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Original Article

OXA-type beta-lactamases among extended-spectrum cephalosporin-resistant *Pseudomonas aeruginosa* isolates in a university hospital in southern Taiwan

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Background and Purpose: Data on the epidemiology of OXA-type extended-spectrum beta (β)-lactamases (ESBLs) are limited due to difficulty of identification by routine microbiology laboratories. We determined the prevalence rate of OXA-type β-lactamases among extended-spectrum cephalosporin (ESC)-non-susceptible *Pseudomonas aeruginosa* isolates at a university hospital in southern Taiwan.

Methods: A total of 1294 ESC-non-susceptible *P. aeruginosa* isolates collected between 1989 and 1996 (n = 42) and between December 1999 and December 2002 (n = 1252) were analyzed by polymerase chain reaction assays with primers specific for bla_{OXA} genes and isoelectric focusing.

Results: Forty five isolates (3.5%) were found to produce an OXA-type β-lactamase. Overall, 2 OXA-type ESBLs, OXA-14 (n = 2) and OXA-17 (n = 35), were detected in 37 (2.9%) isolates, and the OXA-10-type narrow-spectrum β-lactamase was found in 8 (0.6%) isolates. OXA-10 and the 2 OXA-type ESBLs were detected in 6 (14.3%) and 4 (9.5%) of 42 ESC-non-susceptible isolates collected between 1989 and 1996. OXA-10 and OXA-17 were detected in 2 (0.2%) and 33 (2.6%) of 1252 ESC-non-susceptible isolates collected between December 1999 and December 2002.

Conclusions: These data indicate that OXA-17 was the most common OXA-type ESBL and that OXA-type β-lactamases have decreased in ESC-non-susceptible *P. aeruginosa* at this hospital in recent years. Pulsed-field gel electrophoresis revealed clonal diversity among the OXA-producing isolates.

Key words: beta-Lactamases, cephalosporin resistance, microbial sensitivity tests, polymerase chain reaction, *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa is responsible for nosocomial infections and is intrinsically resistant to many antibiotics. *P. aeruginosa* is naturally susceptible to ureido- and carboxypenicillins and some extended-spectrum cephalosporins (ESCs) [1]. ESC resistance in *P. aeruginos*a is often associated with the overproduction of a naturally produced cephalosporinase [2-4]. Extended-spectrum beta (β)-lactamases (ESBLs) that can confer resistance to ESCs are common in *Enterobacteriaceae* and have spread worldwide [5]. Various Ambler's class A ESBLs, such as TEM-, SHV-, VEB-, and PER-type ESBLs, and class D ESBLs, such as OXA-type ESBLs, have been identified in *P. aeruginosa* [2,5-8]. Among these enzymes in *P. aeruginosa*, OXA-type ESBLs have been encountered most commonly, and class A ESBLs are uncommon [2,4,6-8]. Most OXA-type ESBLs are OXA-2 or OXA-10 derivatives [6]. Unlike class A ESBLs, most OXA-type ESBLs are only weakly inhibited by clavulanic acid and cannot be identified at routine microbiology laboratories due to the lack of standard phenotypic detection methods [5]. Thus, the epidemiology of OXA-type ESBLs is not well known.

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In Taiwan, a high proportion of *P. aeruginosa* isolates from patients in intensive care units have been found to be resistant to ceftazidime [9,10]. Moreover, although various β-lactamases of classes A, B, and C that confer resistance to ESCs in Gram-negative bacilli have been identified in Taiwan [9,11], the prevalence of class D ESBLs has not been reported. Thus, the aim of the present study was to determine the prevalence of OXA-type β-lactamases among ESC-non-susceptible *P. aeruginosa* isolates at a university hospital in Taiwan. This is the first report of the prevalence of OXA-type β-lactamases in *P. aeruginosa* in Taiwan.

Methods

Bacterial isolates

A total of 366 *P. aeruginosa* isolates from blood samples were collected between 1989 and 1996, and a total of 8681 *P. aeruginosa* isolates were isolated from various specimens between December 1999 and December 2002 at the Department of Pathology of National Cheng Kung University Hospital, a 900-bed teaching hospital in southern Taiwan. The disk diffusion susceptibility results of these isolates were analyzed according to the guidelines of the National Committee for Clinical Laboratory Standards [12]. Isolates that demonstrated intermediate susceptibilities or resistance to ceftazidime, cefotaxime, cefoperazone, cefepime, or aztreonam were selected for further investigation.

Detection of ESBL genes

All ESC-non-susceptible isolates were subjected to polymerase chain reaction (PCR) assays to detect bla_{OXA} genes. A fresh bacterial colony was suspended in 100 μL of sterile distilled water and boiled at 100°C for 10 min. After centrifugation, the supernatant was removed for PCR. Amplifications were performed with primers OPR1 (5'-GTCTTTCGAGTACGG CATTA-3') and OPR2 (5'-ATTTTCTTAGCGGCAAC TTAC-3') for bla_{OXA-10}-related genes and primers OXA-2,3 (5'-GCCAAAGGCACGATAGTTGT-3') and OXB-2,3 (5'-GCGTCCGAGTTGACTGCCGG-3') for bla_{OXA-2}-related genes under PCR conditions as described previously [8,13]. PCR products were purified with a commercial kit (Roche Molecular Biochemicals, Mannheim, Germany) and both strands of the amplicons were sequenced on an ABI PRISM 310 automated sequencer (PE Applied Biosystems, Foster City, CA, USA). bla_{VEB-1} was amplified by PCR from a *Klebsiella pneumoniae* control strain [14]. PCR products of bla_{OXA} and bla_{VEB-1} and previously obtained PCR products of bla_{TEM-1}, bla_{SHV-12}, $bla_{CTX-M-9}$, and $bla_{CTX-M-14}$ [11] were used as the templates to synthesize the digoxigenin-labeled probes. Colony hybridization was performed with these probes and was detected with Detection Starter Kit II (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

Isoelectric focusing analysis

Crude β-lactamase extracts were prepared using sonication as described previously [15]. Isoelectric focusing was performed by the method of Matthew et al [16] with an LKB Multiphor apparatus on prepared PAGplate gels (pH 3.5 to 9.5; Amersham Pharmacia Biotech, Hong Kong, China) as previously described [11,16].

Susceptibility testing

Minimal inhibitory concentrations (MICs) of various antimicrobial agents were determined by using E-test strips (AB BIODISK, Solna, Sweden).

Pulsed-fielded gel electrophoresis analysis

Pulsed-fielded gel electrophoresis (PFGE) was performed with a CHEF Mapper apparatus (Bio-Rad Laboratories, Hercules, CA, USA) according to the instruction manual. Chromosomal DNA was digested with *Xba*I (New England Biolabs, Beverly, MA, USA) [17], and was separated on 1% agarose gels. PFGE patterns were interpreted according to the Tenover's criteria [17].

Results and Discussion

Forty two (11.4%) of the 366 *P. aeruginosa* isolates collected between 1989 and 1996 and 1647 (19.0%) of the 8681 *P. aeruginosa* isolates isolated between December 1999 and December 2002 demonstrated intermediate susceptibilities or resistance to at least 1 of the 5 ESCs based on the results of disk diffusion tests. Among the 1647 ESC-non-susceptible isolates, 1252 isolates were collected. A total of 1294 ESC-non-susceptible isolates collected during the 2 periods were subjected to PCR and colony hybridization.

All 1294 isolates had negative results on colony hybridization assays with the bla_{VEB}, bla_{CTX-M}, bla_{SHV}, and bla_{TEM} probes. In the PCR assays, all 1294 isolates had negative results with the primers for bla_{OXA-2}-related genes, and only 45 (3.5%) isolates from 21

patients yielded a 720-bp fragment with the primers for bla_{OXA-10}-related genes. The results of colony hybridization were consistent with those of PCR assays for bla_{OXA} genes. Nucleotide sequencing revealed that 8 isolates from 7 patients, 2 isolates from 2 patients, and 35 isolates from 12 patients carried bla_{OXA-10}, bla_{OXA-14}, and bla_{OXA-17}, respectively. OXA-10 is a narrow-spectrum class D β-lactamase [18], and OXA-14 and OXA-17 are two OXA-10-derived class D ESBLs [19,20]. These data suggest that ESC resistance in *P. aeruginosa* at this university hospital was mostly due to mechanisms other than ESBL production, such as hyperproduction of intrinsic class C cephalosporinases and reduced accumulation of antibiotics [2-4]. In isoelectric focusing analysis, all bla_{OXA-10}-positive and bla_{OXA-17}-positive isolates demonstrated 2 bands at pH values for the isoelectric point (pIs) of 6.1 and 7.9, and both bla_{OXA-14}-positive isolates demonstrated 2 bands at pIs of 6.2 and 7.9. The bands at pI of 7.9 were consistent with the expression of intrinsic class C β-lactamases [18], the bands at pI of 6.1 were consistent with the expression of OXA-10 or OXA-17 [18,19], and the bands at pI of 6.2 were consistent with the expression of OXA-14 [20]. Our results indicate that among the ESC-non-susceptible *P. aeruginosa* isolates at this university hospital, OXA-17 was the most common OXA-type ESBL, accounting for 35 (94.6%) of the 37 isolates producing an OXA-type ESBL.

Among the 42 ESC-non-susceptible isolates collected between 1989 and 1996, OXA-10 and OXA-type ESBLs were detected in 6 (14.3%) and 4 (9.5%) isolates (2 OXA-14-producing and 2 OXA-17-producing isolates), respectively. Among the 1252 isolates collected between December 1999 and December 2002, OXA-10 and OXA-17 were detected in 2 (0.2%) and 33 (2.6%) isolates, respectively. Thus, 23.8% of the ESC-non-susceptible isolates collected between 1989 and 1996 and 2.8% of the ESC-non-susceptible isolates collected between December 1999 and December 2002 produced an OXA-type β-lactamase. These results suggest that the prevalence rate of OXA-type ESBLs as well as OXA-type narrow-spectrum β-lactamases decreased among ESC-non-susceptible *P. aeruginosa* isolates at this hospital in recent years. The exact prevalence rate of OXA-type β-lactamases in *P. aeruginosa* could not be determined because ESC-susceptible isolates, which may

Table 1. Antimicrobial susceptibilities and PFGE patterns of 21 non-repetitive OXA-producing *Pseudomonas aeruginosa* isolates

Isolate/year of collection	Specimen type	pIs of beta-lactamases	bla_{OXA} gene	MIC (μg/mL)											PFGE profile
				PIP	TZP	CTX	CAZ	FEP	ATM	IPM	MEM	GEN	AMK	CIP	
4-16/1989	Blood	7.9, 6.1	bla_{OXA-10}	>256	>256	32	2	24	8	1	1.5	>256	>256	0.25	IIIa
32-52/1991	Blood	7.9, 6.1	bla_{OXA-10}	>256	>256	24	1.5	24	1	>32	32	>256	>256	0.125	IV
38-59/1991	Blood	7.9, 6.1	bla_{OXA-10}	128	192	12	1	12	1	1	1	256	>256	0.125	II
39-69/1991	Blood	7.9, 6.1	bla_{OXA-10}	>256	>256	48	2	16	12	1	1	>256	>256	0.094	IIIb
44-67/1992	Blood	7.9, 6.1	bla_{OXA-10}	>256	128	12	1	12	1	1.5	0.75	>256	>256	0.19	II
195/1996	Blood	7.9, 6.1	bla_{OXA-10}	>256	>256	12	1	12	2	>32	8	16	>256	0.032	I
149/2002	Wound	7.9, 6.1	bla_{OXA-10}	>256	>256	48	2	24	6	>32	>32	256	>256	2	NS
176/1996	Blood	7.9, 6.2	bla_{OXA-14}	128	64	>256	>256	24	6	2	0.5	>256	>256	8	Va
178/1996	Blood	7.9, 6.2	bla_{OXA-14}	>256	>256	>256	16	24	8	2	0.75	128	>256	12	Vb
29-1/1991	Blood	7.9, 6.1	bla_{OXA-17}	>256	>256	64	4	48	96	2	12	>256	3	0.5	VI
181/1996	Blood	7.9, 6.1	bla_{OXA-17}	128	64	128	4	12	16	0.75	4	64	3	4	XII
611/2000	Wound	7.9, 6.1	bla_{OXA-17}	>256	>256	64	2	128	12	3	1.5	>256	12	0.125	VII
956/2000	Wound	7.9, 6.1	bla_{OXA-17}	>256	>256	>256	32	96	16	>32	>32	>256	12	0.5	VIII
1110/2000	Sputum	7.9, 6.1	bla_{OXA-17}	>256	>256	>256	32	>256	16	>32	>32	>256	12	0.5	VIII
1390/2000	Urine	7.9, 6.1	bla_{OXA-17}	>256	>256	>256	32	32	32	1.5	6	>256	12	1	IXa
103/2002	Urine	7.9, 6.1	bla_{OXA-17}	>256	>256	>256	128	>256	32	1	1.5	>256	16	>32	X
106/2001	Sputum	7.9, 6.1	bla_{OXA-17}	>256	>256	>256	48	192	16	>32	16	>256	12	0.5	VIII
195/2001	Sputum	7.9, 6.1	bla_{OXA-17}	>256	>256	>256	8	64	6	1	1.5	>256	12	>32	XIII
87/2002	Sputum	7.9, 6.1	bla_{OXA-17}	>256	>256	>256	32	>256	48	>32	>32	>256	12	>32	IXb
312/2002	Urine	7.9, 6.1	bla_{OXA-17}	>256	192	>256	4	32	16	1	1.5	>256	12	1	XI
444/2002	CSF	7.9, 6.1	bla_{OXA-17}	>256	>256	>256	>256	96	48	>32	>32	>256	16	>32	IXc

Abbreviations: PFGE = pulsed-fielded gel electrophoresis; pIs = pH values for the isoelectric point; MIC = minimal inhibitory concentration; PIP = piperacillin; TZP = piperacillin/tazobactam; CTX = cefotaxime; CAZ = ceftazidime; FEP = cefepime; ATM = aztreonam; IPM = imipenem; MEM = meropenem, GEN = gentamicin; AMK = amikacin; CIP = ciprofloxacin; CSF = cerebrospinal fluid; NS = not successful

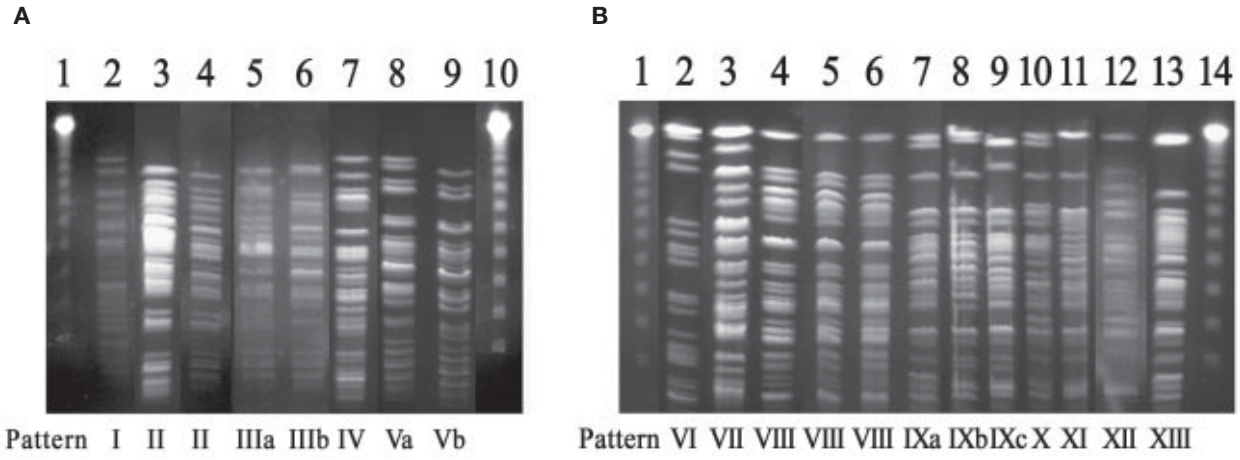

Fig. 1. Pulsed-fielded gel electrophoresis (PFGE) patterns of *Xba*I-digested genomic DNA of 20 *P. aeruginosa* isolates. Numbers designating PFGE patterns are shown below the gels. A) Lanes 1 and 10, a lambda ladder; lanes 2 to 9, isolates 195/96, 38-59/91, 44-67/92, 4-16/89, 39-69/91, 32-52/91, 176/96, and 178/96. B) Lanes 1 and 14, a lambda ladder; lanes 2 to 13, isolates 29-1/91, 611/00, 956/00, 1110/00, 106/01, 1390/00, 87/02, 444/02, 103/02, 312/02, 181/96, and 195/01.

produce narrow-spectrum OXA-type β-lactamases, were not analysed.

One OXA-producing isolate from each patient was selected for further analyses. The results of susceptibility testing are shown in Table 1. All 21 isolates showed high-level resistance to piperacillin and piperacillin/tazobactam and were non-susceptible to cefotaxime (MICs >8 μg/mL) and cefepime (MICs >8 μg/mL). Twelve and 5 of the 14 isolates that produced an OXA-type ESBL showed high-level resistance to cefotaxime (MICs ≥128 μg/mL) and cefepime (MICs ≥128 μg/mL), respectively. The MICs of ceftazidime for the 14 isolates producing an OXA-type ESBL ranged widely, from 2 to >256 μg/mL. All OXA-10-producing and both OXA-14-producing isolates showed high-level resistance to amikacin (>256 μg/mL), while all OXA-17-producing isolates were susceptible to this drug (≤16 μg/mL).

OXA-type β-lactamases have strong activity against oxacillin and cloxacillin and are predominantly penicillinases [18]. The OXA-type ESBLs provide weak resistance to oxyiminocephalosporins when cloned into *Escherichia coli* [5,19]. The OXA-17 β-lactamase confers resistance to cefotaxime and ceftriaxone but provides only marginal protection against ceftazidime [5,19]. In this study, 5 of the 12 $bla_{\text{OXA-17}}$-positive isolates were susceptible to ceftazidime (MICs ≤8 μg/mL). This result indicates that all ESC-resistant isolates rather than ceftazidime-resistant isolates only should be analyzed to determine the prevalence rate of OXA-type ESBLs in *P. aeruginosa*.

Of the 21 non-repetitive OXA-producing isolates, 20 isolates were successfully typed by PFGE, and 13 major patterns were identified. Six typeable OXA-10-producing isolates revealed 4 major patterns (patterns I to IV), among which pattern III had 2 subtypes (Fig. 1A). Two OXA-14-producing isolates differed by two bands and were thus considered to be closely related. Eight major patterns (patterns VI to XIII) were identified among the 12 typeable OXA-17-producing isolates, and 6 of them were represented by a single isolate (Fig. 1B). Three pattern IX isolates were subgrouped into 3 subclones, and pattern VIII was shared by 3 isolates. The data indicate the spread of the OXA-gene among different *P. aeruginosa* clones.

In conclusion, this study indicates that the prevalence rate of OXA-type ESBLs among ESC-non-susceptible *P. aeruginosa* isolates has decreased in recent years at a Taiwanese university hospital. OXA-17 was the most common OXA-type ESBL and its genetic determinant has spread among different clones in *P. aeruginosa*.

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