MICROBIAL DRUG RESISTANCE
Volume 21, Number 3, 2015

DOI: 10.1089/mdr.2014.0172

Mechanisms of Carbapenem Resistance in Multidrug-Resistant Clinical Isolates of *Pseudomonas aeruginosa* from a Croatian Hospital

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Pseudomonas aeruginosa is an opportunistic pathogen, one of the leading causes of nosocomial infections such as pneumonia, urinary tract infections, and bacteraemia. The bacterial resistance to structurally unrelated antibiotics and its spread within hospitals limits the efficient antimicrobial options and patients' outcome. Carbapenems are important agents for the therapy of infections due to multidrug-resistant (MDR) *P. aeruginosa*; hence, the development of carbapenem resistance severely hampers effective therapeutic options. The aim of this investigation was to examine mechanisms of carbapenem resistance and genomic diversity in carbapenem-resistant MDR strains of *P. aeruginosa*, which caused an outbreak among patients in Clinical Hospital Rijeka. Most of the isolates showed decreased expression of porin that is important for the entry of carbapenems (*oprD*). Overexpression of MexAB-OprM, MexCD-OprJ, and MexEF-OprN efflux systems was observed in many of the isolates. Production of metallo-β-lactamases was not detected. Typing by pulsed-field gel electrophoresis discriminated the isolates into five clusters. The clonal distribution of the strains was related to the location of hospital departments where the isolates were collected, which implies that most of the infections were caused by spread of the epidemic strains within the hospital.

Introduction

PSEUDOMONAS AERUGINOSA is an important opportunistic pathogen, characterized by environmental versatility and ability to cause a wide range of human infections in susceptible individuals. In addition to being intrinsically resistant to several antimicrobials, this bacterial species often acquires various mechanisms of resistance to other antibiotics.[38] Antibiotic selection pressure represents the leading risk factor for multidrug-resistant (MDR) acquisition. MDR is defined as ''acquired nonsusceptibility to at least one agent in three or more antimicrobial categories (aminoglycosides, carbapenems, antipseudomonal cephalosporins, fluoroquinolones, antipseudomonal penicillins/β-lactamase inhibitors, monobactams, phosphonic acids, polymyxins).''[27]

The increasing frequency of MDR *P. aeruginosa* strains in hospital environment is a major concern due to limited treatment options. Since carbapenems are important agents for the therapy of infections caused by MDR *P. aeruginosa*, the development of carbapenem resistance additionally reduces effective therapeutic options. Resistance to carbapenems can be due to decreased uptake, increased efflux, inactivation enzymes, or modified target.[25] However, the resistance patterns to individual carbapenems can vary considerably among clinical strains as a result of complex interactions between listed nonenzymatic and enzymatic mechanisms.

Carbapenems (imipenem [IPM], and to a certain degree also meropenem [MEM] and doripenem [DOR]) enter into the periplasmic space of bacteria through the OprD outer membrane porin. Thus, reduced expression of OprD porin mainly leads to imipenem resistance.[30] Although the loss of specific porin channels also increases minimum inhibitory concentrations (MICs) of meropenem and doripenem, clinical resistance to these drugs is thought to require additional mechanisms such as upregulation of efflux pump expression or overproduction/mutation of AmpC cephalosporinase.[18,24] MexAB-OprM, MexCD-OprJ, and MexEF-OprN efflux pump systems have broad substrate specificity, extruding many antibiotic classes, including β-lactams, fluoroquinolones, and aminoglycosides.[36] Besides, redundancy frequently exists among transporters—fluoroquinolones, for example, are universal substrates for the main efflux systems.

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Over the previous decade, an increasing number of *P. aeruginosa* isolates producing carbapenem-hydrolysing enzymes have been reported from various parts of the world, including Europe, and four major families of metallo-β-lactamases (MBLs) have been identified (VIM, IMP, SPM, and GIM families). Although it is still rare, global prevalence of MBL-expressing *P. aeruginosa* strains has increased steadily, and isolates expressing VIM enzymes have been detected in different regions of Croatia.[3,35] In addition, *Klebsiella pneumoniae* carbapenemase (KPC)-producing *P. aeruginosa* isolates were recently reported in Europe.[14]

Recently, an increase in the prevalence of carbapenem-resistant *P. aeruginosa* isolates has been observed at the Clinical Hospital Centre Rijeka. This study was conducted to determine the underlying mechanisms that conferred the carbapenem resistance phenotype, and to examine the clonal relationships of isolates.

Materials and Methods

Bacterial isolates

A total of 38 nonduplicate *P. aeruginosa* isolates with reduced susceptibility to at least one of the carbapenems were collected from hospitalized patients at Clinical Hospital Centre Rijeka, during the second half of 2009 and the beginning of 2010, and identified by API 20NE (bioMérieux, Marcy l'Etoile, France). Isolates were collected from patients admitted to several wards (two intensive care units [ICUs], cardiology, gastroenterology, neurology, neurosurgery, pulmonology, pediatric, and surgery) of three geographically distinct hospital locations. Strains isolated from endotracheal aspirates, sputum, urine, and pus were serotyped by slide agglutination using commercially obtained antisera according to recommendation of the manufacturer's protocol (Bio-Rad, Marnes-la-Coquette, France).

Antimicrobial susceptibility

For antimicrobial susceptibility testing (AST), strains were cultured at 37°C for 24 hr in Mueller–Hinton broth (MHB; BD Difco, Le Pont de Claix Cedex, France), and inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard. *P. aeruginosa* ATCC 27853 was used as a quality control strain. MIC of the antibiotics was determined using Etest according to the manufacturer's and EUCAST guidelines.[39] MIC breakpoints were derived from EUCAST.[39] The following antibiotics were tested: MEM, IPM, DOR, amikacin (AMK), gentamicin (GEN), tobramycin (TOB), ciprofloxacin (CIP), cefepime (FEP), ceftazidime (CAZ), and colistin (CST) from the AB Biodisk (Solna, Sweden). MIC_{50} and MIC_{90} levels were defined as the lowest concentration of the antibiotic at which 50% and 90% of the isolates were inhibited, respectively.

The inducible AmpC activity was tested phenotypically using disk approximation technique. Imipenem (10 μg), cefoxitin (30 μg), and amoxicillin-clavulanate (20/10 μg) disks are used as the inducing antibiotics, and ceftazidime (30 μg) disks (Bio-Rad) as the reporter antibiotic. Disks were applied by use of an applicator at a distance of 20 mm, and any obvious blunting or flattening of the zone of inhibition between the ceftazidime disk and the inducing antibiotic was interpreted as a positive result for AmpC.

Inhibitory effects of CCCP against efflux pumps

Efflux pump overexpression was initially screened for by testing susceptibility to imipenem and meropenem in the presence and absence of an efflux pump inhibitor, carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP). AST was performed by agar dilution method with and without CCCP added in MH agar at concentrations of 12.5 mM. Drugs and CCCP were obtained from Sigma-Aldrich Corp. (St. Louis, MO).

Detection of MBLs

Screening for MBL production was done by Etest® MBL commercial strips according to the manufacturer's instructions (AB Biodisk). The Etest MBL strip contains a double-sided dilution range of imipenem and imipenem combined with EDTA. The test was considered positive if a reduction of imipenem MIC by threefold or more twofold dilutions was observed in the presence of EDTA.

Polymerase chain reaction (PCR) analyses for detection of MBLs and KPC carbapenemase genes were carried out for all strains. Template DNA was prepared by boiling method. Specific primers to detect the presence of: bla_{IMP}, bla_{VIM}, bla_{GIM} and $bla_{SPM-1,}$ $bla_{NDM,}$ and bla_{KPC} were used in PCR mixtures (50 μl final volumes) containing 25 μl of PCR master mix, 20 μl of ultrapure water, 1 μl of each primer (Sigma-Aldrich), and 3 μl of template DNA. All primers are listed in Table 1.

Pulsed-field gel electrophoresis

P. aeruginosa strains were genotyped by pulsed-field gel electrophoresis (PFGE) of *Spe*I restricted fragments. PFGE was performed as previously described,[16] with our modifications. Briefly, bacteria were grown overnight in BHI medium, washed, resuspended in saline EDTA (SE) buffer (75 mM NaCl, 25 mM EDTA), and embedded in agarose by mixing 150 μl of bacterial suspension and 150 μl of 2% low melting point agarose solution in SE buffer. Agarose plugs were incubated overnight in Lysis solution 1 (6 mM Tris-HCl pH 7.5, 100 mM EDTA pH 7.5, 1 M NaCl, 0.5% Brij 58, 0.2% sodium dodecyl sulfate [SDS], 0.5% lauryl sarcosine, and 0.5 mg/ml lysosim), washed twice for 15 min with 50 mM EDTA pH 9.5, and then incubated overnight in Lysis solution 2 (1% lauryl sarcosine, 500 mM EDTA pH 9.5, and 0.5 mg/ml proteinase K). Agarose plugs were washed with TE buffer (10 mM Tris pH 7.5, 10 mM EDTA pH 7.5) thrice for 30 min at 4°C. After this, the wash plugs were sliced in ~1–2 mm slices, that were digested with 20 U of *Spe*I restriction endonuclease (Fermentas, Vilnius, Lithuania) at 37°C overnight, and then transferred to 0.5×TBE buffer, loaded on the comb, and run in 1.2% agarose gel in CHEF-DR® III pulsed-field electrophoresis system (Bio-Rad, Hercules, CA) at 6 V/cm, 120° field angle, 14°C, with initial and final switch times between 3 and 36 sec for 26 hr. Gels were analyzed with GelCompar II software (Applied Maths NV, Sint-Martens-Latem, Belgium). A dendrogram showing clonal relationship of the isolates was constructed using the unweighted-pair-group method of the arithmetic average clustering with the Dice similarity coefficient (optimization 1%, tolerance 1%, and tolerance change 0.5%).

Table 1. Primers Used for Polymerase Chain Reaction and Reverse-Transcription Quantitative PCR

Gene	*Primer*	*Oligonucleotide sequence*	*Reference*
VIM-1[a]	VIM-1-F	CAGATTGCCGATGGTGGTTGG	Lauretti *et al.*[20]
	VIM-1-R	AGGTGGGCCATTCAGCCAGA	
IMP[a]	IMP-A	GAAGGYGTTTATGTTCATAC	Watanabe *et al.*[42]
	IMP-B	GTAMGTTTCAAGAGTGATGC	
SPM[a]	SPM-F	CCTACAATCTAACGGCGACC	Toleman *et al.*[40]
	SPM-R	TCGCCGTGTCCAGGTATAAC	
GIM[a]	GIM-F	AGAACCTTGACCGAACGCAG	Castanheira *et al.*[5]
	GIM-R	ACTCATGACTCCTCACGAGG	
NDM-1[a]	NDM-F	AATGGAATTGCCCAATATTATGC	Yong *et al.*[44]
	NDM-R	CGAAAGTCAGGCTGTGTTGC	
KPC[a]	KPC-F	TGTCACTGTATCGCCGTC	Yigit *et al.*[43]
	KPC-R	CTCAGTGCTCTACAGAAAACC	
Ribosomal protein S12 reference gene[b]	rpsL-1	GCTGCAAAACTGCCCGCAACG	Islam *et al.*[12]
	rpsL-2	ACCCGAGGTGTCCAGCGAACC	
oprD[b]	oprD-F	GCTCGACCTCGAGGCAGGCCA	Rodriguez-Martinez *et al.*[34]
	oprD-R	CCAGCGATTGGTCGGATGCCA	
mexAB[b]	mexB-1	CAAGGGCGTCGGTGACTTCCAG	Islam *et al.*[12]
	mexB-2	ACCTGGGAACCGTCGGGATTGA	
mexCD[b]	MxD-F	GGACGGCTCGCTGGTCCGGCT	Rodriguez-Martinez *et al.*[34]
	MxD-R	CGACGAAGCGCGAGGTGTCGT	
mexEF[b]	mexF-F	CGCCTGGTCACCGAGGAAGAGT	El Amin *et al.*[8]
	mexF-R	TAGTCCATGCCTTGCGGGAAGC	

[a]Primers used for PCR.
[b]Primers used for RT-qPCR.
RT-qPCR, reverse-transcription quantitative polymerase chain reaction.

Real-time RT-PCR

The expression of the genes coding for the efflux pumps proteins MexAB, MexCD, MexEF, as well as outer membrane protein OprD, was analyzed using reverse-transcription quantitative PCR (RT-qPCR). Total RNA was extracted from all strains using the High Pure RNA Isolation kit (Roche, Mannheim, Germany), and reverse transcribed into cDNA using the first-strand cDNA Synthesis Kit for RT-PCR (Roche). The latter was subjected to qPCR using LightCycler FastStart DNA Master SYBR Green I (Roche) in the LightCycler 2.0 LC (Roche). Relative expression of the efflux pumps, and *oprD* genes in clinical isolates was determined by comparing mRNA levels of the genes in the clinical isolates and control *P. aeruginosa* ATCC 27853 strain, which is sensitive to all tested antibiotics. The gene coding for the ribosomal protein S12 (rpsl) was used as a reference gene for normalizing the transcription levels of target genes. Relative quantification was done by comparative C_t method ($2^{-\Delta\Delta Ct}$).[23] According to previously published criteria, isolates were considered as having MexAB efflux pump overexpression if the mRNA level of *mexB* gene was at least threefold higher in the isolate than in reference ATCC 27853 strain, negative if lower than twofold, and borderline if between twofold and threefold.[4] Isolates were considered positive for MexCD and MexEF overexpression if the level of mRNA for *mexD* or *mexF* were at least 10-fold higher than in reference ATCC strain, negative if lower than 5-fold, and borderline if between 5- and 10-fold.[4] Isolates were considered having reduced *oprD* expression if the mRNA levels were more than twofold lower than in the reference ATCC strain 27853.[29] We used primers for RT-qPCR that amplified the region of *oprD* mRNA between nt. 516 and 718 of *oprD* sequence from PAO1 (corresponds to the region between aa. 172 and 238 in the OprD protein). Primers used for qPCR are listed in Table 1.

Results

Clinical characteristics and antimicrobial susceptibility of isolates

Most of the isolates were obtained from patients of the ICUs (70%), and mostly isolated from endotracheal aspirates (87%). Serotype O:11 was the most prevalent, accounting for ~47% of the isolates, followed by serotype O:1 (29%). Six isolates were the rare serotypes O:6, O:7/8, O:15, and O:16, while two isolates were untypeable or polyagglutinable (Fig. 1). All of 38 isolates were resistant to meropenem and doripenem, and in general, intermediary resistant to imipenem (Table 2). Nine isolates were imipenem resistant. Almost all isolates displayed resistance to the other groups of anti-pseudomonas antibiotics, with 79% being MDR (30 out of 38 isolates). All isolates belonging to different serotypes were MDR *P. aeruginosa*, except seven O:1 isolates that showed sensitivity to the most of the tested antibiotics. *P. aeruginosa* strains demonstrated high-level resistance to gentamicin (79%), amikacin (66%), tobramycin (79%), and ciprofloxacin (71%). Ten strains (26%) showed AmpC β-lactamase activity by phenotypic testing. Table 3 depicts the MIC_{50} and MIC_{90} values of clinical isolates. According to MIC_{90} values, colistin was found to be the most active agent (0.38 μg/ml), followed by cefepime (8 μg/ml) and ceftazidime (12 μg/ml). The MIC values of colistin were below the resistance breakpoints.

Detection of MBLs

Although eight isolates were suspected to be MBL positive by the MBL Etest, PCR analyses did not detect MBLs

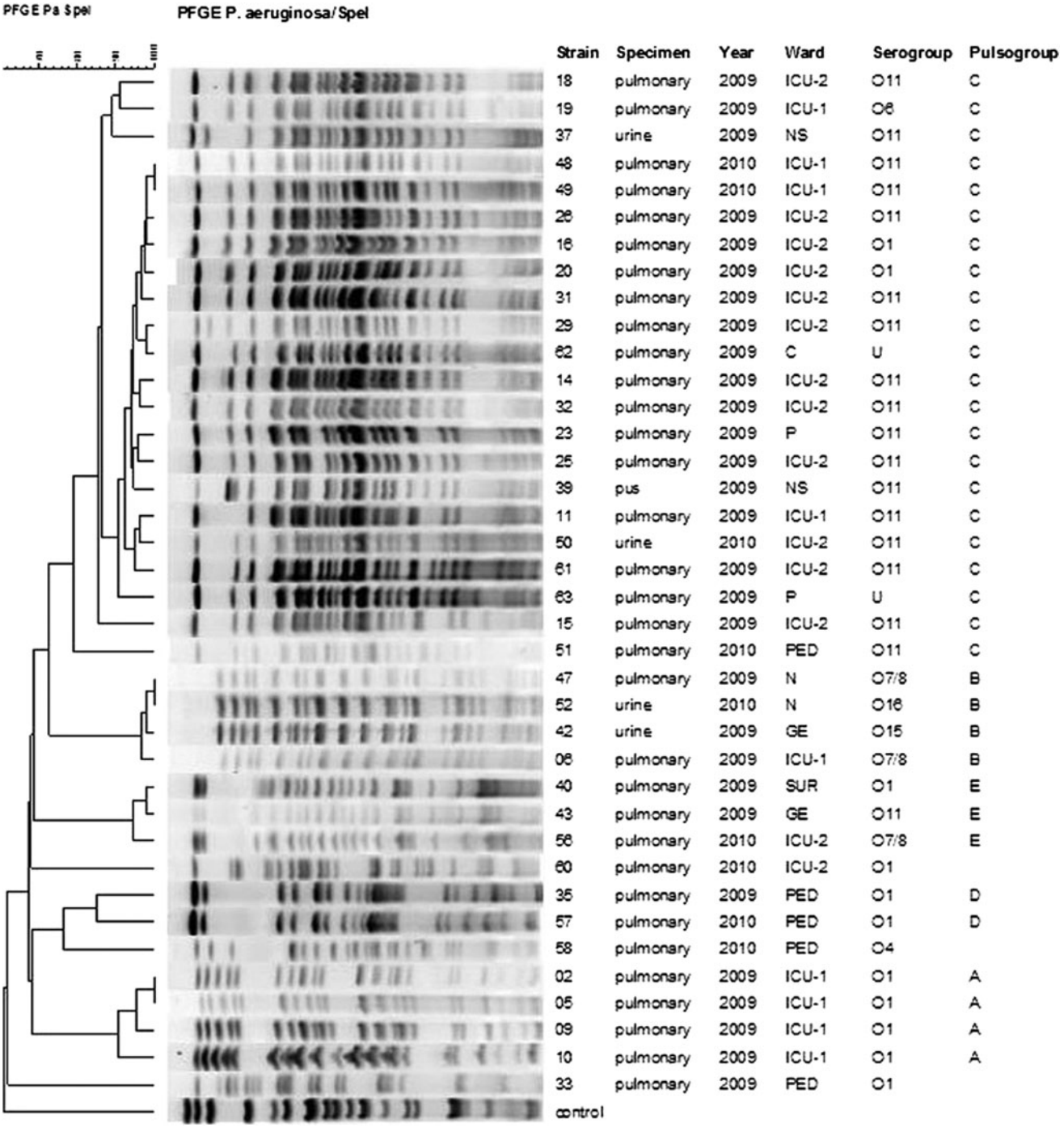

FIG. 1. Dendrogram showing the clonal relationship of *Pseudomonas aeruginosa* clinical strains, generated from the PFGE profiles. C, cardiology; GE, gastroenterology; ICU, intensive care unit; N, neurology; NS, neurosurgery; P, pulmonology; PED, pediatric; PFGE, pulsed-field gel electrophoresis; SUR, surgery; U, untypeable.

of either IMP, VIM, GIM, SPM, or NDM family (data not shown). PCR for KPC genes was also negative.

Synergy between CCCP and carbapenems

A degree of synergy was observed between efflux pump inhibitor and tested carbapenems. The MIC levels of meropenem showed twofold to eightfold reduction in the presence of CCCP in 32 (84%) out of 38 isolates, but CCCP did not allow complete restoration of meropenem activity according to relatively high meropenem MICs (8–64 μg/ml). MIC levels of imipenem showed mainly a twofold reduction in the presence of CCCP in 22 (58%) out of 38 isolates (data not shown).

Pulsed-field gel electrophoresis

Among 38 isolates, obtained mainly from patients of ICU, and mostly isolated from respiratory specimens, PFGE analysis revealed 35 distinct profiles (Fig. 1). Isolates were grouped in five clusters based on $>80\%$ similarity criterion,

TABLE 2. ANTIMICROBIAL SUSCEPTIBILITY OF *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES

		MIC (μg/ml)									
Strain[a]	*Sero type*	*IPM*	*MEM*	*DOR*	*CAZ*	*FEP*	*GEN*	*AMK*	*TOB*	*CIP*	*CST*
2	O1	8	>32	4	4	8	3	4	1.5	0.047	0.19
5	O1	12	>32	6	2	3	1.5	3	0.75	0.19	0.25
6	O7/8	4	>32	4	2	2	24	4	24	0.19	0.38
9	O1	8	>32	6	3	4	3	4	2	0.38	0.25
10	O1	8	>32	6	2	3	1.5	3	1	0.25	0.032
11	O11	8	>32	8	4	4	96	12	24	>32	0.125
14	O11	8	>32	24	3	3	>256	16	32	>32	0.19
15	O11	4	>32	4	3	4	96	192	>256	24	0.19
16	O1	6	16	12	3	4	128	12	32	>32	0.25
18	O11	8	>32	12	6	6	>256	32	96	>32	0.125
19	O6	6	>32	16	6	4	>256	24	48	>32	0.25
20	O1	8	>32	12	24	8	>256	16	48	>32	0.25
23	O11	8	16	8	48	6	>256	16	64	>32	0.38
25	O11	8	>32	32	4	4	>128	12	32	>32	0.25
26	O11	8	>32	12	4	4	>256	24	48	>32	0.19
29	O11	4	>32	12	6	3	64	12	32	>32	0.38
31	O11	8	>32	12	8	4	96	12	48	>32	0.25
32	O11	8	>32	16	6	6	>256	12	48	>32	0.19
33	O1	12	>32	3	2	2	3	8	1.5	0.25	0.125
35	O1	12	>32	32	2	8	2	8	2	0.25	0.25
37	O11	8	>32	12	4	4	>256	24	64	>32	0.19
39	O11	6	>32	8	12	3	>256	24	64	>32	0.125
40	O1	12	12	3	1	2	>256	8	96	>32	0.19
42	O15	8	>32	6	3	3	24	4	16	0.5	0.25
43	O11	6	8	8	1.5	3	>256	4	48	>32	0.25
47	O7/8	6	>32	8	3	2	16	2	16	0.094	0.19
48	O11	8	>32	8	3	3	192	16	64	>32	0.19
49	O11	6	>32	8	3	3	192	12	96	>32	0.25
50	O11	8	>32	8	4	6	64	64	>256	>32	0.19
51	O11	6	>32	4	4	3	64	12	64	>32	0.25
52	O16	6	>32	4	0.25	1.5	16	3	12	0.064	0.125
56	O7/8	64	>32	6	6	6	>256	24	48	>32	1
57	O1	8	>32	4	4	12	3	8	1.5	0.25	0.25
58	O4	12	>32	3	12	8	4	12	2	2	0.094
60	O1	12	>32	24	256	48	>256	12	>256	24	0.125
61	O11	8	>32	8	3	3	64	96	>256	>32	0.25
62	U	16	>32	24	4	6	>256	12	32	>32	0.19
63	U	12	>32	24	8	6	>256	16	64	>32	0.25

[a]AmpC-producing strains are underlined; multidrug-resistant strains are indicated in boldface.
AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; DOR, doripenem; FEP, cefepime; GEN, gentamicin; IPM, imipenem; MIC, minimum inhibitory concentration; MEM, meropenem; TOB, tobramycin; U, untypeable.

TABLE 3. MIC RANGE, MIC_{50}, MIC_{90} (IN μG/ML), AND SUSCEPTIBILITY OF *P. AERUGINOSA* CLINICAL ISOLATES

	MIC (interpretation)			Susceptibility
Antibiotic	*MIC range*	*MIC_{50}*	*MIC_{90}*	*(%)*
IPM	4 to >64	8 (I)	12 (R)	5
MEM	6 to >32	>32 (R)	>32 (R)	0
DOR	2 to >32	8 (R)	32 (R)	0
CAZ	0.25 to >256	4 (S)	12 (R)	87
FEP	1.5 to >48	4 (S)	8 (S)	95
GEN	1.5 to >256	28 (R)	256 (R)	21
AMK	2 to >192	12 (I)	24 (R)	34
TOB	0.75 to >256	48 (I)	256 (R)	21
CIP	0.047 to >32	>32 (R)	>32 (R)	29
CST	<0.032 to 1	0.25 (S)	0.38 (S)	100

with three singleton strains. The largest cluster designated C comprised 22 out of 38 isolates (58%). There were two more clusters (A, B) comprising four isolates, one cluster with three, and one with two isolates. The dominant cluster C consisted mainly of O:11 isolates (77%) found in ICU-2, and neurosurgery, while cluster A consisted entirely of O:1 isolates (100%) observed only in ICU-1. Cluster B was more heterogeneous, consisting of O:7/8, O:15, and O:16 isolates.

Real-time RT-PCR

The expression of *mexB, mexD, mexF*, and *oprD* genes was measured using real-time PCR. Significant (the mRNA level of *oprD* of the isolate less than 50% of the mRNA level in reference ATCC strain) downregulation of *oprD* expression was observed in 87% (31 out of 38) of isolates (Table 4). Whether the lower expression of *oprD* was due to

Table 4. Expression of Genes *mexB*, *mexD*, *mexF*, and *oprD* in *P. aeruginosa* Clinical Strains Relative to the Respective Referent *P. aeruginosa* ATCC 27853 Strain

Strain[a]	*Gene transcription (fold* P. aeruginosa *ATCC)* mexB	mexD	mexF	oprD	*Imipenem susceptibility*
ATCC	1.00	1.00	1.00	1.00	S
2	7.41[b]	2.20	2.84	0.04[b]	I
5	3.68[b]	5.85[c]	12.56[b]	0.21[b]	R
6	0.59	0.68	4.38	0.32[b]	S
9	5.83[b]	2.01	1.62	0.05[b]	I
10	4.75[b]	4.89	18.58[b]	0.48[b]	I
11	2.64[c]	1.28	4.45	0.07[b]	I
14	1.23	1.68	16.26[b]	0.07[b]	I
15	1.22	0.57	3.80	0.12[b]	S
16	2.06[c]	2.04	3.66	0.27[b]	I
18	2.58[c]	3.45	5.33[c]	0.29[b]	I
19	3.11[b]	3.19	3.64	0.34[b]	I
20	2.74[c]	2.39	11.21[b]	0.47[b]	I
23	1.99	1.26	4.65	0.13[b]	I
25	2.57[c]	0.84	3.54	0.12[b]	I
26	1.33	1.46	6.20[c]	0.23[b]	I
29	1.95	1.24	7.32[c]	0.15[b]	S
31	1.31	0.77	1.27	0.13[b]	I
32	2.61[c]	0.59	2.02	0.13[b]	I
33	5.79[b]	1.50	6.72[c]	0.08[b]	R
35	6.66[b]	0.58	4.44	0.86	R
37	1.42	0.86	1.87	0.14[b]	I
39	1.17	0.25	0.81	0.09[b]	I
40	0.70	1.43	4.36	0.33[b]	R
42	0.37	2.23	1.01	0.31[b]	I
43	0.67	2.08	4.29	2.59	I
47	0.98	5.24[c]	2.88	2.63	I
48	1.69	2.56	4.63	0.28[b]	I
49	3.74[b]	2.76	3.80	0.43[b]	I
50	2.74[c]	1.33	4.34	0.14[b]	I
51	2.59[c]	3.96	6.88[c]	0.29[b]	I
52	0.83	4.97	14.89[b]	0.68	I
56	0.56	2.76	4.29	0.84	R
57	2.22[c]	3.21	3.53	1.48	I
58	0.39	6.12[c]	3.40	0.99	R
60	0.80	18.06[b]	2.48	0.04[b]	R
61	0.59	3.45	4.93	0.25[b]	I
62	1.11	0.74	3.99	0.10[b]	R
63	1.70	7.48[c]	4.98	0.23[b]	R

[a]AmpC-producing strains are underlined.
[b]Increases in gene expression of *mexD* and *mexF* >10-fold, increases in gene expression of *mexB* >3-fold, and decreases in gene expression of *oprD* <2-fold were taken as positive.
[c]Increases in gene expression of *mexD* and *mexF* between 5- and 10-fold, and increases in gene expression of *mexB* between 2- and 3-fold were taken as borderline.
I, intermediate; R, resistant; S, susceptible.

the gene ORF mutation, promoter variation, or regulator mutation was beyond the scope of this study. The genes *mexB*, *mexD*, and *mexF* were upregulated in 45%, 11%, and 26% isolates, respectively. Simultaneous upregulation of two efflux pump genes (*mexB* and *mexF*) was identified in five isolates, while one isolate showed overexpression of all three efflux pumps (Table 4). We considered the isolates with the borderline values of overexpression of mexB, mexD, and mexF as having upregulated efflux pump expression, as the results of synergy between efflux pump inhibitor CCCP and carbapenems showed that almost all of the isolates with borderline values of efflux pump mRNAs showed the reduction of MICs for imipenem, meropenem, or both in the presence of CCCP (data not shown). There was no correlation between antibiotic susceptibility phenotype and expression of efflux pump genes.

Discussion

The aim of this study was to detect the resistance mechanisms and clonal relatedness in carbapenem-resistant *P. aeruginosa* that caused hospital outbreak in several wards at the Clinical Hospital Center Rijeka, a tertiary care, university Croatian hospital with 1,191 inpatients and 26 ICU beds. All isolates were resistant to meropenem and doripenem, and in general, intermediary resistant to imipenem, 79% being MDR. Although isolates with acquired carbapenemases have been reported recently in Croatia and adjacent regions,[3,9,15] this resistance mechanism was not detected in clinical strains studied here. Five strains were positive for MBL in phenotypic test but did not yield any product with primers specific for MBL. It is possible that they were false positive in phenotypic test or they posses some rare type of MBL such as AIM, DIM, or FIM that was not sought in this study.

Efflux activity was initially screened for by testing susceptibility to carbapenems with and without an efflux pump inhibitor. Efflux pump inhibitor reverts MICs of resistant strains to those that do not express efflux systems.[41] In this study, synergy observed between CCCP and meropenem suggested that its extrusion by the efflux systems contributes to the resistance. Molecular method (RT-qPCR) used to detect the expression of efflux pumps and OprD porin genes revealed overproduction of one or more putative efflux pumps genes in 63% of the isolates. In fact, most of the examined clinical isolates had simultaneous downexpression of *oprD* and increased efflux pumps expression, in addition to AmpC production detected by phenotypic method. Although resistance to meropenem in *P. aeruginosa* is principally efflux pump mediated, *oprD* downregulation observed in 87% of strains could explain the relatively high meropenem MICs in the presence of CCCP. It could be postulated that both, overexpression of efflux systems, identified in more than half of the isolates, and downregulation of *oprD* expression, observed in the most of the strains, contributed to meropenem resistance. There was no correlation between antibiotic susceptibility phenotype and expression of efflux pump genes. However, in half of the isolates with reduced susceptibility to imipenem, besides the downregulated *oprD* expression, increased expression of at least one of the efflux pumps was observed. Although other studies have shown that beside ceftazidime, imipenem is the least affected among β-lactams by efflux pumps,[28] efflux mechanism and/or AmpC production are possibly involved in the imipenem resistance of several strains with normal *oprD* expression. It is known that imipenem consumption select loss of porins, while meropenem utilization leads to efflux pumps hyperexpression.[18,25,30,36] Before and during the outbreak in our hospital, especially in ICUs, twice higher consumption of meropenem in comparison to imipenem was observed, explaining more frequent meropenem resistance in *Pseudomonas* isolates. Although doripenem was not used in our hospital, all isolates

were resistant to doripenem according to EUCAST breakpoints. In accordance with results of Riera *et al. in vitro* study, we noticed lower doripenem than meropenem MICs, but better activity of doripenem than meropenem could only be speculated.[33]

The susceptibility to ceftazidime and also to cefepime observed in most of the carbapenem-resistant isolates implies the absence of resistance mechanisms against these β-lactams. It has been previously demonstrated that strains with downexpressed OprD porins may remain susceptible to β-lactam agents that penetrate via other porins.[32] Apart from carbapenem resistance, efflux pumps of *P. aeruginosa* are known to be an extremely important cause of multidrug resistance in the hospital settings.[25,36]

An increase in the prevalence of carbapenem-resistant *P. aeruginosa* observed at our hospital during the previous decade can be partly attributed to widespread empirical use of ciprofloxacin, which was the most commonly used antibiotic. Ciprofloxacin consumption, expressed as the number defined daily doses (DDDs) per 100 bed days (BD), increased about 41% from 2006 (2.35 DDD/100BD) to 2010 (3.30 DDD/100BD). We assume that increased consumption of ciprofloxacin in our hospital led to not only increased quinolone but also carbapenem resistance. Previous clinical studies support this concept. Restriction of ciprofloxacin in a teaching hospital was associated to decreased resistance of *P. aeruginosa* isolates to antipseudomonal carbapenems as reported by Lewis *et al.*[22] Cook *et al.* showed in an observational study correlation of ciprofloxacin use with the percentage of *P. aeruginosa* isolates resistant to the meropenem and imipenem.[6] Several other studies have suggested that the use of fluoroquinolones may select strains resistant not only to quinolones but also to other structurally unrelated antibiotics.[7,21] Authors reported selection of mutants that overexpress the MexAB-OprM pump during the course of quinolones or cephalosporins therapy.[45] In *Pseudomonas* spp., induction of efflux pumps is associated with reduced levels of OprD porin. Therefore, quinolone use may lead to cross-resistance to carbapenems, either by increased efflux (meropenem and doripenem) or by decreased porin entry (imipenem, meropenem, and doripenem).

Our study recognized serotype O:11 as an important problem in our hospital because of the resistance to multiple antibiotics. Current evidence suggests that in Croatia, similarly to the other Mediterranean countries, most *P. aeruginosa* strains belong to the O:11 serotype frequently associated with the international epidemic clone ST235.[10,26] The next most frequent serotype in our hospital was O:1. Interestingly, while all isolates belonging to O:11 and other observed serotypes were MDR, the majority of O:1 isolates maintained sensitivity to the most of the antibiotics. In this study, PFGE analysis defined five major clusters of isolates. The largest cluster C correlated with O:11 serotype, and resistance pattern to gentamicin, tobramycin, and ciprofloxacin as all the isolates from the largest cluster C were resistant, while the isolates from clusters A, B, and D were susceptible to those three antibiotics. Cluster A consisted entirely of antibiotic-sensitive O:1 isolates. Unlike clusters A and C, cluster B was more heterogeneous comprising O:7/8, O:15, and O:16 isolates. This suggests that serotyping can lead to a misleading classification of the isolates. The inadequacy of serotyping to confirm a clonal relation among MDR isolates has been previously reported.[13,31,35] It has been demonstrated that antimicrobial therapy was able to induce serotype conversion in *P. aeruginosa*, leading to clustering of isolates with different serotypes.[17]

Although downregulation of OprD porin was found in all isolates belonging to A and C clusters, there was no correlation between PFGE type and antibiotic susceptibility to carbapenems. Our molecular epidemiological study has revealed that two clones, included in clusters A and C, caused outbreaks in ICUs. Distribution of strains showed correlation with the other locations of hospital departments where the isolates were collected, which implies that most of the infections are due to spread of several clones within the hospital.

Many *P. aeruginosa* outbreaks in hospitals of environmental origin have been reported in the previous few years.[1,2,11,19,37] Although we performed extensive environmental sampling, we were unable to identify an environmental source of infection. *P. aeruginosa* strains were isolated from two environmental specimens collected—cleaning mop and hand washing tap, but the isolates differed from the outbreak strains in both antibiotic resistance and PFGE profiles (data not shown). However, clustering of isolates may be due to either patient-to-patient transmission or a common source of acquisition (*e.g.*, contaminated respiratory therapy equipment).

Hospital infections caused by MDR *P. aeruginosa* are frequently life threatening due to lack of effective antimicrobial treatment. Our study showed oligoclonal outbreak of *P. aeruginosa* with several mechanisms involved in carbapenem resistance—the low outer membrane permeability combined with efflux system overexpression. Increased consumption of ciprofloxacin and carbapenems, especially meropenem, has probably contributed to the emergence and spread of these mechanisms. Availability of local epidemiological data and bacterial resistance mechanisms are necessary to decrease the risk of treatment failure, and aid in establishing infection control programs, as well as to prevent global dissemination of the resistant strains.

Acknowledgment

This work was supported by ''Molecular mechanisms of bacterial pathogenesis and response to stress'' (M.A., grant number 13.06.1.1.07. from the University of Rijeka).

Disclosure Statement

All authors disclose no commercial associations that might create a conflict of interest in connection with this study.

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