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Detection of *Pseudomonas aeruginosa* Carried a New Array of Gene Cassettes within Class 1 Integron Isolated from a Teaching Hospital in Nanjing, China

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We report here novel array of gene cassettes found in single variable region of class 1 integron disseminated in *Pseudomonas aeruginosa* isolated from a teaching hospital in Nanjing, Jiangsu Province, China. 29 of 47 (61%) *P. aeruginosa* strains were confirmed haboured class 1 integron, and all the positive strains have the same variable region confirmed by PCR and RFLP methods. The variable region contained an unreported order of four gene cassettes *aac*(6')-II-*aad*A13-*cml*A8-*oxa*-10. Of those, *cml*A8 gene was a variant of *cml*A5 encoding non-enzymatic protein which putatively confer resistance to chloramphenicol. Susceptibility testing revealed multidrug-resistant mechanisms were involved in the class 1 integron positive clinical isolates. And the class 1 integron located on an about 15 kb transferable plasmid was certified by conjugation experiment and plasmid DNA analysis. The macro restriction profile indicated those clinical strains were clonally related.

***Keywords*:** *Pseudomonas aeruginosa*, gene cassette, class 1 integron

An efficient method to acquisition or dissemination of antimicrobial resistance determinants is by mobile elements or mobilizable structures, including plasmids, transponsons, insert sequences, and resistance gene cassettes containing integrons (Collis *et al.*, 2002). Class 1 integrons are the integron that most frequently found among clinical isolates of both Gram-negative and Gram-positive bacteria (Lévesque *et al.*, 1995). Class 1 integrons usually contain two conserved segments (CS) flanking the antibiotic resistance gene cassettes. The 5'-CS includes the *int*I1 gene, which encodes the *Int*I1 integrase; the *att*I1 recombination site; and the Pc promoter, which directs transcription of cassette genes. The 3'-CS can vary in length but usually includes the *sul*1 sulfonamide resistance determinant (Stokes and Hall, 1989). In general, the variable region contains one or more integrated gene cassettes (Partridge *et al.*, 2000), which encode enzymes to several classes of antibiotics, such as β-lactams, aminoglycosides, and chloramphenicol, etc. *Pseudomonas aeruginosa* is one of opportunistic pathogens which mainly results in the infections in immunocomprised patients. The infections are often life threatening and difficult to treat because of its multidrug resistance and high frequency of inducing antibiotic resistance during the therapy (Quale *et al.*, 2006). One of the most important mechanisms is the resistant gene cassettes harbored by integrons encoding such as metallo-enzymes (*bla*VIM, *bla*IMP, *bla*SIM, and *bla*GIM), aminoglycosides modifing enzymes and responsible for the transmission or dissemination of anti-pseudomonas antibiotics resistance (Bissonnette *et al.*, 1991; Danel *et al.*, 1999; Guerra *et al.*, 2001; Shmara *et al.*, 2001; Kehrenberg *et al.*, 2005; Lee *et al.*, 2005; Lolans *et al.*, 2005; Yu *et al.*, 2006; Kadlec *et al.*, 2007). So far, only class 1 and class 3 integron were found in *P. aeruginosa* (Poirel *et al*., 2005). Here, we describe the dissemination and the feature of both groups of integrons in *P. aeruginosa* isolated from our hospital, a 1,800-bed teaching hospital in East China.

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Materials and Methods

Bacterial strains resources

Forty-seven strains of *Pseudomonas aeruginosa* were obtained from 1997 to 2003 and randomly chosen for study. Those isolates are clinically significant because each specimen was isolated from the patient diagnosed with explicit and obvious infection. All isolates were identified by the analytical profile index procedure (API-20NE system; bioMeriuex, France). *P. aeruginosa* ATCC 27853 was used as quality control and *P. aeruginosa* PU21 as recipient cell.

PCR, DNA sequencing, and restriction fragment length polymorphism (RFLP) analysis

All the isolates were candidates for screening class 1 and class 3 integrons by using specific primers located on integron-encoded integrase gene, the *intI*1 gene and *intI*3 gene, respectively (Shibata *et al.*, 2003). Variable region was amplified by using primers described before (White *et al.*, 2000). The PCR conditions were as follows: pre-denature at 94°C for 3 min; 30 cycles of amplification according to denature at 94°C for 30 sec, annealing at 62°C for 30 sec, extension at 72°C for 2 min; final extension at 72°C for 5 min. PCR for amplicons longer than 2 kb were performed with 2.5 U

of Ex *Taq* polymerase (TaKaRa, Japan) and 3.5 mmol/L of final Mg^{2+} concentration. In order to identify the gene cassettes harbored by this integron, the variable region amplicon was then purified by using the TaKaRa PCR Purification Kit and cloned into the pMD18-T vector (TaKaRa, Japan). The sequencing was done by an ABI PRISM 3730 genetic analyzer. PCR amplicons were digested by *Hinf*I, and amplicons with identical digested profiles were considered to contain the same gene cassettes.

Conjugation experiments and plasmids DNA analysis
To investigate the transferable resistance of the integron harbored by isolates, integron-positive wild-type isolates were cultured together with recipient cell rifampin resistant *P. aeruginosa* PU21 according to the description of Poriel (Poirel *et al.*, 2001b). Conjugants were selected on Mueller-Hinton agar plate containing 200 mg/L rifampin and 8 mg/L amikacin. Plasmid DNA was extracted from clinical strains and transconjugants by alkaline lysis. To obtain the good quality of plasmid, the velocity of centrifugal was enhanced to 18,000 rpm (Beckman, USA). For the fingerprinting analysis, about 5 μg of plasmid DNA was digested with 10 U of *Pst*I restriction enzyme (TaKaRa biotechnology, China). The productions were electrophoresed in 0.8% agarose at 80 V for 2 h.

Susceptibility testing
According to the recommendation of Clinical of Laboratory Standards Institution (CLSI), the susceptibility testing of parental strains and transconjugants was determined by agar dilution method (CLSI, 2006). The antibiotics used in this study included piperacillin (PIP), pipercillin-tazobactam (PTZ), ceftazidime (CAZ), imipenem (IMP), ciprofloxacin (CIP), amikacin (AM), gentamicin (GEN), and streptomycin (STR) (National Institute for the Control of the Pharmaceutical and Biological Products, Beijing, China). The constant concentration of tazobactam used in combination with piperacillin was 4 μg/ml.

Pulsed field gel electrophoresis (PFGE) patterns
Pulsed field gel electrophoresis was performed to clarify the relationship of those positive isolates. After overnight culture and adjusting turbidities to 2.5 Mc, all the *P. aeruginosa* were embedded in 2% agarose (Seakem Gold, Cambrex bio, America). The plugs were cultured overnight in 37°C in Gram-positive lysis solution (6 mM Tris-HCl, 100 mM EDTA, 1 M NaCl, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% SLS, pH 7.5) containing 0.5 mg/ml lysozyme and then in 56°C with Gram-negative lysis solution (1% SLS, 500 mM EDTA, pH 9.5) containing 0.5 mg/ml Proteinase K for 24 h. Restriction endoenzyme *Spe*I (TaKaRa, Japan) was used to digest the plug overnight at 37°C. Electrophoresis was performed on a CHEF MAPPER XA apparatus (Bio-Rad laboratory, USA) at 14°C for 19 h under the conditions of 2.5 sec for initial switch time, 5.0 sec for final switch time, 120 V for 19 h with angle of 120° and voltage gradient of 6 V/cm.

Results

Bacterial strains resources
Total of above 300 strains of *P. aeruginosa* were preserved from 1997 to 2003, of those, 47 *P. aeruginosa* strains were blind choosen to perform the afterwards experiments. Among 47 strains of *P. aeruginosa*, one was isolated from oral secretion, one from tracheal cannula, two strains from urine and the remainder were isolated from sputum.

PCR, sequencing, and RFLP
About 61% *P. aeruginosa* (29/47) were positive for class 1 integron by amplifying integrase 1 region and no class 3 integron was found. All the class 1 integron positive isolates possessed a same band of about 4.3 kb amplicon amplified by variable region PCR primers. One of the variable region amplicon of those isolates was cloned into the pMD18-T vector and sequenced. Analysis of the sequence by BLASTN and BLASTX revealed that the 4,300-bp amplicon contained aac(6')-II, aadA13, cmlA5-like, and oxa-10 gene cassettes (Fig. 1). The third cassette contains an allelic variant of the *cmlA* gene known to be responsible for nonenzymatic resistance to chloramphenicol (Bissonnette *et al.*, 1991). The product of the *cmlA* allele of our report different from other known CmlA proteins is most closely related to CmlA1, CmlA5, and CmlA6, from whom it differs by twelve amino acid residues. It shares 95% nucleotide similarity to *cmlA*5 gene by the Blast program (http://www.ncbi.nlm.nih.gov/BLAST/) and putative expresses a 44.6-kDa peptide. By *Hinf*I digestion, all the restriction profiles of amplicons showed the no change compared with before and exhibited single band (data not shown).

Conjugation experiment and plasmids analysis
All class 1 integron positive clinical isolates *P. aeruginosa* were used as donors to perform conjugation experiment and 29 strains of transconjugants resistant to rifampin and amikacin were obtained. Meanwhile all the wild-type strains and the recipient strain *P. aeruginosa* PU21 couldn't be cul-

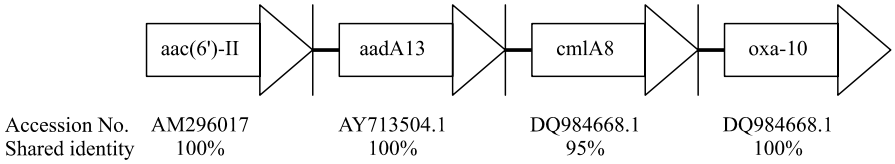

Fig. 1. The array of gene cassettes harbored by class 1 integron in this study. Open reading frames of the various resistance genes and the transcriptional orientation are represented by arrows. Percent homologies of current sequences compared with the GenBank sequences list in the last line.

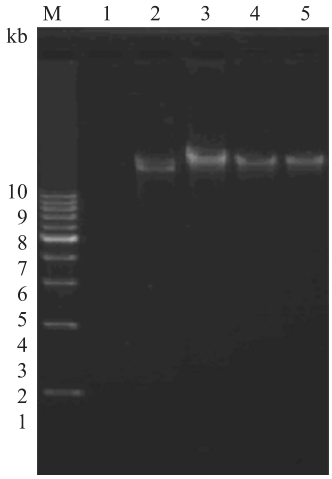

Fig. 2. The electrophoresis pattern of plasmids extracted from clinical strain, the corresponding transconjugant and *Pst*I restriction fragment. [M, 1,000 bp DNA ladder (TaKaRa, Dalian Biocompany, China); 1, *P. aeruginosa* PU21; 2, clinical *P. aeruginosa* strain; 3, the corresponding transconjugant; 4 and 5, *Pst*I digested plasmids of clinical *P. aeruginosa* strain and transconjugant.]

tured on the opposite screening label containing agar. An about 15 kb plasmid was obtained by alkaline lysis method among all the clinical strains and transconjugants. And the *Pst*I restriction profile showed identical among those strains. None of the DNA plasmids of those strains were cut off by great efforts with repeated five times (Fig. 2).

Susceptibility testing

Susceptibility testing revealed that all class 1 integron positive wild-type isolates and the corresponding transconjugants exhibited an overall resistant property to piperacillin, ceftazidime, gentamicin, amikacin, and streptomycin, and the MIC_{90} of piperacillin, ceftazidime, piperacillin/tazobactam, imipenem, ciprofloxacin, gentamicin, amikacin, and streptomycin against class 1 positive clinical strains was ≥512 mg/L, ≥512 mg/L, ≥128 mg/L, 32 mg/L, 64 mg/L, ≥256 mg/L, ≥256 mg/L, and ≥2,000 mg/L, respectively. Compared with the parental *P. aeruginosa* strains, the transconjugants decreased the resistance to piperacillin-tazobactam, ciprofloxacin, and imipenem, and MIC_{90} values of those antimicrobial agents decreased at least 8 folds, 64 folds, and 128 folds, respectively.

PFGE patterns

The macro restriction profiles of the *Spe*I-digested genomic DNAs of the 29 class 1 integron positive *P. aeruginosa* isolates involved in the outbreak were either identical to each other or different by no more than five bands (Fig. 3). According to Tenover, there are total six clones of strains and all those strains were clonal relatedness (Tenover *et al.*, 1995).

Discussion

Class 1 integrons were discovered in *P. aeruginosa* worldwide, and the frequency of class 1 integrons disseminated in *P. aeruginosa* displayed diversity. Compared to the previously reported frequencies (40.8%, 40/98) from our area (Gu *et al.*, 2007), we reported here a higher percentage of class 1 integron prevalence in *P. aeruginosa*. We considered the reason might be that our specimens were isolated from single hospital and different years, for Gu's specimen was collected from four different general hospitals (our hospital is one among them) of Nanjing from 2003∼2005. Analyzing the variable region of class 1 integron, a novel order of gene cassettes *aac*(6')-II, *aad*A13, *cml*A5-like, and *oxa*-10 (Fig. 1) was discovered in our reports. According to the database in GenBank, *aad*A13 is found in five strains of bacterium, including three strains of uncultured bacterium, one strain of *E. coli* and one strain of *Yersinia enterocolitica*. And it has never been reported in *P. aeruginosa* worldwide before. The third cassette contains an allelic variant of the *cmlA* gene known to be responsible for nonenzymatic resistance to chloramphenicol.

Modifying enzymes for aminoglycosides were found either

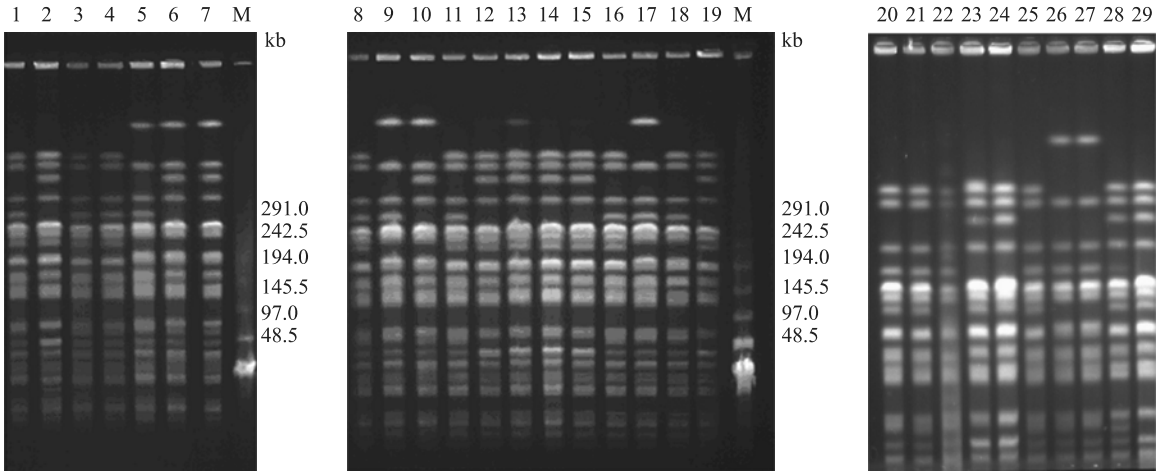

Fig. 3. PFGE pattern for 19 of 29 class 1 integron positive *P. aeruginosa* strains. [Lane 1 to 19, clinical *P. aeruginosa* strains digested by *SpeI*; M, Lambda Ladder PFGE Marker (New England Biolabs)]

on plasmids or on chromosome (Poriel *et al*., 2001a), but most of them were located on transferable plasmid, and our report confirmed that that modifying enzymes carried integron was located on transferable plasmid. Susceptibility testing and conjugation experiment showed the MIC value determined in both clinical isolates and transconjugants exhibited identical resistant pattern of piperacillin, ceftazidime, gentamycin, amikacin, and streptomycin but the resistant patterns of piperacillin-tazobactam were different. Transconjugants were susceptible to piperacillin-tazobactam, while the corresponding clinical isolated *P. aeruginosa* showed MIC90 value of ≥128 μg/ml. Therefore the transferable plasmid(s) was supposed to be not only a vector of class 1 integron, but also a vector for other genes, such as ESBL genes. Furthermore, we inferred that those class 1 integron positive isolates were multidrug-resistant *P. aeruginosa* and resistant mechanisms such as, chromosomal overproduction of AmpC enzyme, mutation of target enzymes DNA gyrase or topoisomerase IV of fluoroquinolones, low outer membrane permeability or drug efflux system maybe involved in via analyzing MIC_{90} values converted between clinical isolates and transconjugants.

By the PFGE pattern, although our strains were minimum part of the clinical isolate at the span of seven years by random selection, it is highly likely that they were clonally related, given the consistency of their resistance phenotype and their overall epidemiological relationships. It has been reported that not only may isolates of the same genotype be associated with different integrons, but also unrelated isolates of different genotypes may contain the same integron. Thus, it is likely that in our report the phenotype and genotype are identical to each other, that is, the same integron has the same resistant property. From the partial isolates macro restriction pattern, we infer that class 1 integron positive *P. aeruginosa* are seem to genetic corelation in our hospital.

Our study provides a new array of *aac*(6')-II-*aad*A13-*cml*A8-*oxa*-10 gene cassettes in a class 1 integron and aligns the putative amino acid sequence of a new CmlA protein and offers information of the epidemiology of those outbreak isolates. Meanwhile the gene transfer experiment discloses the class 1 integron containing novel arrangement of gene cassettes located on transferable plasmid which carried by multidrug-resistant *P. aeruginosa* clinical isolates.

GenBank accession number: The sequence of 3,946 bp was submitted to GenBank (accession no. EU182575).

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