MH)
4 agar plates, which partially inhibits the cephalosporinase activity [17,18]. European Committee
5 on Antimicrobial Susceptibility Testing (EUCAST) methods and interpretation criteria were
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7 used for all antimicrobial agents [19]. The isolates were stored at -80 °C in trypticase soy broth
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9 until molecular tests were performed.
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19 **Phenotypic detection of carbapenemase activity**

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21 Two phenotypic tests, the Carba NP and Carbapenem Inactivation Method (CIM) were
22 applied to all isolates for detection of carbapenem-hydrolyzing activity [20,21]. Both tests were
23 performed as previously described [20,21], and the results were interpreted blindly by two
24 independent microbiologist that ignored all other results of this study.
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34 **Molecular detection of carbapenemase-encoding genes**

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36 *Bla_{KPC}*, *bla_{NDM}*, *bla_{VIM}*, *bla_{IMP}*, *bla_{SIM}*, *bla_{GIM}*, *bla_{GES}*, *bla_{OXA-23}*, *bla_{OXA-48}*, and *bla_{OXA-}*
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38 198 genes responsible for carbapenemase production were investigated by conventional PCR as
39 previously described [22]. The primers used are shown in Table 1. Dream-Taq Green PCR
40 Master Mix (Thermo Fisher Scientific) was used for PCR tests. Amplification of bacterial DNA
41 was performed by using Gene Amp PCR System 9700 (Applied Biosystems, Les Ullis, France)
42 thermal cycler. To confirm the enzyme gene types and determine subtypes, nucleotide
43 sequencing was performed by using the Bigdye Terminator V3.1 cycle sequencing kit with an
44 automated DNA sequencing on ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The
45 nucleotide and deduced amino acid sequences were analysed with software available through
46 the internet (<http://www.ncbi.nlm.nih.gov/>).
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Table 1. Nucleotide sequences of the primers used in the amplification were from Dortet et al. [22].)

Gene	Primer	Nucleotide sequence (5'-3')
<i>bla_{KPC}</i>	KPC-A	CTG-TCT-TGT-CTC-TCA-TGG-CC
	KPC-B	CCT-CGC-TGT-GCT-TGT-CAT-CC
<i>bla_{NDM}</i>	NDM-F	GGT-TTG-GCG-ATC-TGG-TTT-TC
	NDM-R	CGG-AAT-GGC-TCA-TCA-CGA-TC
<i>bla_{VIM}</i>	VIM-2004A	GTT-TGG-TCG-CAT-ATC-GCA-AC
	VIM-2004B	AAT-GCG-CAG-CAC-CAG-GAT-AG
<i>bla_{IMP}</i>	IMP-2004A	ACA-YGG-YTT-GGT-DGT-TCT-TG
	IMP-2004B	GGT-TTA-AYA-AAA-CAA-CCA-CC
<i>bla_{SIM}</i>	SIM-1F	TAC-AAG-GGA-TTC-GGC-ATC-G
	SIM-1R	TAA-TGG-CCT-GTT-CCC-ATG-TG
<i>bla_{GIM}</i>	GIM-1A	GGA-GTA-TAT-CTT-CAT-ACC-TCC
	GIM-1B	TTC-CAA-CTT-TGC-CAT-GCC-CC
<i>bla_{GES}</i>	GES-1A	ATG-CGC-TTC-ATT-CAC-GCA-C
	GES-1B	CTA-TTT-GTC-CGT-GCT-CAG-G
<i>bla_{OXA-48 like}</i>	OXA-48A	TTG-GTG-GCA-TCG-ATT-ATC-GG
	OXA48B	GAG-CAC-TTC-TTT-TGT-GAT-GGC
<i>bla_{OXA-23}</i>	OXA-23A	GAT-GTG-TCA-TAG-TAT-TCG-TCG
	OXA-23B	TCA-CAA-CAA-CTA-AAA-GCA-CTG
<i>bla_{OXA-198}</i>	OXA-198-F	CTC-GAA-TTC-ATG-CAT-AAA-CAC-ATG-AGT-AAG
	OXA-198-R	CTC-AAG-CTT-TTA-TTC-GAT-GAT-CCC-CTT-T

Whole genome sequencing (WGS) and bioinformatic analysis

Total DNA was extracted from colonies using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, US) following the manufacturer's instructions. The DNA concentration and purity were controlled by a Qubit® 2.0 Fluorometer using the dsDNA HS

1 and/or BR assay kit (Life technologies, Carlsbad, CA, US). The DNA library was prepared
2 using the Nextera XT-v3 kit (Illumina, San Diego, CA, US) according to the manufacturer's
3 instructions and then run on Miseq (Illumina) for generating paired-end 150-bp reads. De novo
4 assembly with a minimum contig length of 300 bp was performed by CLC Genomics
5 Workbench v9.5 (Qiagen, Hilden, Germany) after quality trimming ($Q_s \geq 20$) with word size
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12 34. The acquired antimicrobial resistance genes were identified by uploading assembled
13 genomes to the Resfinder server v2.1 (<http://cge.cbs.dtu.dk/services/ResFinder-2.1>) [23]. The
14 multi locus sequence typing (MLST) was also obtained, by uploading assembled genomes to
15 MLST 1.8 [24] respectively from <https://cge.cbs.dtu.dk/services/>. The identification of the
16 plasmids was performed by uploading assembled genomes to PlasmidFinder 1.3 [25],
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Nucleotide sequence accession number

The Whole Genome Shotguns of Pyo24 and Pyo5 isolates have been deposited at
DDBJ/ENA/GenBank under the accession number SUNF00000000 and SUNG00000000,
respectively.

Detection of high-risk clones by MALDI-TOF MS

The presence of 5 main *P. aeruginosa* high-risk clones (ST111, ST175, ST235, ST253, and
ST395) was investigated for all 45 isolates using MALDI-TOF MS (Bruker Daltonics) as
described by Cabrolier *et al.* [26]. Analysis of the mass spectra was performed using the
spectrum view of flexAnalysis 3.4 software (Bruker Daltonics). The presence or absence of the
peak biomarkers specific to the high risk clones was analysed by visual comparison of the
specific spectra.

Results

Bacterial isolates

During the study period, a total of 585 clinical *P. aeruginosa* isolates responsible for infections have been documented in the laboratory information system, and 115 (20%) of them were reported as carbapenem resistant. Among these cases, 45 non-duplicate isolates that were still available in the laboratory's stocks could be included in the study. Of these isolates, 17 (38%) were from female patients, while 28 (62%) were from males. Of these patients, 13, 3, 2, 15, and 12 were aged between 0-5, 6-15, 16-29, 30-59, and >60, respectively. Most of the isolates were obtained from respiratory specimens (42%), followed by urine (20%), wound and soft tissue specimens (27%), blood (9%), and cerebrospinal fluid (2%). Most of the specimens were from ICU (45%), followed by surgical wards (22%), internal medicine wards (22%), and from outpatients (11%) of the hospital.

Antimicrobial susceptibility profile

Using E-tests, the range of MIC values for imipenem and meropenem were 4 - ≥ 32 μg /ml and 2 - ≥ 32 μg /ml, respectively. MIC50 and MIC90 values were determined as ≥ 32 and ≥ 32 $\mu\text{g}/\text{ml}$ for imipenem and 16 and ≥ 32 $\mu\text{g}/\text{ml}$ for meropenem. Among the carbapenem-resistant *P. aeruginosa* isolates, susceptibility to piperacillin-tazobactam was the highest, while susceptibility to aztreonam was the lowest. Antimicrobial susceptibility profiles of the isolates are displayed in Table 2.

Eighteen isolates were found ceftazidime-resistant. Among them, no synergy was evidenced for any isolate in the DDST with cloxacillin-containing agar, suggesting the absence of any ESBL.

Table 2. Antimicrobial Susceptibility Profiles of the Isolates determined using disk diffusion antibiograms

Overall (n = 45)

Antibiotics	S (%)	I (%)	R (%)
Imipenem ^a	2	0	98
Meropenem ^a	16	20	64
Amikacin	76	4	20
Aztreonam	9	42	49
Ciprofloxacin	62	0	38
Levofloxacin	51	0	49
Piperacillin-tazobactam	78	0	22
Ceftazidime	60	0	40
Cefepime	71	0	29
Gentamicin	67	0	33
Ticarcillin	40	0	60
Piperacillin	73	0	27
Netilmicin	53	0	47
Tobramycin	76	0	24
S, susceptible; I, intermediate; R, resistant.			
^a Determined using E-tests			

Twelve highly drug-resistant bacteria were further studied for susceptibility to ceftolozane/tazobactam combination. Three of these isolates were susceptible as revealed by MIC values interpreted according EUCAST criteria [19]. Nine *P. aeruginosa* isolates were resistant to ceftolozane/tazobactam as shown in Table 3.

Table 3. MIC values of 12 selected *P. aeruginosa* isolates for ceftolozane/tazobactam

	Susceptible						Resistant					
MIC (µg/ml)	≤ 0.125	0.25	0.5	1	2	4	8	16	32	64	128	≥ 256
No. of isolates	0	0	0	1	0	2	0	1	1	1	0	6
Total	3						9					

Carba NP and CIM Results

Carba NP and CIM tests were used to detect a carbapenem-hydrolyzing activity among the 45 carbapenem-resistant *P. aeruginosa* isolates. Two isolates (Pyo5 and Pyo24) revealed a positive carbapenemase-activity by both Carba NP and CIM tests, while the remaining 43 isolates gave negative test results with both tests. The isolate Pyo24 was obtained from a urine sample of a 14-year-old boy with neurogenic bladder dysfunction who had been hospitalized several times for recurrent urinary tract infection. The isolate was susceptible to ciprofloxacin, levofloxacin, and colistin, but resistant to all other antimicrobials tested, including ceftolozane/tazobactam with an MIC of >256 µg/ml. The isolate Pyo5 was isolated from a wound specimen of an 81-year-old woman with urinary incontinence and was resistant to all antimicrobials (including ceftolozane/tazobactam) except colistin, piperacillin, and piperacillin-tazobactam.

Molecular detection of carbapenemase genes

As phenotypic tests may sometimes miss weakly expressed carbapenemases, the 45 isolates were screened by PCR for the most common carbapenemases encountered in Gram-negative Bacilli (GNB) (*bla*_{KPC}, *bla*_{NDM}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{SIM}, *bla*_{GIM}, *bla*_{GES}, *bla*_{OXA-23}, *bla*_{OXA-}

198 and *bla*_{OXA-48}) [22]. PCR detection of carbapenemase encoding genes gave a positive result only for *bla*_{VIM} gene in isolate Pyo24, which was identified as carbapenemase-producers using the phenotypic tests. Sequencing of the PCR product of isolate Pyo24 revealed the presence of *bla*_{VIM-5} gene. For the second isolate (Pyo5) that demonstrated carbapenemase activity using phenotypic tests, despite repeated PCR attempts, the molecular results remained negative.

Genetic characterization

WGS data of the Pyo24 and Pyo5 *P. aeruginosa* isolates were analyzed using CLC genomic workbench and revealed genomes of 6932577 bp and 6824239 bp respectively, with a 70X coverage for both genomes.

The Pyo24 *P. aeruginosa* isolate belonged to ST-308 and the Pyo5 *P. aeruginosa* to the ST-357. Resistome was analyzed by searching acquired resistance genes and point mutations involved in resistance. According to <https://cge.cbs.dtu.dk/services/>, Pyo24 *P. aeruginosa* harbored three β -lactamase genes: a novel variant of the chromosomal and natural *bla*_{PAO-like} gene coding for a broad-spectrum cephalosporinase, *bla*_{OXA-488} coding for OXA-50 family oxacillin-hydrolyzing class D β -lactamase and *bla*_{VIM-5} coding for a carbapenemase. The isolate also expressed three aminoglycoside resistance determinants (*aph(3')-IIb*, *aadA1*, *aac(6')-II* genes), a phenicol resistance determinant (*catB7*), a sulfonamide resistant gene (*sul1*) and a chromosomal fosfomycin resistance gene (*fosA*) coding for a Mn(II)-dependent metalloenzyme (FosA) that catalyzes the addition of glutathione (GSH) to the broad-spectrum antibiotic fosfomycin [27].

The Pyo5 isolate presented four β -lactamase genes: chromosomal *bla*_{PAO-like} gene, *bla*_{OXA-50-like} coding for a variant of OXA-50 family oxacillin-hydrolyzing class D β -lactamase, *bla*_{OXA-2} coding for a broad-spectrum oxacillinase and an integron-located *bla*_{IMP-7} metallo-beta-lactamase gene [28]. The isolate also presented the quinolone resistance determinant *aac(6')*-

Ib-cr gene and four aminoglycoside resistance determinants (*aph(3')*-*Ib3*, *aac(6')*-*Ib3*, *aph(3'')*-*Ib* and *aph(6)*-*Id* genes).

Moreover, the Pyo5 isolate contained the recently described plasmid-encoded fluoroquinolone resistance determinant *crpP* gene [29], while Pyo24 isolate carried a novel variant of the *crpP* gene, that we named *crpP-2*. This novel variant coding for the CrpP-2, differed from the CrpP by two amino-acid substitutions, K4R and G7D. The *crpP-2* gene was present in a similar environment to the one described for *crpP* [29] except for the deletion of a phage regulatory *rha-like* gene and other regions upstream the *crpP-2* gene (Figure 1). The genetic environment of *crpP* gene present in the Pyo5 *P. aeruginosa*, also showed differences in respect to that already described by Chavez-Jacobo *et al* [29] (Figure 1).

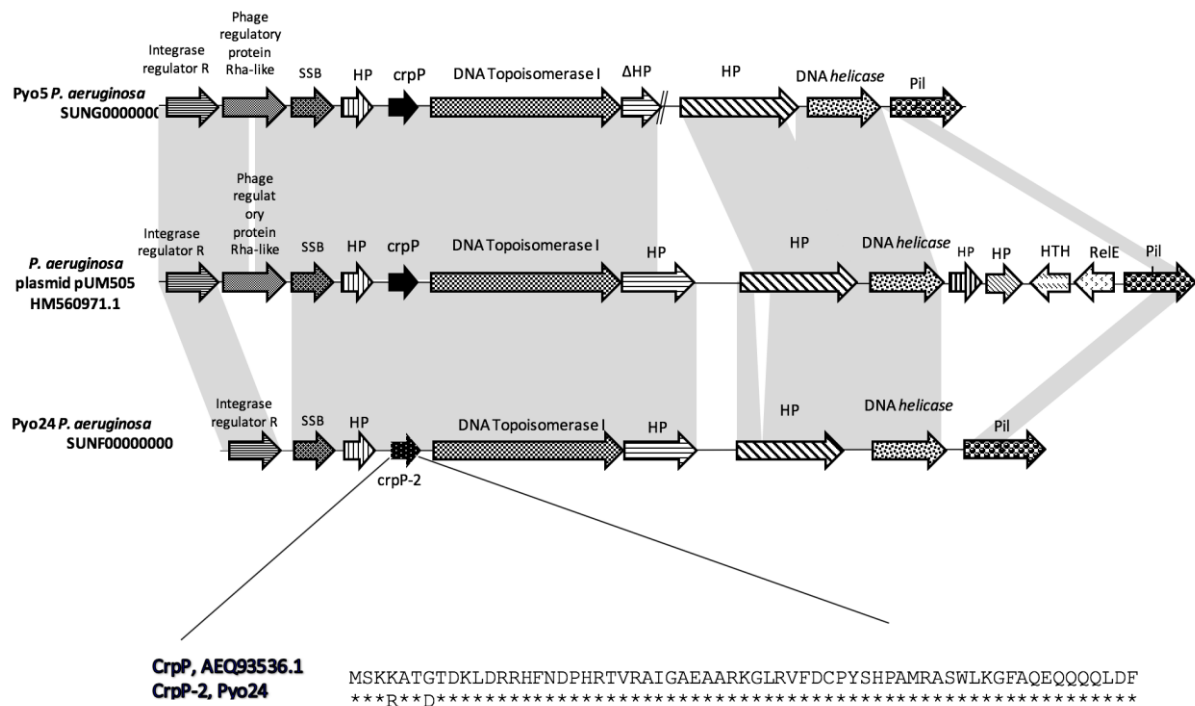
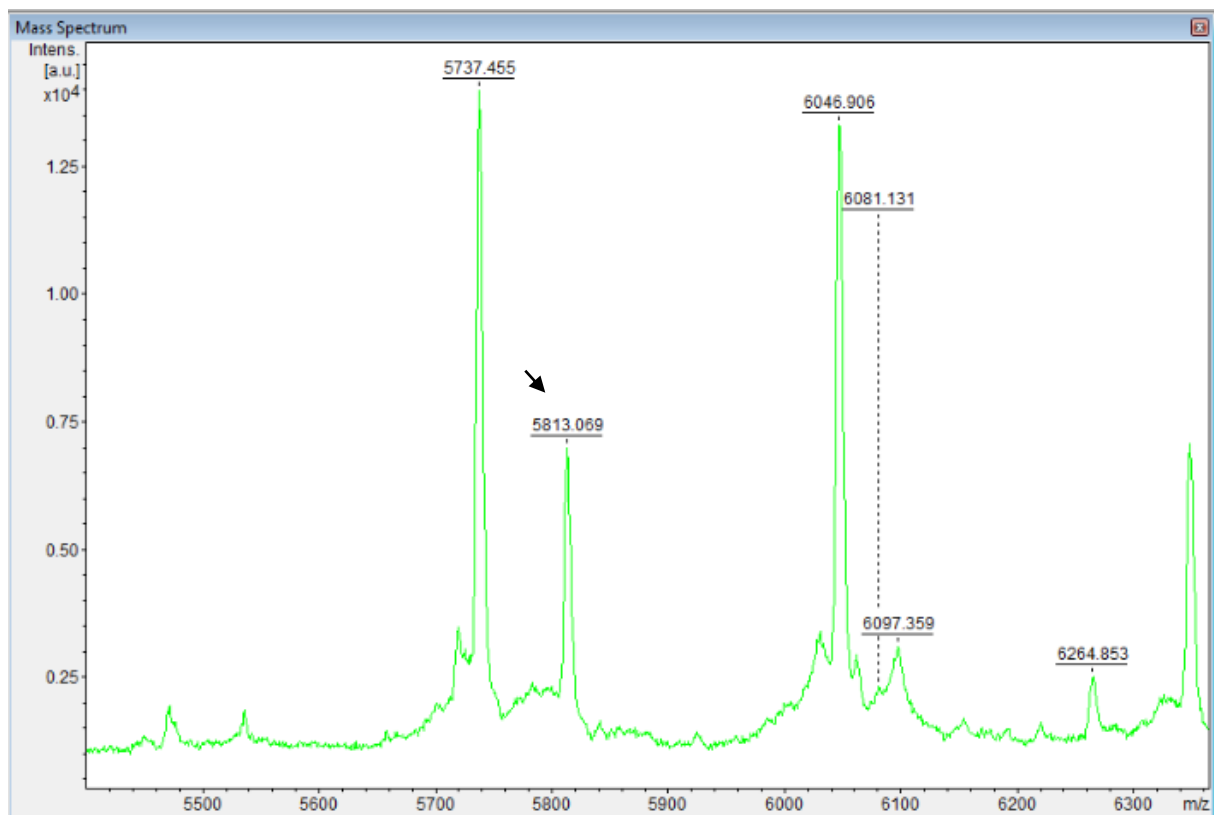


Fig. 1. Genetic environment of *crpP* genes and CrpP-2 amino acid sequence comparison with the CrpP protein. Sequence similarities are indicated by grey boxes.

MALDI-TOF MS analysis for high risk clones

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According to MALDI-TOF MS analysis of the 45 isolates, there were no characteristic peaks of high risk clones (ST111, ST175, ST235, ST253, and ST395) in the VIM-5 and IMP-7-type carbapenemase producing isolates. One non-carbapenemase producing isolate yielded peaks characteristic for ST253. It was recovered from a biopsy specimen of a 84 year-old female patient hospitalized in intensive care unit. The isolate was resistant to all antimicrobials except colistin. The peak biomarkers for ST253 are shown in Figure 2.



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Fig. 2. MALDI-TOF MS peak profile of the ST253 *P. aeruginosa* isolate. The relative intensities of the ions (in a. u. arbitrary units) are shown on the y axis, and the masses of the ions (in Da) are shown on the x axis. The m/z value represents mass to charge ratio. The arrow shows peak at m/z 5,813 which is specific to ST253.

66 Discussion

1 The fight against infectious diseases is probably one of the greatest public health
2 challenges faced by our society, especially with the emergence of carbapenemase-producing
3 gram-negatives being in some cases pan-drug resistant. The pandemic carbapenemase
4 disseminating worldwide in gram-negative organisms threatens to take medicine back into the
5 preantibiotic era since the mortality associated with infections caused by these “superbugs” is
6 very high due to limited treatment options. Resistance to carbapenems in *P. aeruginosa* is
7 mostly due to loss of OprD porins; however, the carbapenemase production is an important
8 mechanism of carbapenem resistance in *P. aeruginosa* that is increasingly identified worldwide.
9 That resistance trait is more worrisome since the majority of carbapenemase encoding genes
10 are in high-mobility genetic elements that may disseminate horizontally and impacts almost all
11 carbapenem molecules that are all hydrolyzed by those enzymes [30,31]. In the present study,
12 of the 45 carbapenem-resistant isolates, two isolates were found as positive for carbapenemase
13 production by phenotypic Carba NP and CIM tests. PCR tests for detection of the most common
14 carbapenemase genes remained negative for one of those two isolates (Pyo5). Carbapenemase
15 activity for this isolate was also detected by a commercial phenotypic test [RAPIDEC®
16 CARBA NP (bioMérieux, France)], suggesting the presence of a new carbapenemase encoding
17 gene or a carbapenemase currently undetectable by our PCR methods. Although molecular
18 detection by PCR is considered the gold standard for carbapenemase gene identification, false
19 negativity can be observed due to the presence of a carbapenemase gene not tested in the PCR
20 reaction, or mutations affecting annealing of primers [32]. At this point, fast and accurate
21 phenotypic methods for detection of carbapenemase activity are important for early
22 implementation of infection-control measures. Furthermore, these phenotypic methods don't
23 require expensive specialized equipment and can be easily performed in all medical
24 microbiological laboratories, making them more suited for screening purposes. Our results

1 showed that phenotypic Carba NP and CIM tests could represent an important contribution to
2 carbapenemase detection that was missed by PCR tests.
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5 Different classes of carbapenemases have been identified in *P. aeruginosa* mainly of the
6 VIM and IMP type metallo- β -lactamases [6]. So far, IMP-1-[10,11], IMP-9-[12], VIM-1-[9],
7 VIM-2-[9,10,12,13], VIM-5-[7], VIM-38-type metallo- β -lactamases [8,33], GES-1-[10],
8 GES-5-type class A carbapenemases [8,9] and OXA-10-, OXA-14- [10], OXA-23-, OXA-
9 40-, OXA-58-type [14] class D carbapenemases have been identified in *P. aeruginosa* from
10 Turkey. The VIM-5 that differs from VIM-1 by five amino acid substitutions was firstly
11 identified in a *P. aeruginosa* isolate in Turkey in 2004 [7]. After this report, there have been a
12 few reports of VIM-5-producing *P. aeruginosa* in India [34], Denmark (from a patient
13 previously hospitalised in India) [35], Myanmar [36] and Bangladesh [37]. To our knowledge,
14 this is the second report of a clinical isolate of *P. aeruginosa* carrying the *bla*_{VIM-5} gene in
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31 The IMP-7 identified in *P. aeruginosa* strain Pyo5 could not be detected by PCR tests
32 since the genes are very different from IMP-1. The *bla*_{IMP-7} gene was originally identified in *P.*
33 *aeruginosa* isolates in a nosocomial outbreak in Canada and was not detected with standard
34 *bla*_{IMP}-specific primers, due to mismatches in the forward primer [28]. Kouda *et al.* [38], who
35 reported first isolation of the *bla*_{IMP-7} gene in *P. aeruginosa* in Japan, also found the presence
36 of metallo- β -lactamase by a phenotypic test but could not detect *bla*_{IMP} gene using the PCR
37 with the *bla*_{IMP}-specific primers and needed further molecular methods such as pulsed-field gel
38 electrophoresis, southern hybridization, and DNA sequencing to identify the *bla*_{IMP-7} gene.
39 Some automated real-time PCR systems such as Xpert Carba-R Assay (Cepheid, Toulouse,
40 France) are unable to detect *bla*_{IMP-7} gene expressing bacterial strains [39], suggesting silent
41 spread of IMP-7 producers due to undertetection. Importantly, this type of IMP in *P. aeruginosa*
42 has never been previously reported from Turkey. Unlike most of the IMP types, which mostly
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1 have a defined geographical distribution, IMP-7 shows a global dissemination. So far, IMP-7
2 has been identified in patients in Canada [28], the Czech Republic [40], Japan [38], Malaysia
3 [41], Slovakia [42], Denmark [43], Saudi Arabia [44] and Australia [45]. Moreover, regional
4 spread of *P. aeruginosa* ST357 producing IMP-7 has been reported in central Europe [46,47].
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9 Our study would indicate a wider spread of the IMP-7-producing *P. aeruginosa* of ST357.
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14 In *P. aeruginosa*, international high-risk clones such as ST235, ST111, ST175, and
15 ST357 are responsible for epidemics of nosocomial infections by MDR strains worldwide [46-
16 48]. Another high-risk emerging clone, ST308, has been described in France, Germany, and
17 Singapore [51-54]. Here we report the presence of ST308 and ST357 epidemic clones among
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P. aeruginosa isolates for the first time from Turkey. Since high risk clones of *P. aeruginosa* play a major role in the spread of resistance, early detection of these clones should have major consequence for infection control, helping to prevent their spread in the hospital environment. A method described by Cabroler et al [26] was able to accurately and quickly detect several *P. aeruginosa* high-risk clones, including ST235, ST111, ST175, ST253 and ST395, using the MALDI-TOF MS. With this method, one non-carbapenemase producing isolate in our collection yielded peaks characteristic for ST253. The ST253 is known as a globally spread clone that previously had been detected in *P. aeruginosa* from various animal, human and environmental samples [55-61].

66 In the present study, genetic characterization of the carbapenemase producing *P.*
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aeruginosa strains also revealed the presence of novel resistance determinants such as *crpP* (ciprofloxacin resistance protein, plasmid encoded) and *crpP-2* genes. The CrpP enzyme encoded by the pUM505 plasmid was recently described by Chavez-Jacobo *et al* [29]. It was isolated from a clinical isolate of *P. aeruginosa* and was shown to confer an increase in ciprofloxacin MIC value when conjugated to *E. coli* J53-3 strain. It is suggested that the CrpP

1 decreases ciprofloxacin susceptibility through the enzymatic phosphorylation of the antibiotic
2 [29]. In a subsequent study, Chavez-Jacobo *et al.* reported the presence of the *crpP* gene in
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4 ESBL-producing enterobacterial isolates and ESBL-producing transconjugants with plasmids
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6 from clinical isolates, suggesting the spread of *crpP* genes among hospital bacteria including
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8 other Gram-negative species [62].
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11 Ceftolozane/tazobactam is a new second-generation cephalosporin/ β -lactamase
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13 inhibitor combination with activity against *P. aeruginosa*, including drug-resistant strains, and
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15 other common gram-negative bacteria, including ESBL producing Enterobacteriaceae strains
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17 [63]. Ceftolozane/tazobactam was cleared in 2014 by the Food and Drug Administration for the
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19 treatment of complicated intraabdominal infections and complicated urinary tract infections. In
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21 many studies, it has been reported that ceftolozane-tazobactam retained good activity against
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23 MDR isolates of *P. aeruginosa* [63-67]. In the present study, ceftolozane/tazobactam retained
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25 activity against 3 of the 12 carbapenem resistant *P. aeruginosa* isolates, with MIC50 and MIC90
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27 values of 64 and 256 μ g/ml, respectively. Carbapenemase-producing isolates (VIM-5 and IMP-
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29 7) were highly resistant (MIC > 256 mg/L) to ceftolozane/tazobactam, which supports the stated
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31 lack of antimicrobial activity of the antibiotic against carbapenemase producers by Cho *et al.*
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33 and Pazzini *et al.* [68-69]. Despite the limited number of isolates tested for ceftolozane-
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35 tazobactam, our results are useful for evaluating the efficiency of ceftolozane-tazobactam
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37 therapy on carbapenem-resistant *P. aeruginosa* isolates in Turkey. Further extensive studies are
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39 needed to define ceftolozane-tazobactam's activity against *P. aeruginosa* isolates in our area.
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41 Carbapenem resistant *P. aeruginosa* isolates should be further tested for
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43 ceftolozane/tazobactam susceptibility as it can be an alternative therapy for infections with
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45 multidrug resistant and extremely drug resistant GNB.
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56 In conclusion, this study reveals new information about carbapenemases, high-risk
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58 clones, and other resistance determinants among *P. aeruginosa* isolates in Turkey. Detection of
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1 carbapenemase producers by reliable phenotypic methods can provide effective infection
2 control surveillance with the ability of detecting unknown carbapenemases. Although we
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4 detected a low prevalence of carbapenemase genes among the study strains, detection of these
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6 carbapenemases is still important as they can easily disseminate among the strains of the
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8 bacterium. As this is the first report of IMP-7 producing *P. aeruginosa* from Turkey, caution
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10 must be taken against further spread of this type of carbapenemase in our area. Our findings
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12 illustrates the particular threat of *P. aeruginosa* high-risk clones with multiple resistance
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14 determinants in a tertiary care hospital in Turkey in a time of dearth of new antibiotics for the
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16 treatment of infections caused by these bacteria.
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