Diagnostic Microbiology and Infectious Disease 79 (2014) 373–377

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Molecular epidemiology of *Pseudomonas aeruginosa* clinical isolates from Korea producing β-lactamases with extended-spectrum activity

Il Kwon Bae [a], Borum Suh [b], Seok Hoon Jeong [c,*], Kang-Kyun Wang [d], Yong-Rok Kim [d], Dongeun Yong [c], Kyungwon Lee [c]

[a] Department of Dental Hygiene, Silla University, Busan, Republic of Korea
[b] Department of Laboratory Medicine, MizMedi Hospital, Seoul, Republic of Korea
[c] Department of Laboratory Medicine and Research Institute of Bacterial Resistance, Yonsei University College of Medicine, Seoul, Republic of Korea
[d] Department of Chemistry, Yonsei University, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 30 October 2013
Received in revised form 11 February 2014
Accepted 9 March 2014
Available online 17 March 2014

Keywords:
Metallo-β-lactamase
Extended-spectrum β-lactamase
Extended-spectrum oxacillinase
Clonal complex 235 (CC235)

ABSTRACT

This study was performed to investigate the prevalence and molecular epidemiology of *Pseudomonas aeruginosa* isolates from Korea that produce enzymes with extended-spectrum (ES) activity to β-lactams. A total of 205 non-duplicate *P. aeruginosa* clinical isolates were collected from 18 university hospitals in Korea. PCR and sequencing experiments were performed to identify genes encoding β-lactamases. PCR mapping and sequencing of the regions surrounding the β-lactamase genes were performed. Multilocus sequence typing experiments were performed. The most common sequence type (ST) was ST235 (n = 96), and 2 single-locus variants of ST235, ST1015 (n = 1) and ST1162 (n = 1), were also identified. These 3 STs were grouped as a clonal complex (CC), CC235. The remaining 107 isolates were identified as 59 different STs. Isolates belonging to CC235 showed higher rates of non-susceptibility to imipenem (85.4% versus 47.7%) and meropenem (92.7% versus 52.3%) compared to non-CC235 isolates. All the metallo-β-lactamase (MBL)–producing isolates were identified as CC235, except for 1 ST591. Genes encoding OXA-17 and OXA-142 were detected in 1 isolate and 4 isolates of CC235, respectively; while the bla_{SHV-12} gene was detected in 4 non-CC235 isolates. Class A and D β-lactamases with ES activity play a role in acquiring ceftazidime resistance in *P. aeruginosa* in Korea. Production of IMP-6 and VIM-2 MBLs is the main mechanisms in acquiring resistance to ceftazidime and carbapenems in *P. aeruginosa* isolates in Korea. Clonal spread of *P. aeruginosa* CC235 may be an important conduit for the dissemination of MBL genes in Korea.

1. Introduction

Pseudomonas aeruginosa is an important nosocomial pathogen and is notorious for its ability to develop resistance to multiple classes of β-lactam drugs. Multidrug resistance has been associated with hyperproduction of derepressed AmpC enzymes, reduced permeability or overexpression of drug efflux systems, and the acquisition of genes encoding transferable β-lactamases (Livermore, 2002). β-Lactamases with extended-spectrum (ES) activity have been increasingly detected in clinical isolates of *P. aeruginosa*. These enzymes are classified as class A clavulanic acid–inhibited ES β-lactamases (ESBLs), class B metallo-β-lactamases (MBLs), and class D ES-oxacillinase (ES-OXAs) (David et al., 2008; Fournier et al. 2010; Juan et al., 2009; Ryoo et al., 2010; Weldhagen et al., 2003).

Previous studies that documented multilocus sequence typing (MLST) experiments on *P. aeruginosa* clinical isolates have shown that the worldwide increase of multidrug-resistant (MDR) clinical isolates is caused by the dissemination of a few MDR clones. MDR clones belonging to sequence type (ST) 111, ST175, ST235, and ST357 are widespread in European countries (Cabot et al., 2012; Edalucci et al., 2008; Hrabák et al., 2011; Koutsogiannou et al., 2013), and strains belonging to ST235, ST298, and ST773 have been identified in Asian countries (Kim et al., 2013; Kitao et al., 2012; Yousefi et al., 2013). In order to gain a better perspective on the prevalence and molecular epidemiology of *P. aeruginosa* producing enzymes with ES activity to β-lactams, we carried out a nationwide investigation of *P. aeruginosa* isolates in Korea.

2. Materials and methods

2.1. Bacterial isolates

A total of 205 non-duplicate *P. aeruginosa* clinical isolates were collected from 18 university hospitals in 8 provinces of Korea between July and August 2008. Species identification was performed using the Vitek GNI card (bioMérieux, Marcy l'Étoile, France) and 16S rRNA gene sequencing.

* Corresponding author. Tel.: +82-2-2019-3532; fax: +82-2-2057-8926.
E-mail address: kscpjsh@yuhs.ac (S.H. Jeong).

http://dx.doi.org/10.1016/j.diagmicrobio.2014.03.007
0732-8893/

2.2. Antimicrobial susceptibility testing

Susceptibility to piperacillin, piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, amikacin, gentamicin, tobramycin, and ciprofloxacin were tested by disk diffusion assays following CLSI guidelines (CLSI, 2012). MICs of ceftazidime, imipenem, and meropenem were determined using the Etest (bioMérieux). *P. aeruginosa* ATCC 27853 was used as the control strain. The presence of carbapenemase was screened using the modified-Hodge (MH) test, and MBLs were screened by imipenem and EDTA–sodium mercaptoacetic acid double-disk synergy (IEDDS) tests on MacConkey agar plates as described previously (Lee et al., 2003).

2.3. Identification of β-lactamase genes

Genes encoding β-lactamases were amplified using primers described previously (Seok et al., 2011). Whole cell lysates were used as templates for PCR amplification, and amplified products were directly sequenced using both DNA strands with an automatic sequencer (model 3730*xl*; Applied Biosystems, Weiterstadt, Germany). Comparison of experimentally determined nucleotide sequences to sequence databases was performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast). PCR mapping and sequencing of the regions surrounding genes encoding ESBLs, MBLs, and ES-OXAs were performed using the primers described in Table 1.

2.4. MLST experiments

MLST experiments were performed as previously described (Curran et al., 2004). PCR and sequencing experiments for 7 housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) were performed. Nucleotide sequences were determined for both strands and compared with pre-existing sequences in the MLST database (http://pubmlst.org/paeruginosa/) for assignment of allelic numbers and STs. When different STs shared alleles at 5 or 6 out of 7 loci, the STs were grouped as a clonal complex (CC).

2.5. GenBank accession numbers

Nucleotide sequence data reported in this paper are available in the GenBank nucleotide database under accession numbers JF795487 (class 1 integron carrying the $bla_{OXA-210}$ gene cassette), KF257849 (class 1 integron carrying the $bla_{OXA-142}$ gene cassette), KC960556, KF512017, KF512018 (class 1 integrons carrying the bla_{IMP-6} gene cassette), and KC960557 (class 1 integron carrying the bla_{VIM-2} gene cassette).

3. Results

3.1. Strain type

A total of 205 *P. aeruginosa* clinical isolates were identified as 62 different STs by MLST experiments. The most common ST was ST235 (n = 96), and 2 single-locus ST235 variants, ST1015 (n = 1) and ST1162 (n = 1), were also identified. Clinical isolates belonging to these 3 STs were grouped as CC235. The remaining 107 isolates were identified as 59 different STs, sharing alleles at less than 5 out of the 7 loci with ST235, and were grouped as non-CC235 isolates.

3.2. Antimicrobial susceptibility

The collected isolates exhibited high rates of non-susceptibility to ceftazidime (188/205, 91.7%), imipenem (135/205, 65.9%), and meropenem (147/205, 71.7%). Sixty-one imipenem- and/or meropenem-non-susceptible isolates showed positive results in both MH and IEDDS tests indicating MBL production. Interestingly, the isolates belonging to CC235 (n = 98) showed higher rates of non-susceptibility to imipenem (85.4% versus 47.7%) and meropenem (92.7% versus 52.3%) compared to the isolates belonging to non-CC235 groups (n = 107) (Table 2).

3.3. β-Lactamase genes

PCR and sequencing experiments detected genes encoding SHV-12 ESBL (n = 4), 2 types of ES-OXAs (OXA-17, n = 1; and OXA-142, n = 4), and 2 types of MBLs (IMP-6, n = 45; VIM-2, n = 17). One isolate carried 2 genes encoding MBLs, IMP-6 and VIM-2 (Table 3). Two isolates simultaneously carried the gene encoding IMP-6 MBL and the gene encoding ES-OXA, OXA-17 or OXA-142. Genes encoding broad-spectrum β-lactamases (BSBLs), TEM-1 (n = 3), OXA-1 (n = 15), OXA-2 (n = 10), OXA-4 (n = 4), and OXA-10 (n = 58), were also identified. A novel OXA-2 variant, OXA-210 with 1 amino acid substitution [Y158C], was found. The MIC range of imipenem and meropenem for MBL-producing isolates was 8 mg/L to >32 mg/L and 32 to >32 mg/L, respectively. The MIC range of ceftazidime for ESBL-producing or ES-OXA–producing isolates was 24 mg/L to >32 mg/L.

Table 1
Oligonucleotide sequences used in this study.

Primer name	Target gene	Nucleotide sequence (5′ to 3′)	Reference
INT1-F	*IntI1*	GGC ATC CAA GCA GCA AG	16
INT1-R		AAG CAG ACT TGA CCT GA	
N3CS		ATC AAG CTT TTG CCC ATG AA	
IMP-1F	bla_{IMP-1} cluster	AAG GCG TTT ATG TTC ATA CTT CG	16
IMP-1R		TTT AAC CGC CTG CTC TAA TGT AA	
IMP-1bF		GTA GTG GTT TGG TTG CCT GAA	
IMP-1mR		TTA TAG CCA CGC TCC ACA AA	
VIM-2F	bla_{VIM-2} cluster	ATC ATG GCT ATT GCG AGT CC	
VIM-2R		ACG ACT GAG CGA TTT GTG TG	
OXA-1F	bla_{OXA} cluster	TAT CTA CAG CAG CGC CAG TG	16
OXA-1R		TGC ACC AGT TTT CCC ATA CA	
OXA-2F		CGA TAG TTG TGG CAG ACG AA	This study
OXA-2bF		TAC GCC AAA CAG AAT GGA TG	
OXA-2R		CTT GAC CAA GCG CTG ATG T	
OXA-7F		CAA AGA GTT CTC TGC CGA AGC	
OXA-7R		CCA CCA ATG ATG CCC TCA C	
aacA4-mF	*aacA4* gene	GGT TCG AGC TCT GGT TGA GT	16
aacA4-R		CTG GCG TGT TTG AAC CAT GT	
aacA7-F	*aacA7* gene	CAG GCC TGT TGA AAC TAC CG	This study
aacA7-R		CTT GAG CAA CCT CCG TGA AT	
aadA1-mF	*aadA1* gene	ACA TCA TTC CGT GGC GTT AT	
aadA1-mR		AGG TTT CAT TTA GCG CCT CA	
aadA2-mF	*aadA2* gene	AGC TGC AAT TTG GAG AAT GG	
aadA2-mR		GCT GCG AGT TCC ATA GCT TC	
qac-F	*qac* gene	CAA TCT TTG GCG AGG TCA TC	16
qac-R		CGC TGA CCT TGG ATA GCA G	
catB2-F	*catB2* gene	GGG AAG CTT CTG ACT GAG CA	This study
catB2-R		TTA GGC GCT TGT GCC TTG	
catB3-F	*catB3* gene	AAG GCA AGC TGC TTT CTG AG	
catB3-R		AAC GAT AGC GTA AGG CTC CA	
IS26-mF	IS*26*	CAA CGT GAA GAA GTG GCA GA	
SHV-R	bla_{SHV} cluster	AGG TGC TCA TCA TGG GAA AG	

Table 2
Antimicrobial susceptibility rates (%) of *P. aeruginosa* clinical isolates by strain type.

Antimicrobial agents	CC235 (n = 98)	Non-CC235 (n = 107)	Total (n = 205)
Piperacillin	4.2	10.1	7.3
Piperacillin-tazobactam	8.3	16.5	12.7
Ceftazidime	1.0	2.8	1.9
Cefepime	3.1	11.9	7.8
Imipenem	14.6	52.3	34.6
Meropenem	7.3	47.7	28.7
Amikacin	5.2	41.3	24.4
Gentamicin	2.1	31.2	17.6
Tobramycin	2.1	45.9	25.4

Table 3
Characteristics of β-lactamase-producing *P. aeruginosa* clinical isolates in Korea.

Strain type (no. of isolates)	ST	No. of isolates	β-Lactamase gene encoding:			MIC (mg/L)								
						Ceftazidime			Imipenem			Meropenem		
			MBL	ESBL/ES-OXA	BSBL	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
CC235 (98)	ST235	14	bla_{IMP-6}			24 to >32	32	>32	8 to >32	16	>32	32 to >32	>32	>32
		3	bla_{IMP-6}		bla_{OXA-1}	16 to >32			24 to >32			>32		
		22	bla_{IMP-6}		bla_{OXA-10}	24 to >32	>32	>32	12 to >32	>32	>32	32 to >32	>32	>32
		1	bla_{IMP-6}	bla_{OXA-17}		>32			>32			>32		
		1	bla_{IMP-6}	$bla_{OXA-142}$		>32			>32			>32		
		3	bla_{VIM-2}			>32			>32			>32		
		1	bla_{VIM-2}		bla_{OXA-2}	>32			>32			>32		
		12	bla_{VIM-2}		bla_{OXA-10}	32 to >32	>32	>32	24 to >32	>32	>32	32 to >32	>32	>32
		1	bla_{IMP-6}+bla_{VIM-2}		bla_{OXA-10}	>32			>32			>32		
		3		$bla_{OXA-142}$		32 to >32			4–24			8–32		
		6			bla_{OXA-1}	12–32			4–12			16–32		
		4			bla_{OXA-4}	8–24			0.25–32			0.125–32		
		23			bla_{OXA-10}	6 to >32	32	32	0.125 to >32	12	16	0.25 to >32	16	32
		2				16			0.5–2			2–3		
	ST1015	1	bla_{IMP-6}		bla_{OXA-1}	>32			>32			>32		
	ST1162	1	bla_{IMP-6}			32			>32			>32		
Non-CC235 (107)[a]	ST170	1		bla_{SHV-12}		>32			12			4		
		2		bla_{SHV-12}	bla_{TEM-1}	>32			12			4–6		
	ST591	1	bla_{IMP-6}		bla_{OXA-1}	16			24			32		
	ST773	1		bla_{SHV-12}		>32			0.5			1		
	ST829	1			$bla_{OXA-210}$	24			2			1		
	ST111	1			bla_{OXA-1}	16			12			32		
		4			bla_{OXA-2}	6–32			0.19–8			0.25–16		
		1			bla_{TEM-1}+bla_{OXA-2}	8			6			6		
	ST244	1			bla_{OXA-2}	4			0.5			2		
	ST641	2			bla_{OXA-1}	12–32			2–12			16–24		
	ST1166	1			bla_{OXA-1}	12			12			24		
	ST708	1			bla_{OXA-2}	32			0.25			3		
	ST983	1			bla_{OXA-2}	8			16			16		
	ST1154	1			bla_{OXA-2}	16			4			12		

Abbreviation: ND = not done.
[a] Genes encoding β-lactamases were not detected in remaining 88 isolates of non-CC235 identified as following STs (no. of isolates): ST16 (1), ST244 (3), ST245 (12), ST251 (1), ST253 (1), ST260 (1), ST270 (1), ST274 (2), ST316 (1), ST319 (1), ST357 (3), ST377 (2), ST446 (3), ST554 (1), ST557 (1), ST594 (7), ST606 (1), ST641 (2), ST676 (1), ST773 (3), ST782 (1), ST934 (1), ST1029 (1), ST1044 (1), ST1074 (1), ST1101 (1), ST1123 (1), ST1150 (1), ST1151 (1), ST1153 (11), 1154 (1), ST1155 (1), ST1156 (1), ST1157 (1), ST1158 (1), ST1159 (1), ST1160 (1), ST1161 (1), ST1163 (1), ST1164 (1), ST1165 (1), ST1167 (2), ST1168 (1), ST1169 (1), ST1170 (1), ST1211 (2), ST1212 (1), ST1213 (1).

3.4. Strain type versus β-lactamase gene

All the MBL-producing isolates were identified as CC235, except for 1 that was designated as ST591. Genes encoding OXA-17 and OXA-142 ES-OXAs were detected in 1 isolate and 4 isolates of CC235, respectively, while the SHV-12 ESBL gene was detected in 4 non-CC235 isolates. The genes encoding OXA-4 and OXA-10 BSBLs were exclusively detected in 4 and 58 CC235 isolates, respectively, while OXA-1 and OXA-2 BSBLs were detected in both CC235 and non-CC235 isolates. The novel $bla_{OXA-210}$ gene was detected in a ST829 isolate.

3.5. Genetic environments surrounding the β-lactamase genes

Genes encoding IMP-6 or VIM-2 MBL were located on class 1 integrons as a gene cassette (Table 4). Class 1 integrons containing the bla_{IMP-6} gene cassette showed 4 different gene cassette array types. Among the 45 IMP-6-producing isolates, 33 CC235 isolates shared an identical class 1 integron (InIMP-6A) that had a novel gene cassette array (*bla*$_{IMP-6}$-*aacA7-aadA1*) between the 5′ and 3′ conserved sequences (5′CS and 3′CS; GenBank accession no. KF512017). Seven CC235 isolates also shared an identical class 1 integron (InIMP-6B) with a novel gene cassette array (*bla*$_{IMP-6}$-*aacA4-aadA1*) between the 5′CS and 3′CS (GenBank accession no. KF512018). Integrons InIMP-6A and InIMP-6B differed by a second gene cassette substitution, from *aacA7* to *aacA4*. Three CC235 isolates and 1 ST591 isolate shared an identical class 1 integron (InIMP-6C) with a gene cassette array (*bla*$_{IMP-6}$-*qac-aacA4-bla*$_{OXA-1}$-*aadA1*) identical to GenBank accession no. EU117233 (Ryoo et al., 2009). The 1 remaining CC235 isolate carried a class 1 integron (InIMP-6D) that had a novel gene cassette array (*bla*$_{IMP-6}$-*qac-aacA4-catB3-aacA4-bla*$_{OXA-1}$-*aadA1*; GenBank accession no. KC960557).

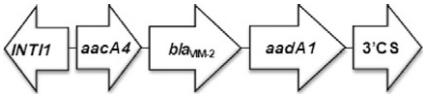
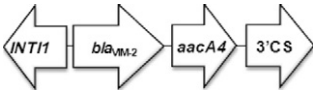
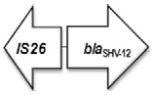
Class 1 integrons containing the bla_{VIM-2} gene cassette showed 3 different gene cassette array types. Ten CC235 isolates shared an identical class 1 integron (InVIM-2A) that had a gene cassette array (*aacA4-bla*$_{VIM-2}$-*aadA1*) identical to GenBank accession no. JF429900 (Seok et al., 2011). Six CC235 isolates also shared an identical class 1 integron (InVIM-2B) with a gene cassette array (*bla*$_{VIM-2}$-*aacA4*) identical to GenBank accession no. EF125009 (Lee et al., 2010). The 1 remaining CC235 isolate carried a class 1 integron (InVIM-2C) with a novel gene cassette array (*bla*$_{VIM-2}$-*aacA4- bla*$_{OXA-2}$; GenBank accession no. KC960556).

Genes encoding ES-OXAs were also located on class 1 integrons as a gene cassette. One CC235 isolate producing OXA-17 carried a class 1 integron (InOXA-17) with a gene cassette array (*aacA7-catB2-bla*$_{OXA-17}$-*orfD*) identical to GenBank accession no. DQ393782 (Yan et al., 2006). Four CC235 isolates producing OXA-142 shared a class 1 integron (InOXA-142) with a gene cassette array (*aacA4-bla*$_{OXA-142}$-*aadA2*) identical to GenBank accession no. KF257849, and 1 ST829 isolate producing OXA-210 carried a class 1 integron (InOXA-210) with a novel gene cassette array (*aadB-qac-aacA4-catB3-bla*$_{OXA-210}$-*orfD*; GenBank accession no. JF795487). The gene encoding SHV-12 ESBL was identically associated with the IS*26* insertion sequence in 3 ST170 isolates and 1 ST773 isolate (Kassis-Chikhani et al., 2013).

4. Discussion

Two single-locus variants of ST235, ST227 and ST230, were previously classified as CCBG1 (Empel et al., 2007); therefore, ST235 and

Table 4
Schematic representation of genetic environment s surrounding the β-lactamase genes in *P. aeruginosa* clinical isolates.

	Genetic environment	No. of isolates	GenBank accession no. (reference)
InIMP-6A		33	KF512017 (This study)
InIMP-6B		7	KF512018 (This study)
InIMP-6C		4	EU117233 (Ryoo et al., 2009)
InIMP-6D		1	KC960557 (This study)
InVIM-2A		10	JF429900 (Livermore, 2002)
InVIM-2B		6	EF125009 (Seok et al., 2011)
InVIM-2C		1	KC960556 (This study)
TnSHV-12		4	JX461340 (Yan et al., 2006)
InOXA-17		1	DQ393782 (Weldhagen et al., 2003)
InOXA-142		4	KF257849 (This study)
InOXA-210		1	JF795487 (This study)

its 2 single-locus variants ST1015 and ST1162 identified in this study also belong to the BG11 complex. However, we named the CC as CC235 because the founder ST235 clone is a true international MDR clone that has been disseminating worldwide over a long period. Isolates belonging to CC235 comprised a large portion (47.8%, 98/205) of our collection, and they exhibited higher rates of non-susceptibility to multiple classes of antibiotics, including penicillins, expanded-spectrum cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones, compared to non-CC235 isolates.

Our data show that production of IMP-6 and VIM-2 MBLs is the main mechanism for acquiring resistance to ceftazidime and carbapenems in *P. aeruginosa* isolates in Korea. Many MBL-producing CC235 isolates shared an identical class 1 integron containing the MBL gene cassette, indicating clonal spread (Yum et al., 2012). However, the fact that 4 different isolates belonging to different STs (3 CC235 isolates and 1 ST591 isolate) shared identical class 1 integron carrying the bla_{IMP-6} gene cassette suggests that MBL genes might also be spread by horizontal transfer. Four isolates were identified as international clone ST111, but none of these isolates produced MBL. Furthermore, clone ST175 was not found in our collection. Our results indicate that the 2 ST111 and ST175 international clones may not play a role in the dissemination of MBL genes in Korea.

This study detected 2 types of OXA-10 ES-OXA variants, including OXA-17 [A218G] and OXA-142 [A218G, G470A], and SHV-12 ESBL. The ES-OXAs and ESBLs were shown to play a role in acquiring ceftazidime resistance in *P. aeruginosa* strains isolated in Korea, although this was less frequent than MBLs. Four ceftazidime-resistant CC235 isolates shared an identical class 1 integron containing the $bla_{OXA-142}$ gene cassette, indicating clonal spread. The bla_{SHV-12} gene associated with the IS*26* insertion sequence was detected in 3 ST170 isolates and 1 ST773 isolate, indicating both clonal spread of the strain and horizontal spread of the ESBL gene.

In conclusion, IMP-6 and/or VIM-2 MBL-producing CC235 *P. aeruginosa* has wide dissemination in Korea due to clonal spread. *P. aeruginosa* clones producing ES-OXAs or SHV-12 ESBL are also spreading. Furthermore, horizontal transfer also spreads the genes encoding IMP-6 MBL and SHV-12.

Acknowledgments

We would like to thank Jung OK Kim for laboratory assistance.

Funding: This work was supported by a grant of the Korea Healthcare technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A121133).

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