Journal of Microbiology, Immunology and Infection (2012) 45, 200—207

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ORIGINAL ARTICLE

Phenotypic detection and polymerase chain reaction screening of extended-spectrum β-lactamases produced by *Pseudomonas aeruginosa* isolates

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Received 30 April 2011; received in revised form 14 July 2011; accepted 9 August 2011

KEYWORDS
Cloxacillin-containing double disc synergy test;
Extended-spectrum β-lactamase;
Polymerase chain reaction;
Pseudomonas aeruginosa

Background/Purpose: A growing number of β-lactamases have been reported in *Pseudomonas aeruginosa* isolates. The aims of this study were to survey the types of extended-spectrum β-lactamases (ESBLs) by polymerase chain reaction (PCR), to evaluate the reliability of phenotypic tests for ESBLs, and to identify the clonal distribution by pulsed-field gel electrophoresis (PFGE) among *P. aeruginosa* isolates resistant to expanded-spectrum cephalosporins (ceftazidime, aztreonam, or cefepime).
Methods: The antimicrobial susceptibility of 57 *P. aeruginosa* isolates from blood specimens were examined according to the recommendations of the Clinical Laboratory Standards Institute. ESBL phenotypes were determined by using cloxacillin-containing double disc synergy test (DDST). The existence of 11 β-lactamase genes was detected by PCR.
Results: Of the 57 *P. aeruginosa* isolates, 35 (61.4%) isolates were PCR-positive for β-lactamase genes. Twelve of 35 isolates were PCR-positive for combination of *ampC* and ESBL genes, including TEM, GES, SHV, VEB and OXA-I genes. The sensitivity and specificity of cloxacillin-containing DDST (using the criteria of ceftazidime zone diameter increased ≧5 mm) were 84.1% and 54.5%, respectively. Nine clusters were classified among 35 PCR-positive isolates by PFGE. Isolates of clusters B and C were distributed in different wards of this hospital during a period of 3—4 years.
Conclusion: ESBL genes are not uncommon in *P. aeruginosa* isolates. Cloxacillin-containing DDST can enhance the sensitivity and has a potential role for phenotypic detection of

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doi:10.1016/j.jmii.2011.11.015

ESBL-producing *P. aeruginosa*, and PCR is also helpful for the identification of specific β-lactamase genes. These *P. aeruginosa* isolates were classified into several diverse clones which could continue to spread in the hospital over a long period of time.

Introduction

Pseudomonas aeruginosa is a pathogen commonly implicated in serious nosocomial infections such as pneumonia, urinary tract infection, and sepsis. *P. aeruginosa* is also notably resistant to many classes of antimicrobial agents, including β-lactams, aminoglycosides and fluoroquinolones. The development of β-lactam resistance in this opportunistic pathogen can be caused by several mechanisms: (1) genetic mutations that lead to stable overexpression of AmpC, a chromosome-mediated cephalosporinase; (2) acquisition of transferable genes that code for a variety of β-lactamases; (3) overproduction of efflux systems; and (4) reduced permeability.[1] A growing number of Ambler class A extended-spectrum β-lactamases (ESBLs), class B carbapenemases [metallo-β-lactamases (MBLs)], and class D extended-spectrum oxacillinases (OXAs) have been reported in clinical strains of *P. aeruginosa*.[2–4]

ESBLs are a rapidly growing group of β-lactamases that hydrolyze broad-spectrum cephalosporins as well as aztreonam. ESBL expression also confers penicillin and narrow-spectrum cephalosporin resistance. ESBLs are inhibited by β-lactamase inhibitors such as clavulanic acid. Over 200 different ESBLs have been identified in Gram-negative bacilli. Thirty-two ESBLs have been detected in *P. aeruginosa*, which can be categorized into two molecular classes A and D, and further divided into eight subgroups: TEM-, SHV-, CTX-M-, PER-, VEB-, GES-, BEL-, and OXA-type β-lactamases.[5] TEM- and SHV-type enzymes are the most common ESBLs found in enterobacterial species, whereas OXA- and PSE-types are most frequently encountered in *P. aeruginosa*.[2]

AmpC β-lactamases are class C cephalosporinases and have been identified in Enterobacteriaceae and *P. aeruginosa*. Class C molecules contribute to the intrinsic resistance of Enterobacteriaceae and *P. aeruginosa* to penicillins and cephalosporins, as well as a combination of β-lactams and β-lactamase inhibitors.[5] In *P. aeruginosa*, resistance to ceftazidime mostly results from overexpression of its naturally occurring AmpC-type cephalosporinase.[6]

Resistance to various β-lactamases of class A, B and C among Enterobacteriaceae has been reported in Taiwan.[7–13] Only one study has ever reported ESBLs (focusing on OXA-types) of *P. aeruginosa* in Taiwan.[14] Three ESBLs belonging to the OXA-types were identified in 1294 *P. aeruginosa* isolates, including OXA-14 (0.2%), OXA-17 (2.8%) and OXA-10 (0.6%). Surveillance of the various types of ESBLs found in *P. aeruginosa* has not been reported in Taiwan. The double disc syngery test (DDST) has been recommended by the Clinical Laboratory Standards Institute (CLSI) as a phenotypic test of ESBL in *Escherichia coli* and Klebsiella pneumoniae.[15] However, due to sensitivity of the phenotypic test, the application of DDST to *P. aeruginosa* has only been reported in a small subset of studies.[16] Phenotypic detection of ESBLs can be obscured by the chromosomal AmpC cephalosporinase in *P. aeruginosa* because AmpC is not inhibited by β-lactamase inhibitors (e.g., clavulanic acid).[17] With the addition of cloxacillin, ESBL detection in *P. aeruginosa* by DDST can be enhanced through the inhibition of AmpC enzyme activity.

The aims of this study were to survey the antimicrobial susceptibility and types of ESBLs in *P. aeruginosa* by polymerase chain reaction (PCR) and to evaluate the reliability of phenotypic tests for ESBLs among *P.* aeruginosa, and to identify the clonal distribution of *P. aeruginosa* by pulsefield gel electrophoresis (PFGE).

Materials and methods

Bacterial strains

A total of 765 *P. aeruginosa* isolates from blood samples were collected from 2005 to 2009 at the *Microbiology Laboratory of* Taichung Veterans General Hospital; a 1000-bed medical center in central Taiwan. Fifty-seven *P.* aeruginosa isolates resistant to expanded-spectrum cephalosporins (ceftazidime, aztreonam or cefepime) as determined by disc diffusion method were selected from the total sample for this study.

Antimicrobial susceptibility tests and detection of ESBLs by phenotypic tests

Susceptibility test

The minimum inhibitory concentrations (MICs) of nine antimicrobial agents: piperacillin/tazobactam (Wyeth, Philadelphia, USA), piperacillin (Wyeth), aztreonam (Bristol–Myers Squibb, New York, NY, USA), amikacin (Bristol–Myers Squibb), cefepime (Bristol–Myers Squibb), imipenem (MSD, Rahway, NJ, USA), ceftazidime (GlaxoSmithKline, London, UK), ceftazidime/clavulanate (GSK) and ciprofloxacin (Bayer, Leverkusen, Germany) were assessed using the broth microdilution method according to CLSI recommendations.[15] The antibiotics were serially diluted twofold in 5 mL cation-adjusted Mueller–Hinton broth (CAMHB). The final range of antibiotic concentrations was 0.125–256 μg/mL. The bacterial suspension was prepared from actively growing bacteria in 4 mL CAMHB, and diluted to a bacterial cell density of 1×10^6 colony forming units (CFU)/mL. Five microliters of bacterial suspension was then added to wells containing 100 μL serially diluted antimicrobial agents to yield a final inoculum of approximately 5×10^4 CFU/mL. The MICs were read after overnight incubation (18–24 hours) at 35°C. The susceptibility was interpreted according to the breakpoints suggested by the CLSI.[18] All MICs were

determined twice. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as the quality control strains.

DDST and cloxacillin-based DDST

To detect possible ESBL production, conventional DDST was performed with discs containing ceftazidime (30 mg) and ceftazidime (30 mg)/clavulanic acid (10 mg).[18] ESBL production was inferred if the zones produced by the disks with ceftazidime (30 mg)/clavulanic acid (10 mg) were ≧5 mm larger than that of ceftazidime (30 mg). Phenotypic detection of ESBLs can be obscured by the chromosomal AmpC cephalosporinase in *P. aeruginosa*, hence cloxacillin-containing DDST was performed. Cloxacillin (250 ug/mL; Sigma, St Louis, MO, USA) was added in Mueller–Hinton agar to inhibit cephalosporinase activity.[16] After overnight culture, test isolates were suspended to 0.5 turbidity using McFarland standards as a reference, and used to inoculate a Mueller–Hinton agar plate containing 250 μg/mL cloxacillin. After drying, discs containing ceftazidime, ceftazidime/clavulanic acid, aztreonam and cefepime were placed 2 cm from a disc containing amoxicillin/clavulanic acid.[19] After 18 hours incubation, cephalosporinase inhibition was considered positive using two criteria: the ceftazidime zone diameter increased ≥5 mm and >10 mm.[16] The sensitivity and specificity were compared between these two criteria.

Molecular techniques

PCR for detection of Ambler class A, C and D β-lactamases

P. aeruginosa DNA was prepared by guanidium thiocyanate extraction as previously described.[20] Two bacterial pellets (3 mm in diameter) were taken from nutrient agar and dispersed in 100 μL 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA. The cells were lysed with 500 μL GES reagent [5 M guanidiumthiocyanate (Sigma), 0.1 M EDTA, and 0.5% (w/v) sarcosyl (Sigma)]. After addition of 250 μL 7.5 M ammonium acetate, the suspension was kept on ice for 10 minutes. For deproteination, 500 mL chloroform–isoamyl alcohol (24:1) was added and the mixture was centrifuged at 13,000 *g* for 10 minutes. The DNA was precipitated from the upper phase with 100% ethanol at −20°C for 1 hour. The extracted DNA (0.1 mg) was used as a template for amplification. Eleven primer pairs for screening the Ambler class A ESBLs (bla_{VEB}, bla_{PER}, bla_{GES}, bla_{TEM}, bla_{SHV}, bla_{CTX}, bla_{BEL}), Class D βlactamases ($bla_{OXA\text{-}group\ I}$, $bla_{OXA\text{-}group\ II}$, $bla_{OXA\text{-}group\ III}$), and class C β-lactamases (bla_{AmpC}) genes were used in the PCR[2,19,21,22] (Table 1). Amplification was performed in 50 μL of final reaction mixture containing 2 U *Taq* polymerase (Super Taq; HT Biotechnology Ltd., Cambridge, UK), 10 mM Tris–HCl, 200 μM deoxynucleotide triphosphates, and 0.5 μM of each primer. Amplification was performed in a PTC-200 Thermal cycler (MJ Research, Watertown, MA, USA) with temperatures as follows: 95°C for 5 minutes to denature the template; 30 cycles of 95°C for 1 minute, 50°C or 62°C for 1 minute, and 72°C for 1 minute; finally, 72°C for 10 minutes. A negative control was run for each amplification. The amplified products were analyzed by electrophoresis on a 1.6% agarose gel containing ethidium bromide (1 mg/mL) at 50 V/cm for 80 minutes, and were detected by UV transillumination.

PFGE

PFGE was performed with a contour-clamped homogeneous electric field DRII apparatus from Bio-Rad Laboratories (Richmond, CA, USA) as described previously.[23] The chromosomal DNA was digested overnight with *Spe*I (GIBCO-BRL, Life Technologies, Gaithersburg, MD, USA). DNA was electrophoresed in 1.2% SeaKem GTG agarose (FMC Bioproducts, Rockland, ME, USA) at 6 V/cm for 24 hours; the pulse time was increased from 5 seconds to 35 seconds. The PFGE patterns were also analyzed with the computer software Gelcompar for Windows version 3.1b (Applied Math, Kortrijk, Belgium). The PFGE patterns were compared by the UPGMA (unweighted pair group method with arithmetic averages) clustering method by using the Dice coefficient, based on the instructions of the Gelcompar manufacturer. A tolerance in the band position of 1.2% was applied during the comparison of PFGE fingerprinting patterns. Isolates were consider as a cluster if the relatedness was >80%.

Results

Suspectibility

The activity of antimicrobial agents against 57 isolates of *P. aeruginosa* is summarized in Table 2. The MIC for 50% and 90% of *P. aeruginosa* isolates (MIC_{50}/MIC_{90}) of amikacin, aztreonam, cefepime, ceftazidime, ceftazidime/clavulanic acid, imipenem, piperacillin, piperacillin/tazobactam and ciprofloxacin was 2/8, 32/256, 4/32, 32/256, 0.25/32, 1/16, ≧256/≧256, ≧256/≧256 and 0.25/2 μg/mL, respectively. The susceptibility rates of ceftazidime and piperacillin determined by disc diffusion differed from those performed by broth microdilution. Amikacin had the highest susceptibility rate, whereas aztreonam had the lowest.

PCR of Ambler A, C and D β-lactamases

Table 3 shows the PCR results of Ambler A, C and D β-lactamases in 57 *P. aeruginosa* isolates. Of all the 57 *P. aeruginosa* isolates, 22 (38.6%) were PCR-negative for Ambler A, C or D β-lactamases. Thirty-five (61.4%) isolates were PCR-positive for β-lactamase genes, including 22 (38.6%) isolates for *ampC* alone, one (1.6%) for TEM alone, and 12 (21.1%) for multiple β-lactamase genes. Of the 13 isolates with class A ESBL genes, TEM-type enzyme was most common (13 isolates). Only two isolates carried the OXA group I gene. CTX-M, PER, BEL, OXA group II and OXA group III genes were not detected in this study. The MIC ranges and susceptible rates of ceftazidime, ceftazidime/clavulanic acid, piperacillin and piperacillin/tazobactam in various combinations of β-lactamase genes are shown in Table 3. The MIC of ceftazidime and piperacillin was not decreased in the presence of β-lactamase inhibitors (clavulanic acid and tazobactam).

PFGE of *P. aeruginosa* isolates

The PFGE patterns of 35 PCR-positive *P. aeruginosa* isolates are shown in Fig. 1. A dendrogram was constructed to show

Table 1 Primers used in this study

Class of -lactamases	Primer name	Sequence (5′ to 3′)	Product size (bp)	Target	Reference
Class A	VEB-1A	cgacttccatttcccgatgc	643	bla_{VEB}	[19]
	VEB-1B	ggactctgcaacaaatacgc			
	PER-A	atgaatgtcattataaaagc	920	bla_{PER}	[19]
	PER-B	aatttgggcttagggcagaa			
	GES-1A	atgcgcttcattcacgcac	864	bla_{GES}	[19]
	GES-1B	taatcagtgaggcacctatctc			
	TEM-A	gagtattcaacatttccgtgtc	851	bla_{TEM}	[19]
	TEM-B	taatcagtgaggcacctatctc			
	SHV-A	aagatccactatcgccagcag	231	bla_{SHV}	[19]
	SHV-B	attcagttccgtttcccagcgg			
	CTX-MA	cgctttgcgatgtgcag	550	CTXM-1,-2,-9 group	[20]
	CTX-MB	accgcgatatcgttggt			
	BEL-1FW	cgacaatgccgcagctaacc	449	bla_{BEL-1}	[21]
	BEL-1RV	cagaagcaattaataacgccc			
Class D	OXA-10F	tctttcgagtacggcattagc	760	OXA group I	[20]
	OXA-10B	ccaatgatgccctcactttcc			
	OXA-2F	gccaaaggcacgatagttgt	700	OXA group II	[20]
	OXA-2B	gcgtccgagttgactgccgg			
	OXA-1A	agccgttaaaattaagccc	908	OXA group III	[20]
	OXA-1B	cttgattgaagggttgggcg			
Class C	AmpC-PA1	atgcagccaacgacaaagg	1243	bla_{ampC}	[22]
	AmpC-PA2	cgccctcgcgagcgcgcttc			

the degree of relatedness among the strains of nine clusters (A—I). There were nine clusters among the 35 PCR-positive isolates. The largest cluster, C, consisted of six isolates that were isolated during 2007—2009. The second cluster, B, included four isolates that were recovered from 2005 to 2009. Three closely related clusters (D, G and H) were isolated within a shorter duration of time. Cluster D included two isolates that were found in June 2008 and May 2009. Cluster G included two isolates that were isolated in September and October 2005. Cluster H included two isolates that were recovered in May and June 2006.

Table 2 Activity of nine antimicrobial agents against 57 isolates of *Pseudomonas aeruginosa*

Antimicrobial agents	MIC (μg/mL)		Susceptible (%) according to	
	MIC_{50}	MIC_{90}	MIC diffusion	disc
Amikacin	2	8	93.0	91.2
aztreonam	32	256	17.5	17.5
Cefepime	4	32	66.7	59.6
Ceftazidime	32	256	24.6	56.1
Ceftazidime/clavulanic acid	32	256	24.6	ND
Imipenem	1	16	75.4	70.2
Piperacillin	256	256	35.1	ND
piperacillin/tazobactam	256	256	36.8	52.6
Ciprofloxacin	0.25	32	68.4	71.9

MIC = minimum inhibitory concentration; MIC_{50} = MIC for 50% of isolates; MIC_{90} = MIC for 90% of isolates; ND = not done.

DDST and cloxacillin-based DDST screening

Table 4 shows the sensitivity and specificity of DDST and cloxacillin-containing DDST screening for detection of ESBL. The sensitivity and specificity in the DDST for ESBL-producing *P. aeruginosa* was 7.7% and 100%, respectively. According to the experimental criteria, the cloxacillin-based DDST was considered positive if the ceftazidime zone diameter increased >10 mm, and the sensitivity and specificity were 38.5% and 84.1%, respectively. A cloxacillin-based DDST was negative according to the experimental criteria if the ceftazidime zone diameter increased ≧5 mm, and the sensitivity increased to 76.9%; however, the specificity decreased to 54.5%.

Discussion

According to the criteria of CLSI, all isolates with MICs ≧2 μg/mL for ceftazidime, ceftriaxone, cefotaxime or aztreonam are potential ESBL producers in Enterobacteriaceae.[15] The CLSI recommends that a confirmatory clavulanic acid inhibition test be performed on positive screening test clinical isolates.[15] Detection of ESBLs by DDST with clavulanic acid and extended-spectrum cephalosporins is sensitive and specific for the detection of ESBLs in the Enterobacteriaceae.[24,25] The Enterobacteriaceae with elevated MIC, positive screen test and positive confirmatory test may cause ESBL genes; while a negative ESBL confirmatory test, indicates the presence of ampC or a combination of ampC and ESBL in the Enterobacteriaceae.[7,26]

Similarly, the DDST may not be sensitive enough for the detection of ESBLs in *P. aeruginosa*.[16] These false-negative

Table 3 PCR results and susceptibility of Ambler A, C, and D β-lactamase genes for 57 *Pseudomonas aeruginosa* isolates

β-lactamases	No. (%) of isolates	MIC range (μg/mL) / susceptible (%)			
		Ceftazidime	Ceftazidime/ clavulanic acid	Piperacillin	Piperacillin/ tazobactam
PCR-negative	22 (38.6)	4–256/40.9	4–256/40.9	8 to ≥256/31.8	4 to ≥256/36.3
PCR-positive:					
AmpC alone	22 (38.6)	8–256/22.7	8–256/22.7	8 to ≥256/50.0	8 to ≥256/50.0
AmpC+TEM	8 (14.0)	32–256/0.0	16–256/0.0	32 to ≥256/25.0	32 to ≥256/25.0
AmpC+TEM+SHV	1 (1.6)	64/0.0	128/0.0	≥256/0.0	≥256/0.0
AmpC+TEM+GES	1 (1.6)	32/0.0	64/0.0	≥256/0.0	≥256/0.0
AmpC+TEM+OXA-I	1 (1.6)	32/0.0	64/0.0	≥256/0.0	≥256/0.0
AmpC+TEM+OXA-I+VEB	1 (1.6)	256/0.0	256/0.0	≥256/0.0	≥256/0.0
VEB					
TEM alone	1 (1.6)	64/0.0	64/0.0	≥256/0.0	≥256/0.0

MIC = minimum inhibitory concentration; PCR = polymerase chain reaction.

results could be due to several factors: (1) chromosome-encoded *ampC* genes that may be overexpressed; (2) simultaneous presence of MBLs with carbapenem-hydrolyzing activities; (3) relative resistance to inhibition by clavulanate, as exemplified by GES-2; and (4) combined mechanisms of resistance, such as impermeability and efflux.[2]

There is no standardized method for the detection of ESBLs in *P. aeruginosa*. Current ESBL detection methods, based on the inhibitory effect of clavulanic acid on the activities of ESBLs against extended-spectrum cephalosporins, are inadequate for the detection of ESBLs in *P. aeruginosa*.[27,28] The inhibition of AmpC enzyme activity with the addition of cloxacillin could enhance the abilities of DDST to detect ESBLs in *P. aeruginosa*.[16] De Champs et al[16] have revealed that the cloxacillin-containing DDST had a low sensitivity rate involving the detection of ESBL-producing *P. aeruginosa* when utilizing experimental criteria with a ceftazidime zone diameter increase of >10 mm. Jiang et al29 also have compared various screening methods for detecting ESBLs in 34 clinical isolates of *P. aeruginosa* whose β-lactamases were well characterized.[29] Among the total of 34 isolates, only 10 (29.4%) strains were positive for a conventional DDST. The sensitivity was increased to 82.4% in DDST that contained 250 μg/mL cloxacillin, when using the experimental criteria of a ceftazidime zone diameter increase of ≧5 mm.[29] In our study, the sensitivity for detecting ESBLs in *P. aeruginosa* was only 7.7%, and the specificity was 100% by conventional DDST; the sensitivity was increased to 38.5% and the specificity was 84.1% by cloxacillin-containing DDST (experimental criteria, ceftazidime zone diameter increase >10 mm). Furthermore, the cloxacillin-based DDST was considered positive by adjusting the experimental criteria (the ceftazidime zone diameter increase of ≧5 mm), the sensitivity was increased to 76.9%; however, the specificity was decreased to 54.5%. The sensitivity of cloxacillin-containing DDST is higher than that of conventional DDST, especially using the experimental criteria of a ceftazidime zone diameter increase of ≧5 mm. The cloxacillin-containing DDST was helpful for the phenotypic detection of ESBLs in *P. aeruginosa*.

The MICs to ceftazidime and piperacillin were not decreased in the presence of β-lactamase inhibitors (clavulanic acid and tazobactam) in PCR-positive strains. In this study, 35 (61.4%) of 57 *P. aeruginosa* isolates were PCR-positive for β-lactamase genes. Among these 35 PCR-positive isolates, 34 (97.1%) were positive for the *ampC* gene. AmpC β-lactamases are cephalosporinases, which contribute to intrinsic resistance to most β-lactams and β-lactamase inhibitors. Of the 34 AmpC-producing *P. aeruginosa* isolates, 29 (85.3%) isolates were not susceptible to ceftazidime. In *P. aeruginosa*, the overexpression of the naturally occurring AmpC is associated with decreased susceptibility or resistance to expanded-spectrum cephalosporins such as ceftazidime.[16,22,30] However, *ampC* genes were not detected in 23 isolates by PCR. Further studies using different primer pairs of *ampC* genes are necessary to identify the AmpC subtypes.

Of the 13 isolates with class A ESBL genes, TEM-type enzymes were most prevalent (12 isolates, 92.3%). Only two isolates carried the OXA group I gene, and three isolates carried SHV, GES and VEB, respectively. CTX-M, PER, BEL, OXA group II and OXA group III genes were not detected in this study. These findings are different from those outside Taiwan. The β-lactamases PER-1 was the first ESBL identified and fully characterized in *P. aeruginosa*, which occurred in 1993.[31,32] ESBLs in *P. aeruginosa* are now reported worldwide. ESBLs of TEM and SHV types are rarely observed in *P. aeruginosa*,[2] however, ESBLs of other types have spread in this species within specific geographical locations: PER-1β-lactamases are most widespread in Turkey,[33] VEB-1 in Southeast Asia,[34] GES-like β-lactamases have been identified in French, Greek, and South African isolates,[35–37] and BEL in Belgium.[38]

Besides ESBLs, the MBLs are also being reported in growing numbers in *P. aeruginosa* isolates. Surveillance of β-lactamases production by *P. aeruginosa* in Taiwan has focused on MBLs.[39–41] Yan et al have indicated that both IMP- and VIM-type MBLs have emerged in *Pseudomonas* spp. In Taiwan, the VIM-2-related enzymes are the most prevalent types.[39,40] Huang et al also have described the higher prevalence of the MBL genes among *P. aeruginosa* isolates in Taiwan (36.0% in 2000 and 17.0% in 2002) compared with neighboring countries.[41] However, MBL genes were beyond the scope of this study; instead, we only focused on ESBL and *ampC* genes. Further surveillance of the β-lactamases

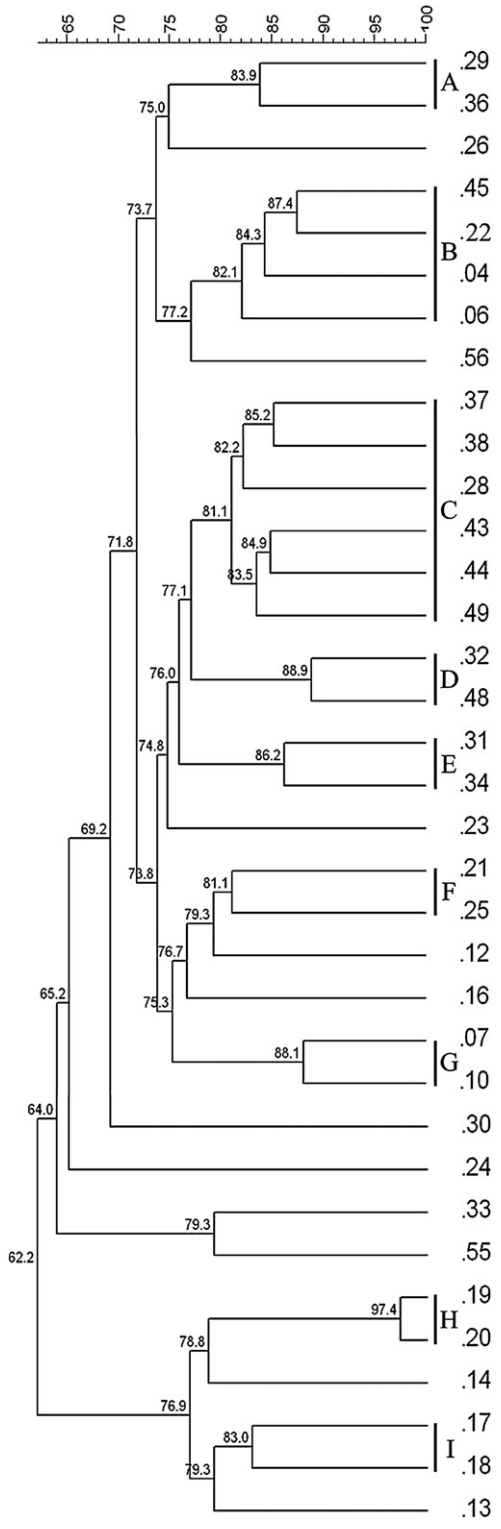

Figure 1. Dendrogram of 35 PCR-positive *Pseudomonas aeruginosa* isolates. The numbers on the right side indicated the isolate numbers. Nine clusters (A—I) were classified.

carried by *P. aeruginosa* could identify the change and spread of the β-lactamase genes.

Three isolates with GES, OXA and VEB belonged to three different clusters (C, D and F, respectively). This finding could be explained by horizontal transfer of resistance gene carried by mobile genetic elements. Isolates of clusters B and C were distributed in different wards of this hospital during a period of 3—4 years. They were isolated from sporadic cases of infections rather than outbreak. However, these sporadic isolates actually originated from common ancestors.

Table 4 Sensitivity and specificity in DDST and cloxacillin-based DDST for ESBL-producing *Pseudomonas aeruginosa*

	Sensitivity		Specificity	
	Criteria of zone diameter difference		Criteria of zone diameter difference	
	≥5 mm	>10 mm	≥5 mm	>10 mm
DDST	7.7%	—	100%	—
Cloxacillin-DDST	76.9%	38.5%	54.5%	84.1%

DDST = ouble disc synergy test.

In conclusion, ESBL genes are not uncommon in *P. aeruginosa* isolates. The fact that the *P. aeruginosa* isolates carried a combination of *ampC* and ESBL genes could result in the failure of detection of an ESBL phenotype by conventional DDST. Cloxacillin-containing DDST can enhance the sensitivity and has a potential role for phenotypic detection of ESBL-producing *P. aeruginosa*. PCR is also helpful for the identification of specific β-lactamase genes. These *P. aeruginosa* isolates were classified into several diverse clones which could continue to spread in the hospital over a long period of time.

Acknowledgments

We thank the staff in the Microbiology Laboratory at Taichung Veteran General Hospital for the collection of the bacterial isolates.

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