Detection and Characterized of Class Ⅰ Integrons in Multidrug Resistant *Pseudomonas aeruginosa* in Intensive Care Unit in Guangzhou, China*

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Abstract: To characterize class 1 integrons in multi-drug resistant (MDR) *P. aeruginosa* in an intensive care unit of a teaching hospital. 23 strain of MDR *P. aeruginosa* recovered from patients in intensive care unit (ICU) were collected during 2008. The gene cassettes and antibiotic susceptibility of the isolates were characterized. All isolates were also subjected to pulsed-field gel electrophoresis (PFGE). Class 1 integrons were detected in 14 isolates (60. 9%). Three types of gene cassettes were identified among these strains, including one that has not been reported previously. The vast majority of the cassettes encoded aminoglycoside resistance gene, including aacA4, aadA2, aadB, aac6-Ⅱ. Most of isolates were classified into five PFGE patterns, type A ($n=6$); type B ($n=6$); type C ($n=4$); type D ($n=2$) and type E ($n=2$). The present study illustrates that integrons appear to be a common feature among MDR *P. aeruginosa* in Guangzhou of China, and associated with a high prevalence of antibiotic resistance. Our data also suggested that cross-infection of MDR *P. aeruginosa* was a challenging work in ICU.
Key words: nosocomail infection; multidrug resistant; *Pseudomonas aeruginosa*; Class 1 integrons; PFGE

重症监护病房多重耐药铜绿假单胞菌Ⅰ型整合子的检测及鉴定

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摘 要：整合子是介导细胞多重耐药的重要机制之一，鉴定了 2008 年某医院重症监护病房分离的 23 株多重耐药铜绿假单胞菌 1 型整合子，并应用脉冲场电泳分析其同源性。1 型整合子的阳性率达成 60.9%。3 种 1 型整合子基因盒被鉴定，其中整合子 blaOXA-10-acc6-Ⅱ-cmlA8 为首次发现报道。基因盒主要编码氨基糖苷类耐药基因，包括 aacA4，aadA2，aadB，aac6-Ⅱ。脉冲电泳结果表明，23 株多重耐药铜绿假单胞菌分为 5 个基因型，A 型（$n=5$）、B 型（$n=6$）、C 型（$n=4$）、D 型（$n=2$）和 E 型（$n=2$）。研究表明编码 1 型整合子是多重耐药铜绿单胞菌较为普遍的特征，且 1 型整合子与多重耐药表型存相关。研究结果同时也表明防止多重耐药铜绿单胞菌交叉感染仍然是 ICU 的一项挑战性工作。
关键词：院内感染；多重耐药；铜绿假单胞菌；1 型整合子；脉冲场电泳
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P. aeruginosa is mostly a nosocomial pathogent hat are capable of causing sever invasive disease in critically ill patients. Intensive Care Units (ICU) constitutes important reservoirs for *P. aeruginosa* that is becoming increasingly multidrug resistant. In recent years, several nosocomial spreads of multidrug-resistant (MDR) *P. aeruginosa* in ICU have been documented[1-5]. Because of the multiple antibiotic resistance exhibited by *P. aeruginosa*, nosocomial infections caused by this organism are difficult to treat[6-7]. A major contributing factor in the emergence of resistance is the acquisition and transfer of antibiotic resistance genes on plasmids, transposons and integrons[8-9]. Integrons form an important source for the spread of antibiotic resistance, especially in gram-negative bacteria[10-11]. Integrons are elements that contain the components of a site-specific recombination system that recognizes and captures mobile gene cassettes. An integron includes a gene encoding an integrase flanked by an attI recombination site. Gene cassettes are not necessarily part of the integron, but when integrated, they become part of the integron, often comprising antibiotic resistance genes. Three classes of antibiotic-resistance - encoding integrons have been described. Class 1 integrons are the most prevalent among Gram-negative bacteria, including *P. aeruginosa* strains[12].

The purposes of the present study were to detect class 1 integrons and to characterize their content and to investigate the possibility of nosocomial transmission of epidemic strains.

1 Material and Methods

1.1 Clinical setting and study period

The internal medicine ICU of the 1000-bed teaching hospital of the Guangzhou medical college, china, consist six rooms, including one single room, four two-bed rooms and one room with a maximal capacity seven patients. During 2008, no repetitive 23 strains of MDR *P. aeruginosa* recovered from patients in ICU were collected. All of the strains isolated from sputum.

1.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using a standard disk-diffusion method according to the Clinical and Laboratory Standards Institute guidelines. The following antibiotics were tested: imipenem, meropenem, cefoperazone/sulbactam, ceftazidime, cefotaxime, ceftriaxone, cefepime, aztreonam, piperacillin, piperacillin/tazobactam, ticarcillin-clavulanic acid, ciprofloxacin, levofloxacin, amikacin, gentamicin. Two control strains of *E. coli* (ATCC25922) and *P. aeruginosa* (ATCC27853) were included in the test.

1.3 Pulsed-field gel electrophoresis (PFGE)

Preparation and lysis of agarose-embedded cells, followed by pulsed-field gel electrophoresis of the *spe* Ⅰ-digested genomic DNA, were carried out as described previously[4]. The gels were run on a CHEF-DR Ⅱ system (Bio-Rad Laboratories, USA) over 10 h with 5 to 15 s, then 10 h with 15 to 45 s of linear ramping at 6 V/cm, 14 ℃. Gel images were analyzed by BioNumerics (Applied Maths, Belgium). The percentage similarity of profiles was calculated by the Dice coefficient and the unweighted-pair group method with arithmetic averages was used for clustering.

1.4 PCR amplification of class Ⅰ integrase

Isolates were grown overnight in Luria-Bertani broth cultures at 37 ℃ with aeration. Total genomic DNA were extracted and purified was performed as described previously[11]. All isolated were screened for class Ⅰ integron by PCR using primer int Ⅰ-F (5'-GCTTACGAACCGAACAGGC), int Ⅰ-R (5'-CCGAGGATGCGAACCACT), which targeting to class Ⅰ integrase. Use the following program: 95 ℃ for 5 min, 30 cycles of 95 ℃ for 30 s, 50 ℃ for 30 s, 72 ℃ for 30 s, with a final extension for 2 min at 72 ℃. PCR amplifications were carried out in 50 μL volumes containing 0.4 μg template DNA, 0.2 mmol/L dNTP, 5 μL of 10 × PCR buffer, 5 U of taq polymerase (Takara, Japan). PCR amplification was performed with Bio-rad thermal cycle (Bio-rad, USA). Amplification products were resolved by electrophoresis at 90 V for 45 min on $w = 1\%$ agarose gels with 0.5 × TBE buffer. Then gels were stained by ethidium bromide and were visualized under UV light.

1.5 Cloning and sequencing of the variable region

Amplification of The variable region of class Ⅰ was performed in class Ⅰ integrase-positive isolates, using the primers 5'-CS/3'-CS according to the method previously[13]. Cassettes PCR products with the same size were digested with 15 U of *Hea*Ⅲ (NEB, USA) and 25 U of *Hinf* Ⅰ at 37 ℃ 2 h. Products showing different sizes or different digestion profiles were subsequently purified from agarose gels and ligated with the Pt simple vector (Takara, Japan). The ligation mix were transformed into *E. coli* DH5α, and then selecting with 50μg/ml ampicillin MacConkey agar plates. Recombinant plasmid DNA was purified using Qiaquick purification columns (QIAGEN, USA) according to the standard methods and subjected to sequencing on ABI3730xl DNA analyzer (applied biosystems, USA). The nucleotide sequences were analyzed and compared with those in GenBank using the BLAST algorithm (http://www.ncbi.nlm.nih.gov)

2 Results

2.1 Antimicrobial resistance

Of 23 MDR *P. aeruginosa* isolates obtained from the sputum samples of ICU-hospitalized adults in 2008, all isolates were selected for determination of antimicrobial resistance patterns. 6 (26.1%) isolates were resistant to all antibiotics in antimicrobial susceptibility tests. All isolates resistant to 5 antibiotics, including imipenem, meropenem, cefotaxime, ceftriaxone and ticarcillin-clavulanic acid. The high resistance to cefoperazone/

sulbactam (82.6%), ceftazidime (91.3%), cefepime (87.0%), levofloxacin (95.6%) were found (Table 1). (91.3%), piperacillin (95.6%), piperacillin/tazobactam

Table 1 Characteristics of MDR *P. aeruginosa* isolates examined in the present study

strain no	first isolated time[1)]	resistance phenotype[2)]	Treatment[2)]	PFGE	intI1[3)]	gene cassettes[4)]
336	1/31/08	IMP MEM CAZ CTX CRO FEP PIP TZP TIM CIP LEV AK GN	SCF, MXF	D	+	aadB-cmlA1
339	1/31/08	IMP MEM SCF CAZ CTX CRO FEP PIP TZP TIM CIP LEV AK GN	IMP, TZP, VA, LEV, Cefotiam, Ornidazole, metronidazole, Piperacillin/sulbactam	A	+	blaIMP-9-accA4-blaOXA-10-aadA2
389	1/31/08	IMP MEM SCF CAZ CTX CRO FEP PIP TIM CIP LEV AK GN	SCF, IMP, TZP, VA	A	+	blaIMP-9-accA4-blaOXA-10-aadA2
507	2/17/08	IMP MEM CAZ CTX CRO FEP ATM PIP TZP TIM CIP LEV AK GN	SCF, IMP, TZP, VA	D	+	aadB-CmlA1
513	2/14/08	IMP MEM SCF CAZ CTX CRO FEP ATM PIP TZP TIM CIP LEV AK GN	SCF, IMP, TZP, VA	A	+	blaIMP-9-accA4-blaOXA-10-aadA2
531	2/14/08	IMP MEM SCF CAZ CTX CRO FEP ATM PIP TZP TIM CIP LEV AK GN	SCF, CIP, TZP, VA	A	+	blaIMP-9-accA4-blaOXA-10-aadA2
793	2/19/08	IMP MEM SCF CAZ CTX CRO FEP PIP TZP TIM CIP LEV AK GN	SCF, TZP, MXF	A	+	blaIMP-9-accA4-blaOXA-10-aadA2
685	3/08/08	IMP MEM SCF CAZ CTX CRO FEP ATM PIP TZP TIM CIP LEV AK GN	SCF, CIP, TZP, VA	sporadic		
1559	6/16/08	IMP MEM CTX CRO ATM TIM CIP LEV	CIP, IMP, VA	B		
2379	6/25/08	IMP MEM SCF CAZ CTX CRO FEP ATM PIP TZP TIM CIP LEV AK GN	SCF, CIP, IMP, TZP, LEV, VA, metronidazole, CC	B	+	blaOXA-10-acc6-Ⅱ-cmlA8
2385	7/10/08	IMP MEM SCF CAZ CTX CRO FEP PIP TZP TIM	CIP, IMP, TZP, LEV, SCF, metronidazole	C	+	blaIMP-9-accA4-blaOXA-10-aadA2
2773	7/28/08	IMP MEM SCF CAZ CTX CRO FEP ATM PIP TZP TIM CIP LEV	TZP, LEV	B		
3087	8/15/08	IMP MEM SCF CAZ CTX CRO FEP PIP TZP TIM CIP LEV GN	CIP, TZP	C	+	blaIMP-9-accA4-blaOXA-10-aadA2
3098	8/19/08	IMP MEM SCF CTX CRO FEP ATM PIP TZP TIM CIP LEV	SCF, IMP, TZP, AK, VA	B		
3391	9/5/08	IMP MEM SCF CAZ CTX CRO FEP PIP TZP TIM CIP LEV GN	SCF, IMP, TZP	C	+	blaIMP-9-accA4-blaOXA-10-aadA2
3825	9/28/08	IMP MEM SCF CAZ CTX CRO FEP ATM PIP TZP TIM CIP LEV AK GN	SCF, CIP, IMP, TZP, Piperacillin/sulbactam	sporadic		
3739	9/28/08	IMP MEM SCF CAZ CTX CRO FEP ATM PIP TZP TIM LEV	SCF, CIP, IMP, TZP, Piperacillin/sulbactam	E		
3980	9/28/08	IMP MEM SCF CAZ CTX CRO FEP ATM PIP TZP TIM LEV	SCF, CIP, IMP, TZP, Piperacillin/sulbactam	sporadic		
3984	10/6/08	IMP MEM SCF CAZ CTX CRO FEP PIP TZP TIM LEV AK GN	CIP, AZM	C	+	blaIMP-9-accA4-blaOXA-10-aadA2
3843	10/6/08	IMP MEM SCF CAZ CTX CRO FEP ATM PIP TIM LEV GN	CIP, AZM	A	+	blaIMP-9-accA4-blaOXA-10-aadA2
3808	10/6/08	IMP MEM SCF CAZ CTX CRO FEP ATM PIP TZP TIM LEV	TZP, metronidazole	E		
4159	12/3/08	IMP MEM CAZ CTX CRO ATM PIP TZP TIM LEV	SCF, CIP, IMP, metronidazole	B		
158	12/6/08	IMP MEM SCF CAZ CTX CRO FEP ATM PIP TZP TIM CIP LEV AK GN	SCF, IMP, TZP, LEV, TEI	B	+	blaOXA-10-acc6-Ⅱ-cmlA8

1) month/day/year; 2) IMP: imipenem; MEM: meropenem; SCF: cefoperazone/sulbactam; CAZ: ceftazidime; CTX: cefotaxime; CRO: ceftriaxone; FEP: cefepime; ATM: aztreonam; PIP: piperacillin; TZP: piperacillin/tazobactam; TIM: ticarcillin/clavulanic acid; CIP: ciprofloxacin; LEV: levofloxacin; AK: amikacin ; GN: gentamicin; VA: Vancomycin; CC: clindamycin; TEI: Teicoplanin; AZM: azithromycin; 3) Class 1 integrase gene; 4) Class 1 integron gene cassette array dClass 1 integron gene cassette array

2.2 Epidemiology typing of *P. aeruginosa* isolates by PFGE

The molecular epidemiology of MDR *P. aeruginosa* was studied in the medical ICU during 2008. To determine whether the increase of *P. aeruginosa* isolation was due to the spread of epidemic strains, MDR P. aeruginosa were genotyped by *spe* Ⅰ digestion, PFGE and dendrogram analysis. Genotypic analysis identified five PFGE clones, named from A to E, differed in migration of at least four DNA fragment and showed a similarity of $<85\%$ at dendrogram analysis. The remainder had unique PFGE profiles, therefore classified as sporadic. PFGE clones A and PFGE clones B were each responsible for six strains (Fig. 1).

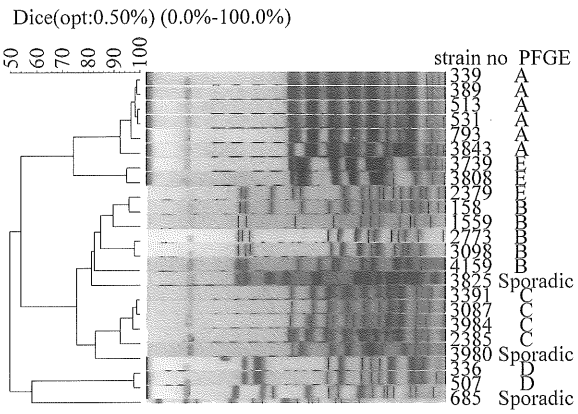

Fig. 1 Pulsed-field gel electrophoresis (PFGE) analysis by *spe* Ⅰ restriction enzyme digestion of 23 MDR *P. aeruginosa* (Clone A include 339, 389, 513, 531, 793, 3843; clone B include 1559, 2379, 2773, 3098, 4159, 158; clone C include 2385, 3087, 3391, 3984; clone D include 336, 507; clone E include 3739, 3808; 685, 3825, 3980 were belong to sporadic strains)

2.3 Detection of class Ⅰ integrons by integrase gene PCR

PCR detection of the *intI*1 genes demonstrated the presence of integrons in part of *P. aeruginosa*. The integrase gene PCR resulted in a frequency of integron-positive isolates of 60.9% (14 of 23). The PCR product size was 292 bp, and all of PCR products were confirmed by DNA sequencing, which were 100% identical to previously published sequences of the *intI*1.

2.4 Characterization of antibiotic resistance gene cassettes in class Ⅰ integron-positive strains

To further investigate the mechanisms of antibiotic resistance in *P. aeruginosa*, we sought to determine whether antibiotic resistance genes might be located in mobile gene cassettes. To address this issue, *P. aeruginosa* isolates were analyzed for class Ⅰ integrons variable region. The variable regions were amplified with the primers 5' CS and 3' CS, which annealed with DNA regions flanking the recombination site. Amplification of these intI-positive isolates gave PCR products of various sizes. Two isolates of integron cassettes PCR products were 2.3 kb, the remainder strains of amplicons were approximately 3.8 kb. To further differentiate between the gene cassette amplification products, they were digested with *Hea*Ⅲ and *Hinf*Ⅰ. After endonuclease analysis and sequencing analysis of the amplification of the integron gene cassettes, all these gene cassettes were divided into eight different gene cassettes. Among these gene cassettes, we have identified three different gene arrays in class Ⅰ integron gene - cassette system (Fig. 2).

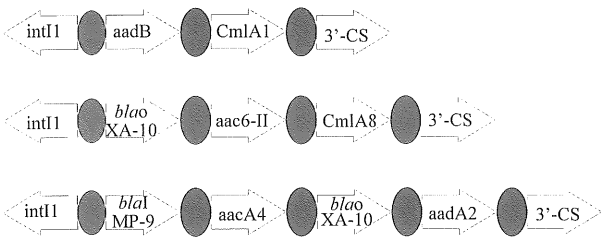

Fig. 2 Schematic structures of the class 1 integrons found in this study

(Gray ellipses represent *attI*1 site; black ellipses represent the 59-base elements. 5' -CS and 3' -CS stand for the 5' and 3' conserved sequences. The open reading frames are shown as arrows, which also indicated the direction of transcription)

3.5 kb cassette array of integron associated with PFGE A, C clones contained blaIMP-9, encoding metallo-beta-lactamse (MBL), which confer resistance to imipenem and broad-spectrum β-lactam antibiotics; oxa-10 β-lactam, encoding an extended-spectrum β-lactamase (ESBL), which confer resistance to to ampicillin and cephalothin, and characterized by the fact that they are poorly inhibited by clavulanic acid; aadA2, encoding an aminoglycoside adenylytranferase which mediates resistance to streptomycin and spectinomycin. 3.5 kb cassette array of integron associated with PFGE B clone consist of oxa-10β-lactamase,

aac6-Ⅱ encoding an aminoglycoside 6' -N-acetyltransferase, which confers resistance to amikacin and other aminoglycosides; cmlA8, encoding chloramphenicaol exporter, which mediates resistance to chloramphenicol. 2.5 kb cassette array of integron associated with PFGE D clone contained cmlA1 and aadB which encoding aminoglycoside 2" -O-nucleotidyltransferase, confers resistance to pathogenic bacteria against several aminoglycoside antibiotics including gentamicin, kanamycin, tobramycin. The gene cassette array blaIMP-9-accA4-blaOXA-10-aadA2 was the most prevalent in isolates.

3 Discussion

Since the ICU patients with more invasive procedures and the hands of healthcare personnel than in other hospital wards, ICUs are becoming an area of considerable antibiotic use. It is well known that multidrug-resistant bacteria are prevalent in ICU[14]. Class Ⅰ integron was the most common class of integrons detected in clinical isolates. Significant association of multi-resistance with the presence of an integron was demonstrated recently[15-16]. In the present study, a molecular diversity of gene cassettes in class Ⅰ integrons was characterized among MDR *P. aeruginosa* isolated from 23 ICU patients. The antibiotic susceptibilities were depicted, and PFGE homology analysis was performed to investigate the clonal relationships among the isolates.

In recent decades, MDR *P. aeruginosa* has increasingly been recognized as being responsible for hospital outbreaks. During the study period, an outbreak had occurred in the ICU caused by MDR *P. aeruginosa*. A total of six isolates recovered form six patients were belong to PFGE clone A. Among those isolates, 5 strains were isolated during the spring festival. After six months later, one isolates belong to PFGE clone A were still recovered. From June to December, strains belong to clone B has been discontinuous isolated. This data suggested that cross-infection of MDR *P. aeruginosa* was still a challenging work in ICU.

The outbreak strains were multi-resistant and were found to contain 3.5 kb integrons (blaIMP-9-accA4-blaOXA-10-aadA2) encoding multiple of antibiotic resistance genes. Most of the non-epidemic strains, which did not harbored class 1 integron, were also multi-resistant. 8 (34.78%), 11 (47.83%) strains were respective susceptible to gentamicin and amikacin, which were conferred by the resistance gene encoded in the integrons of the epidemic strains. Those resistant genes encoded in integrons may confer properties relevant to epidemicity. So antibiotic resistance is an important factor in nosocomial spread, but we can not rule out the possibility that the health care workers did not strictly comply with the medical practices during the holiday, may also contribute to the outbreak of MDR *P. aeruginosa*[5].

Previous study had suggested that detection of the integrase gene can act as a rapid and simple technique for screening the epidemic isolates. There was a significant correlation between the integrons and epidemicity in *Acinetobacter baumannii*, about 75% of isolates harbored integrons were epidemic [17]. In out study, 70% isolates harbored class 1 integrons were epidemic. So we suggested that integrons have also been considered as marker for epidemic potential in MDR *P. aeruginosa*.

Among all integron carrier, 3 integron cassette types were identified. To the best of our knowledge, blaOXA-10-acc6-Ⅱ-cmlA8 array was a novel class 1 integron gene (GU367339). aadB-cmlA1 array and blaIMP-9-accA4-blaOXA-10-aadA2 array found in *P. aeruginosa* have been submitted (DQ266448.1and GU122165.1). blaIMP-9- accA4- blaOXA -10-aadA2 array was the most prevalent in our study. Compared with another report about class 1 integrons in *P. aeruginosa* [18-19], the gene cassettes array have significant characteristics of the geographical prevalence. Epidemiological information can also been provided by characterizations of class 1 *P. aeruginosa* integrons[20].

The same gene cassettes array was found in *P. aeruginosa* stains belong to PFGE clone A and clone C. And six strains of PFGE clone B, two of those isolates harbored oxa-10-acc6-Ⅱ-cmlA8 gene array; the remains were no detected class 1 integron. Taken together, those data in partial support the hypothesis that antimicrobial resistances might have been acquired through horizontal gene transfer and a widely accepted view that the acquisition of entire integrons may be easier than that of individual cassette genes[21].

In summary, our data demonstrate that integrons appear to be a common feature among MDR *P. aeruginosa*, and associated with a high prevalence of antibiotic resistance. The horizontal and vertical transfer of antibiotic resistance gene cassettes may contribute to the wide dissemination of integrons.

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