

Study on drug resistance of *Pseudomonas aeruginosa* plasmid-mediated AmpC β -lactamase

BIN ZHU¹, PING ZHANG¹, ZHEN HUANG¹, HAI-QING YAN¹,
AN-HUA WU², GANG-WEN ZHANG¹ and QIANGUO MAO³

¹Department of Infectious Disease, The First Affiliated Hospital of Xinxiang Medical University, Xinxiang 453100; ²Center of Infectious Control, Xiangya Hospital Center South University, Xiangya 410008; ³Department of Hepatology, Xiamen Traditional Chinese Medicine Hospital, Xiamen 361004, P.R. China

Received July 25, 2012; Accepted November 9, 2012

DOI: 10.3892/mmr.2012.1235

Abstract. The aim of the present study was to investigate the resistance of plasmid-mediated AmpC β -lactamase in *Pseudomonas aeruginosa*, to detect and identify the AmpC genotype and to provide evidence for antibiotic applications in the clinic. Resistance phenotype in 108 strains of clinically isolated *P. aeruginosa* was determined by Kirby-Bauer disk test and cefoxitin three dimensional test in AmpC-positive strains. Plasmids were extracted from AmpC-positive strains using the SDS-alkali splitting technique. The depurated plasmid was used to amplify AmpC β -lactamase genes by PCR. Positive PCR products were sequenced by the Shanghai Sangon Biological Engineering Technology Company. Gene homology of PCR products with other index sample gene sequences was compared. In the present study, 28 AmpC enzyme-positive *P. aeruginosa* strains among 108 were identified. Multidrug-resistance to antibiotics was observed in positive AmpC *P. aeruginosa* strains and a new *P. aeruginosa* strain of plasmid-mediated CMY-7 type AmpC enzyme was identified. In addition, AmpC type β -lactamases were revealed to be important in the resistance mechanism to antibiotics in *P. aeruginosa*. This is the first report of CMY-7 plasmid-mediated AmpC enzyme expression in *P. aeruginosa*.

Introduction

The increasing use and application of broad-spectrum antibiotics has led to increased reports of bacterial drug resistance and clinical infection. At present, drug-resistant bacteria, including methicillin-resistant *Staphylococcus aureus*, multidrug-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus*, multidrug-resistant *Baumannii*,

Klebsiella pneumoniae, *Escherichia coli* and *Pseudomonas aeruginosa* are increasingly being detected in clinical and laboratory settings (1). Appearance of pan-resistant bacteria, including *Cray Borrelia*, which produces New Delhi Metallo β -lactamase-1, an enzyme responsible for generating resistance (2), is causing an even greater challenge to humans. Therefore, study of clinical bacterial drug resistance and mechanisms and the development of solutions to bacterial resistance is important. Previously, Gram-negative bacilli AmpC β -lactamase was understood to be generated by chromosome mediation only. However, Papanicolaou *et al* (3) identified plasmid-mediated AmpC β -lactamase (MIR-1) in a strain of *Cray Borrelia* bacterium isolated from a patient in Rhode Island Hospital (USA) in 1989. Following this, the plasmid-mediated AmpC enzyme was rapidly identified in a number of other countries. At present, more than 20 plasmid-mediated AmpC enzymes have been identified. The plasmid-mediated AmpC enzyme is resistant to antibiotics, enabling the plasmid to continue to express the protein at high levels and carry multiple drug-resistant genes. Plasmid-mediated drug resistance is transmitted between members of the same or different bacterial species. Transmission is rapid and performed at a range of distances. Therefore, understanding of the mechanism and elucidation of solutions to plasmid-mediated AmpC enzyme resistance is crucial.

P. aeruginosa is one of the main bacterial strains associated with hospital infections, including burns, cystic pulmonary fibrosis and ventilator-associated infections. In addition, *P. aeruginosa* is resistant to multiple antibiotics and is associated with high rates of mortality (4-6). A number of drug resistance mechanisms have been identified in *P. aeruginosa*, including production of β -lactamase (extended-spectrum β -lactamases or AmpC) (7), alteration of penicillin-binding protein expression (8), formation of biomembranes, expression of cytomembrane permeability barriers (9,10) or active multidrug efflux pumps, including mex-ABoprM (11) and alterations in channel protein expression, including oprD (11,12). At present, *P. aeruginosa* plasmid-mediated AmpC β -lactamase has not been studied. The present study aimed to investigate plasmid-mediated AmpC in clinically-isolated *P. aeruginosa* through identification of the genotype of a drug-resistant strain, to aid clinical antibiotic applications.

Correspondence to: Professor Bin Zhu, Department of Infectious Disease, The First Affiliated Hospital of Xinxiang Medical University, Jinshuida Road, Xinxiang 453100, P.R. China
E-mail: zbahcn@163.com

Key words: *Pseudomonas aeruginosa*, plasmid, ampC enzyme resistance

Materials and methods

Specimens. In the present study, 108 strains of *P. aeruginosa* were clinically isolated from the sputum, urine, feces, ascites, hydrothorax, arthroedema, cerebrospinal fluid and other specimens of hospitalized patients in the ICU, respiratory, cardiology, infection, burns and pediatrics departments in the First Affiliated Hospital of Xin Xiang Medical University and Xiang Ya Hospital Center South University. Bacterial isolation, culture and identification was conducted according to the National Guide to Clinical Laboratory Procedures (2nd edition) and bacterial identification was conducted using an automatic bacterial identification analyzer. *E. coli* ATCC25922 and *P. aeruginosa* ATCC27853 were used as quality-control strains and *Aerobacter cloacae* 029M was used as a positive control strain as it is known to express the AmpC enzyme. The study was evaluated and approved by the ethics committees of Xin Xiang Medical University (XinXiang, China) and Xiang-Ya Medical College of Central South University (Changsha, China). Informed patient consent was obtained from the patient or the patient's family.

Primary screening test of specimens. Isolated *P. aeruginosa* was inoculated onto a blood plate and cultured at 37°C overnight. The following day, cultures were suspended in 0.9% stroke-physiological saline solution (Maxwell turbidity, 0.5) and coated uniformly onto M-H plates with sterile cotton swabs. Cefoxitin (FOX), ceftazidime (CAZ), cefotaxime/clavulanic acid (CTX/CA) and imipenem (IMP) drug-sensitive slips were symmetrically placed onto the M-H plates with bacterial suspension using sterile forceps and cultured overnight at 37°C. The next day, bacteriostatic rings of drug-sensitive slips were observed and the diameters of bacteriostatic rings were measured with a vernier caliper. According to criteria prepared by the National Clinical Laboratory Standardization Committee of America (13), bacteriostatic ring diameters of ≤ 18 , ≤ 21 , ≤ 22 and ≥ 13 mm for FOX, CTX/CA, CAZ and IMP, respectively, were indicative of strains expressing the AmpC enzyme.

Preparation of AmpC enzyme. *P. aeruginosa* stains identified by screening to express the AmpC enzyme were inoculated into 10 ml LB broth and cultured overnight in a thermostat shaker at a speed of 80-100 rpm at 37°C. Subsequently, the culture mixture was centrifuged in a refrigerated centrifuge at 4°C to remove the supernatant. Bacterial pellets were frozen for 30 min at -80°C and then thawed in a water bath. The freeze-thaw cycle was performed 5 times. Following this, bacteria were suspended in 1.5 ml PBS buffer (0.01 M) in an EP tube, mixed uniformly by agitation and centrifuged at low temperature to obtain the supernatant. The supernatant contained the AmpC enzyme and was filtered using a 0.22- μ m microporous membrane. Next, the filtrate was inoculated onto the blood plate and cultured overnight at 37°C. Bacterial growth was observed and negative filtrates were stored at -20°C.

Three-dimensional test. *E. coli* strain ATCC25922 was suspended in solution (Maxwell turbidity, 0.5) and uniformly coated with sterile cotton swabs onto M-H plates. A FOX drug-sensitive slip (30 μ g) was applied to the center of the

plate and a sterile surgical blade was used to cut a small groove with a width of ~1-2 cm, 3 mm away from the slip edge. In each small groove, 10-20 μ l AmpC enzyme extract of each test bacterium was added, avoiding extract overflow. The extract was cultured overnight at 37°C for 18-24 h prior to observation. The *Bacillus levans* (0.29 M) strain was used as a positive control as it is known to produce the AmpC enzyme. If the cefoxitin three-dimensional test shows a positive result, it suggests that the test bacterium generated the AmpC enzyme.

Plasmid extraction and electrophoresis. *P. aeruginosa* plasmids with positive three-dimensional test results were extracted by the SDS alkaline lysis method and then run on a 1% agarose gel. Electrophoresis was performed using 0.5X TBE electrophoresis buffer and images were captured under UV lamp.

PCR amplification. Plasmid-mediated AmpC β -lactamase-positive strains were divided into 6 populations according to chromosome source: i) ACC (*Hafnia alvei*); ii) FOX (unknown source); iii) MOX (unknown source); iv) DHA (*Morganella morganii*); v) CIT (*Citrobacter freundii*); or vi) EBC population (*A. cloacae*). Primer design was performed using AmpC enzyme gene sequences identified in previous reports and primer 5.0 software (Table I).

PCR system. Total PCR volume was 50 μ l and contained the following: 5 μ l 10X PCR buffer solution, 1 μ l dNTP (10 mmol/l), 2 μ l positive and reverse primers (100 pmol/ μ l), 4 μ l MgCl₂ (25 mmol/l), 0.4 μ l *Taq* enzyme (2 units), 4 μ l template and 31.6 μ l ddH₂O. For each run, 6 reactions were performed. With the exception of primers, all other reaction components and volumes were constant. PCR conditions were as follows: initial denaturation for 3 min at 94°C; 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 58°C, extension for 30 sec at 72°C; and a final extension for 2 min at 72°C. Agarose gel electrophoresis of the PCR amplification products was performed and images were captured using a gel imaging instrument.

Sequencing and analysis. Sequencing of PCR amplification products was performed by the Shanghai Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). PubMed BLAST sequence alignments were analyzed with DNAssist software.

Results

Three-dimensional test. Primary screening tests of K-B drug-sensitive slips of 108 strains of clinically-isolated *P. aeruginosa* demonstrated that 30 strains were AmpC enzyme-positive. Three-dimensional test of FOX confirmed that 28 strains were AmpC enzyme-positive.

Electrophoresis. The 28 positive strains of *P. aeruginosa* were analyzed by electrophoresis following plasmid extraction. Electrophoretic analysis revealed bands in 22 strains. Single and double bands were identified corresponding to 2 kb and/or 5 kb. Single and double bands were observed in 7 and 15 strains, respectively (Fig. 1).

Table I. AmpC enzyme primers.

AmpC enzyme	Primer	Sequence (5'-3')	Length (bp)	Genbank no.
MOX-1 MOX2 CMY-1 CMY-8 CMY-11	MOX	GCT TGA GCG GTA AAC GAG TG GAA TGT GCT GCC TGG GTG	171	D 13304
LAT-1-LAT-4 CMY-7 BIL-1	CIT	AGG GAT TAG GCT GGG AGA TG GAC ACG GAC AGG GTT AGG ATA G	251	X 78117
DHA-1 DHA-2	DHA	CCG TCT CCG TAA AGG GTA AGC CGG TCA GAG CAC CAA ACA GG	396	Y 1641
ACC	ACC	GTT ATC CGT GAT TAC CTG TC TAC TCA GCG AAC CCA CTT	248	AJ 133121
MIR-1 ACT-1	EBC	GAA ATC CGC CAC ATC CTG CCG TAA TAG CAG TTC AAG GGA	462	M 37839
FOX-1-FOX-5b	FOX	CT ACA GTG CGG GTG GTT CGG GCT TAT CTT CCT TCG	297	X 77455

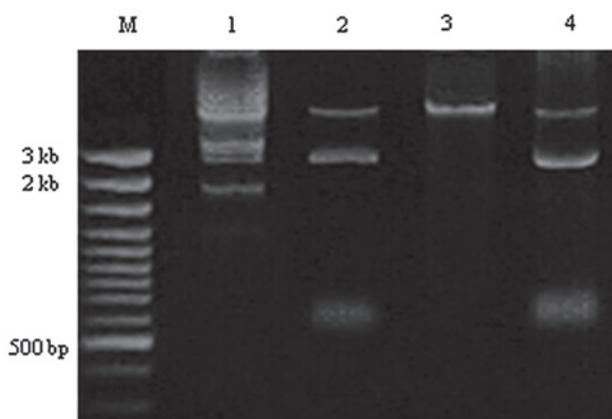
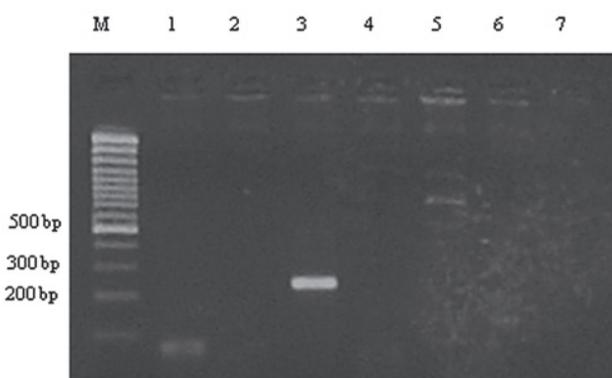
Figure 1. Plasmid DNA electrophoretogram. M, DNA marker; lane 1, *Aerobacter cloacae* positive control; lanes 2-4, *Pseudomonas aeruginosa*.

Figure 2. Electrophoretogram of PCR amplification products. M, DNA marker; lane 1, negative control; lane 3, positive product (251 bp); lanes 2, 4-7, negative.

PCR amplification. Purified plasmid DNA was used for PCR amplification templates. For each DNA template, 6 reactions were performed. An amplification product was obtained using one pair of primers of a total of 6 primer pairs. This primer was of CIT type and the target length was 251 bp (Fig. 2). Within 22 strains of plasmid-positive *P. aeruginosa*,

a single purified PCR amplification product was positive for this band.

Sequencing analysis. The positive strain revealing a target band of 251 bp was sequenced. The sequencing results are shown in Fig. 3.

Homology comparison. The sequenced product (2-220 bp) was identified to exhibit 99.5% (218/219) homology to the *A. cloacae* K9973 β -lactamase AmpC gene (881-1099 bp). The total length of the β -lactamase AmpC gene sequence in *A. cloacae* is 1165 bp (Fig. 4).

Discussion

P. aeruginosa is one of the main bacterial species responsible for severe hospital infections (14) and is resistant to a number of antibacterial drugs. *P. aeruginosa* has several drug-resistant mechanisms: 1) the generation of β -lactamase enzymes; 2) efflux pumps (e.g. mex2oprM); 3) target-site or outer membrane modifications. Multiple drug resistance is usually the result of the chromosomal and plasmid-encoded AmpC enzymes (15-17). Drug resistance cannot currently be detected by routine antibiotic sensitivity tests and the detection of the process is complex (18). At present, no universal test exists for the detection of AmpC β -lactamase; however, the three-dimensional test was previously reported to be the most suitable detection method available (19).

In the present study, 108 strains of *P. aeruginosa* were screened using K-B drug-sensitive slips. An AmpC enzyme-positive result was observed in 30 strains and the three-dimensional test of FOX was performed, identifying 28 strains as AmpC enzyme-positive. The detection coincidence rate of the two methods for AmpC enzyme-positive stains was 93.3%.

The three-dimensional test identified 28 AmpC enzyme-positive strains of *P. aeruginosa*. Following this, electrophoresis of plasmid extracts identified representative plasmid bands in 22 strains. Six strains were negative and had lost or did not carry plasmids initially. PCR amplification analysis identified positive plasmid-mediated AmpC enzyme expression in a

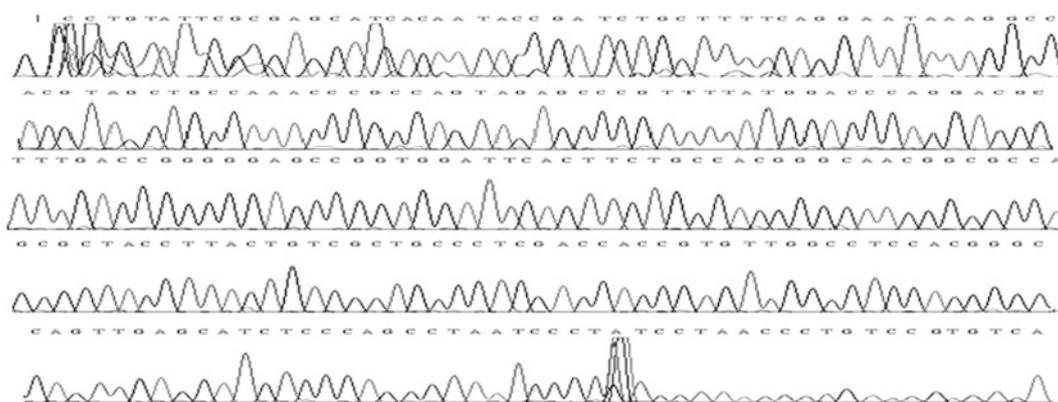


Figure 3. Sequencing of PCR products (251 bp).

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Query: 2      tgtattcgcgagcatcacaataccgatctgcttttcaggaataaaggccacgtagctgcc 61
            |||
Sbjct: 1099   tgtattcgcgagcatcacaataccgatctgcttttcaggaataaaggccacgtagctgcc 1040

Query: 62      aaaccgccagtagagcccgtttatggaccaggacgctttgaccggggagccggtgg 121
            |||
Sbjct: 1039   aaaccgccagtagagcccgtttatggaccaggacgctttgaccggggagccggtgg 980

Query: 122     attcacttctgccacgggcaacggcgccagcgctacctactgtcgctgcctcgaccac 181
            |||
Sbjct: 979     attcacttctaccacgggcaacggcgccagcgctacctactgtcgctgcctcgaccac 920

Query: 182     cgtgttggcctccacgggcccagttgagcatctcccagcc 220
            |||
Sbjct: 919     cgtgttggcctccacgggcccagttgagcatctcccagcc 881

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Figure 4. Sequence comparison of PubMed Bla.

single strain, demonstrating that the plasmid-mediated AmpC enzyme was present in *P. aeruginosa*; however, the proportion of plasmid-positive strains carrying the AmpC enzyme gene was low. It is possible that the *P. aeruginosa* strains that the three-dimensional test showed as positive could produce the majority of the chromosomal-mediated AmpC enzyme.

Previous studies on plasmid-mediated AmpC β -lactamase are largely restricted to the bacterial strains *A. cloacae* and *Cray Borrelia* (20-22). A limited number of studies have analyzed drug resistance in *P. aeruginosa* plasmid-mediated AmpC β -lactamase. In 2003, Shahid *et al* (23) isolated *P. aeruginosa* strains exhibiting plasmid-mediated AmpC enzyme from burns patients in an intensive care hospital in Northern India. In the domestic environment, a single strain of *E. coli* was previously observed to exhibit ACT-1 plasmid AmpC enzyme. However, no domestic reports on *P. aeruginosa* plasmid-mediated AmpC enzyme expression currently exist. The present study identified a single strain of *P. aeruginosa* plasmid-mediated AmpC enzyme expression using plasmid extraction, PCR amplification, product sequencing and BLAST homology comparison. A 99.5% homology with the *A. cloacae* K9973- β -lactamase AmpC gene sequence was identified. The overall length of *A. cloacae* K9973- β -lactamase AmpC gene sequence was 1,165 bp, consistent with the gene

sequence of *A. cloacae* K9973-generating CMY-7 AmpC enzyme previously identified by Lee *et al* (24). The present study is the first reported identification of plasmid-mediated AmpC β -lactamase expression in *P. aeruginosa*.

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