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AmpC and MexAB-OprM Hyperexpression are Most Common Mechanisms Among Ceftazidime and Imipenem Resistant *Pseudomonas aeruginosa* from Chinese Hospitals

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SENTRY ANTIMICROBIAL SURVEILLANCE

AMENDED ABSTRACT

Objective: To evaluate ß-lactam resistance mechanisms among ceftazidime (CAZ)- and imipenem (IMI)-resistant *P. aeruginosa* (PSA) strains collected during 2011 in Chinese hospitals. CAZ- and IMI-resistance rates among PSA from China (30.3 and 26.5%, respectively) were remarkably greater than rates noted in the United States (16.9 and 19.8%, respectively).

Methods: 212 PSA clinical isolates collected in 2011 from 11 Chinese hospitals were susceptibility tested. Isolates were further evaluated if CAZ and IMI MICs were ≥16 and 1 mg/L, respectively. MICs for CAZ, IMI, other carbapenems, aztreonam and cefepime were determined ± of PAßN (efflux inhibitor) and/or cloxacillin (AmpC inhibitor). Screening for ß-lactamases was performed by PCR/sequencing. Expression of chromosomal (c) *ampC*, *mexA*, *mexC*, *mexE* and *mexX* and *oprD* was determined using high quality RNA in triplicate reactions by qRT-PCR using an endogenous control and values compared to *P. aeruginosa* PAO1. Clonality was evaluated by PFGE and MLST.

Results: 12 isolates collected in 7 hospitals were analyzed showing variable resistance rates against ß-lactams. Lower MICs were noted with PAßN, but smaller differences were observed using cloxacillin. Five isolates exhibited lower MICs when efflux and AmpC inhibitors were tested together. Four isolates possessed metallo-ß-lactamases (MBLs; 1 IMP-9, 3 VIM-2; Table) and 2 VIM-producers hyperexpressed cAmpC ± *mexA* and had decreased *oprD* expression. One PSA had high levels of *mexC* and low *oprD* transcripts and another of *mexX* + *cAmpC*, but the majority (8) had elevated *cAmpC* ± *mexA* transcription, 7 with decreased *oprD* expression. No isolates had significant differences in *mexE*. ESBLs PER, PSE, and OXA-30 were detected among 3 PSA. Ten sequence types (STs) were identified among 12 isolates displaying 11 unique PFGE types. Two PSA from one hospital had the same PFGE and ST profile and two PSA from different hospitals had the same ST profile despite the distinct PFGE patterns.

Conclusions: AmpC ± MexAB-OprM hyperexpression, usually associated with decreased *oprD* transcription were the most prevalent resistance mechanisms among genetically diverse PSA from Chinese hospitals, but other resistance determinants such as MBLs and increased MexCD-OprJ or MexXY-OprM were also observed. These results emphasize the prevalence of intrinsic resistance mechanisms among Chinese PSA, but also highlights that multiple factors might contribute to elevated PSA resistance rates in this country.

PFGE	ST	ß-lactamase	Pump effect[a]	AmpC effect[b]	Relative expression (compared to PAO1)[c]					
					ampC	*mexA*	*mexC*	*mexE*	*mexX*	*oprD*
F	1335		+++	-	2.689E+00	7.759E+00	4.248E+01	1.264E-01	1.780E+00	2.217E-02
A	1336		++	+	1.035E+02	4.157E+01	8.133E-01	1.805E-01	1.513E+00	6.985E-02
D	257		++	+	2.640E+02	8.760E+01	8.913E+00	2.672E-01	2.590E+00	8.221E-02
C	1026	IMP-9, OXA-30	-	-	9.808E-01	1.070E+00	1.308E+00	3.947E-02	5.472E-01	3.552E-02
A	1336	VIM-2	+[d,e]	+[d,e]	1.174E+03	1.551E+01	4.567E-01	3.862E-02	8.420E+00	4.947E-03
G	1338	VIM-2, PER-1/-5, PSE-1	++	-	1.814E+00	7.238E+00	NT[g]	1.249E-01	1.065E+01	1.556E-01
K	1339		+[d,e]	+[d,e]	2.953E+03	7.499E+00	5.059E-01	3.091E-01	1.651E+01	2.139E+00
H	1212		++	+[f]	4.676E+01	3.439E+00	NT[g]	1.053E-01	9.357E+00	4.127E-01
J	697		++[d,e]	+[d,e]	1.752E+02	4.183E+01	6.532E-01	2.044E-01	5.852E-01	1.347E-01
B	1337		++[e]	+[e]	3.865E+01	1.469E+01	1.754E+00	1.152E-01	1.576E+00	1.336E+00
E	360		++[d,e]	++[d,e]	1.082E+02	1.416E+01	8.495E-02	8.277E-02	8.282E+00	7.396E-01
I	697	VIM-2, PER-1/-5	+	+	1.563E+01	2.879E+00	1.717E-01	4.503E-02	2.327E+00	4.850E-02

a. Difference between MIC$_4$PA8N.
b. Difference between MIC$_4$cloxacillin.
c. Values highlighted are >10-fold and considered significant.
d. Cephalosporins and aztreonam only.
e. Combined effect.
f. Carbapenems only.
g. NT=not tested.

INTRODUCTION

Carbapenem resistance among *Pseudomonas aeruginosa* clinical isolates has increased worldwide and this problem has become a significant public health concern, due to the limited therapeutic options available to treat infections caused by multi-drug resistant *P. aeruginosa* isolates.

Acquired carbapenem resistance in *P. aeruginosa* is often associated with acquired Ambler class B metallo-ß-lactamase (MßL) production and more recently, KPC serine-carbapenemases have been detected in this species. However, among the majority of *P. aeruginosa* strains, carbapenem resistance is considered to be the result of the interplay between low permeability, activity of the efflux pumps, as well as activity of an inducible or derepressed chromosomal ß-lactamase (AmpC). Earlier studies proved that the outer membrane protein OprD in *P. aeruginosa* could be utilized by imipenem and meropenem but could not be significantly utilized by other ß-lactams, quinolones or aminoglycosides, and its reduced expression is frequently noted in carbapenem-resistant isolates. The MexAB-OprM system is expressed in almost all *P. aeruginosa* isolates, and substrates for this pump include fluoroquinolones, tetracycline, chloramphenicol and ß-lactams. *P. aeruginosa* strains overexpressing MexAB-OprM are reported to be more resistant to meropenem but not to imipenem. Additionally, inducible AmpC can be upregulated by subinhibitory concentrations of certain ß-lactam antibiotics and further mutations can occur in the regulatory components of AmpC leading to stable hyperproduction of AmpC with concomitant high-level resistance to many classes of ß-lactam antibiotics.

In this study, we evaluated acquired and intrinsic ß-lactam resistance mechanisms among twelve *P. aeruginosa* isolates displaying elevated MIC values for ceftazidime and imipenem collected during 2011 in six Chinese hospitals.

MATERIALS AND METHODS

Bacterial strains. A total of 212 *P. aeruginosa* isolates were collected from 11 Chinese hospitals and susceptibility tested using the broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI). Categorical interpretations for all antimicrobials were those found in M100-S23 and quality control (QC) was performed using *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. All QC results were within specified ranges as published in CLSI documents. Twelve isolates displaying ceftazidime and imipenem MIC values ≥16 and 1 mg/L were further evaluated.

Screening for acquired ß-lactamases. Isolates were screened for the presence of bla_{KPC}, bla_{SME}, bla_{GES}, bla_{NMC-A}, bla_{IMI}, bla_{IMP}, bla_{VIM}, bla_{SPM-1}, bla_{GIM-1}, bla_{SIM-1}, bla_{AIM-1}, bla_{KHM-1}, bla_{NDM}, bla_{DIM-1} and bla_{BIC-1} in four separate multiplex polymerase chain reactions. PCR reactions for bla_{GES}, bla_{VEB}, bla_{PER}, bla_{PSE} and oxacillinases with ESBL spectrum (bla_{OXA-2}-, bla_{OXA-10}- and bla_{OXA-30}-group, bla_{OXA-18} and bla_{OXA-45}) were also performed. All PCR experiments included reactions containing target DNA templates for each screening primer set utilized. Amplicons generated were sequenced on both strands; nucleotide and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Madison, Wisconsin, USA). Amino acid sequences were compared with those available through the internet using NCBI/BLAST.

Phenotypic detection of hyperexpression of efflux pumps and cephalosporinase activity. Doripenem, imipenem and meropenem MIC values were determined in the presence of efflux pump inhibitor (EPI) phenyl-arginine-ß-naphthylamide (PAßN; at 100 mg/L) and the cephalosporinase (AmpC) inhibitor cloxacillin (concentration at 250 mg/L). Susceptibility testing was performed by broth microdilution method according to the CLSI guidelines.

Expression analysis of the chromosomally encoded *AmpC*, efflux pumps and porins. The expression of *ampC*, *mexA (*MexAB-OprM), *mexC* (MexCD-OprJ), *mexE* (MexEF-OprN), *mexX* (MexXY-OprM) and *oprD* was determined by quantitative real-time PCR (qRT-PCR) using DNA-free RNA preparations. Total RNA was extracted from mid-log-phase bacterial cultures (cell density at OD_{600} of 0.3-0.5) using RNA Protect Reagent and RNeasy Mini Kit (Qiagen, Hilden, Germany) in the Qiacube workstation (Qiagen) and residual DNA was eliminated with RNase-free DNase (Promega, Wisconsin, USA). Quantification of mRNA and sample quality was assessed using the RNA 6000 Pico kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) according to manufacturer instructions. Only preparations with RNA integrity number (RIN) >6.5 that showed no visual degradation were used for experiments. Relative quantification of target genes was performed in triplicate by normalization to an endogenous reference gene (*rpsL*) on the StepOne Plus instrument (Life Technologies, Carlsbad, California, USA) using custom designed primers showing efficiency >98.0%. Transcription levels were considered significantly different if at least a 10-fold difference was noted compared with *P. aeruginosa* PAO1.

Molecular typing. Pulsed-field gel electrophoresis (PFGE) of the *P. aeruginosa* isolates was performed using previously described procedures. Genomic DNA was prepared in agarose blocks and digested with SpeI (New England, Beverly, Massachusetts, USA) and resolved in the CHEF-DR III (BioRad, Richmond, California, USA) using running conditions described elsewhere. Results were analyzed by GelCompar II software (Applied Math, Kortrijk, Belgium). Percent similarities were identified on a dendrogram derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 1.2% and 0.5%, respectively. Multilocus sequence typing (MLST) method, including PCR amplification, bi-directional sequencing, and ST assignment was performed in accordance with the *P. aeruginosa* PubMLST website (http://pubmlst.org/paeruginosa/).

RESULTS

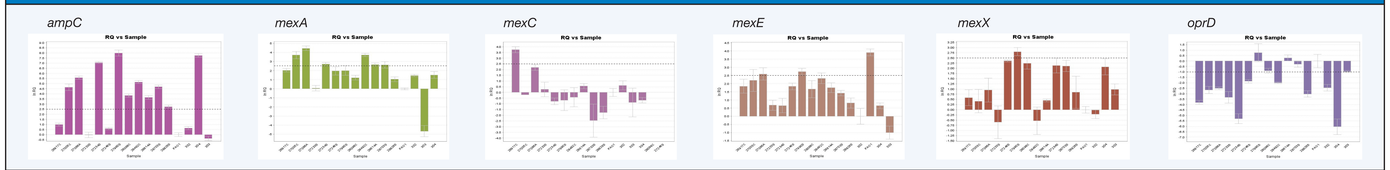
- Twelve isolates collected from Chinese hospitals during 2011 showing variable resistance to carbapenems and cephalosporins were analyzed. Isolates displayed 11 unique PFGE types and 10 ST types (**Table 1**). Two PSA from one hospital had the same PFGE and ST profile and two PSA from different hospitals had the same ST profile despite the distinct PFGE patterns.
- Four isolates possessed MßLs: one IMP-9 and three VIM-2-producers. These strains were from Shenzhen or Shanghai (one VIM-2). The IMP-9-producing strain also carried OXA-30 and VIM-2-producing *P. aeruginosa* harboured this MßL alone or with PER-1/-5 with or without PSE-1.
- Imipenem and meropenem MIC values decreased in the presence of PAßN for one strain. One strain displayed a modestly lower MIC for meropenem in the presence of this efflux inhibitor. PAßN showed greater inhibition when tested combined with ceftazidime, cefepime and aztreonam for 8, 6 and 7 strains, respectively. Cloxacillin, an AmpC inhibitor, produced no MIC reductions when compared to the ß-lactam tested alone.
- Several isolates displayed lower MIC values when tested against the antimicrobial agent in the presence of PAßN and cloxacillin combined. In 22 instances, both inhibitors enhanced the effect of PAßN alone and this was more frequent with the carbapenems (13 occurrences; **Table 1**).
- Hyperexpression of AmpC was observed in 9 isolates (**Figure 1**), five also displaying elevated transcription levels of MexAB-OprM and decreased expression of *oprD*, including one VIM-2-producing strain. The remaining isolates had elevated AmpC expression associated with hyperexpression of MexAB-OprM, MexXY-OprM or reduced expression or *oprD* alone (2, 1 and 1 isolates, respectively).
- One strain showing remarkable MIC reductions in the presence of PAßN displayed high MexCD-OprJ (**Figure 1**) expression levels and low OprD transcription.
- Isolates harbouring genes encoding IMP-9 and VIM-2 with PER-1/-5 and PSE-1 from Shenzhen displayed reduced transcription levels of *oprD* that were significantly lower than the baseline strain (**Figure 1**).
- Two isolates produced no amplification with MexCD-OprJ primers and were not analyzed. Furthermore, *P. aeruginosa* PAO1 had high levels of MexEF-OprN and using a different baseline strain, two isolates evaluated had elevated expression of this efflux system (**Figure 1**).

Table 1. Molecular typing and susceptibility patterns of ceftazidime and/or carbapenem-resistant isolates from Chinese hospitals. Testing using cloxacillin is not displayed due to no significant differences observed.

				MIC (mg/L):[a]													
Isolate	PFGE	ST	City	Imipenem	Imipenem + PAßN	Imipenem + PAßN + cloxacillin	Meropenem	Meropenem + PAßN	Meropenem+ PAßN + cloxacillin	Ceftazidime	Ceftazidime+ PAßN	Ceftazidime + PAßN + cloxacillin	Cefepime	Cefepime + PAßN	Cefepime + PAßN + cloxacillin	Aztreonam	Aztreonam + PAßN	Aztreonam + PAßN + cloxacillin
26917	F	1335	Beijing	8	≤0.03	≤0.03	8	≤0.03	≤0.03	16	≤0.03	≤0.03	32	≤0.03	≤0.03	64	≤0.03	≤0.03
27086	A	257	Beijing	32	8	1	32	8	4	64	4	1	32	4	2	64	4	2
28614	J	1337	Beijing	16	4	0.5	16	4	2	64	8	1	16	4	1	64	4	2
28703	I	360	Beijing	1	1	256	2	2	64	128	8	NT	32	4	NT	64	8	NT
27005	D	1336	Shenzhen	16	4	0.5	32	4	4	64	4	1	16	4	1	64	4	2
27233[b]	C	1026	Shenzhen	2	4	2	32	32	32	>256	128	128	>256	32	16	4	1	0.5
27234[c]	A	1336	Shenzhen	32	8	1	32	8	4	128	16	2	32	8	4	128	16	8
27246[d]	G	1338	Shenzhen	256	64	64	128	32	16	>256	>256	256	256	32	16	>256	256	256
28098	K	1212	Hangzhou	16	4	1	16	4	2	256	32	2	32	8	1	256	16	4
28492	H	697	Zhengzhou	1	1	0.5	1	2	1	128	16	2	32	8	2	64	16	4
27990	B	1339	Jilin	1	1	0.5	1	2	1	64	32	2	16	8	2	128	32	2
28826[e]	E	697	Shanghai	>256	256	0.06	128	64	0.5	64	32	NT[f]	32	16	NT	32	8	NT

a. MIC differences of 8-fold are highlighted in blue and >8-fold are also in red.
b. Isolate harboured genes encoding IMP-9 and OXA-30.
c. Isolate harboured gene encoding VIM-2.
d. Isolate harboured genes encoding VIM-2, PER-1/-5 and PSE-1.
e. Isolate harboured genes encoding VIM-2 and PER-1/-5.
f. NT= not tested.

Figure 1. Relative expression of genes encoding chomossomal AmpC, efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM and OprD. *P. aeruginosa* strain PAO1 was used as baseline for all genes, except for *mexE* for which expression levels on PAO1 seem too elevated. Expression levels were considered significantly different from baseline if changes >10X or <10X (*oprD* only) were observed and the threshold is represented by dotted lines.



CONCLUSIONS

- Resistance mechanisms were very diverse among 12 ceftazidime- and carbapenem-non-susceptible *P. aeruginosa* from Chinese hospitals. Four isolates harboured acquired ß-lactamases, including MßLs and in most cases, hyperexpression of AmpC ± MexAB-OprM and *oprD* decreased expression was present.
- Phenotypic results of efflux pump and cloxacillin inhibitors did not correlate with the genotypes established by quantitative RT-PCR, but a much better correlation was noted when both inhibitors were tested in combination.

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