International Journal of Infectious Diseases (2009) **13**, 717—721

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Identification and characterization of class 1 integrons among *Pseudomonas aeruginosa* isolates from patients in Zhenjiang, China

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Received 6 July 2008; received in revised form 22 September 2008; accepted 26 November 2008
Corresponding Editor: J. Peter Donnelly, Nijmegen, The Netherlands

KEYWORDS
Pseudomonas aeruginosa;
Integrons;
Gene cassettes;
Multi-drug resistance

Summary
Objectives: The role of integrons in the spread of antibiotic resistance has been well established. The aim of this study was to investigate the resistance profiles of *Pseudomonas aeruginosa* isolated from patients in Zhenjiang to 13 antibiotics, and to identify the structure and dissemination of class 1 integrons.
Methods: The Kirby—Bauer disk diffusion assay was used to determine the rate of *P. aeruginosa* resistance. Class 1 integrons from multidrug-resistant isolates were amplified by PCR, and their PCR products were sequenced. We also analyzed the integron structures containing the same gene cassettes by restriction fragment length polymorphism (RFLP). Isolates were genotyped by pulsed-field gel electrophoresis (PFGE).
Results: The resistance rates were between 29.6% and 90.1%. The prevalence of class 1 integrons was 38.0%. These integrons included five gene cassettes (*aadB*, *aac6-II*, *blaPSE-1*, *dfrA17*, and *aadA5*). The *dfrA17* and *aadA5* gene cassettes were found most often.
Conclusions: Class 1 integrons were found to be widespread in *P. aeruginosa* isolated from clinical samples in the Zhenjiang area of China. The antibiotic resistance rates in class 1 integron-positive strains of *P. aeruginosa* were noticeably higher than those in class 1 integron-negative strains. PFGE showed that particular clones were circulating among patients.
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1201-9712/$36.00. Published by Elsevier Ltd on behalf of International Society for Infectious Diseases.
doi:10.1016/j.ijid.2008.11.014

Introduction

The heavy selective pressure of antibiotics has promoted the rapid development of bacterial resistance. This bacterial resistance has become a severe and universal problem, leading to ineffective clinical treatment and increased economic burden, and also directly threatens the life of the patient.

Pseudomonas aeruginosa is an important opportunistic nosocomial pathogen, particularly evident in hospital-acquired pneumonia, especially in immunocompromised patients and those with a tracheal cannula, tracheotomy, or under mechanical ventilation.[1] Its resistance mechanism is extremely complicated. Generally, bacterial resistance is caused by chromosomal gene mutations or plasmid acquisition, however, recently, more and more bacterial resistance has been found to be associated with integrons.[2] Integrons, which consist of a 5′ conserved segment, a 3′ conserved segment, and a variable region between the two conserved segments, have been found to be mobilizable elements that capture external drug resistance gene cassettes and mediate bacterial resistance or multidrug resistance. These have caused the rapid spread of resistance in bacteria, especially among the Gram-negative.[3]

To date, four classes of integrons have been described in Gram-negative bacterial isolates, however class 1 integrons have been found to be the most prevalent in clinical isolates, carrying single or multiple gene cassettes, which confer resistance to aminoglycosides, β-lactams, chloramphenicol, carbapenems, and macrolides.[4–7]

In this study, we investigated the prevalence of resistance in 71 *P. aeruginosa* isolates to 13 antibiotics, and sought to identify the characteristics of class 1 integrons. At the same time, we also analyzed whether a particular clone was circulating among patients.

Materials and methods

Bacterial isolates

From April 2006 to March 2007, 71 *P. aeruginosa* isolates were collected from different patients hospitalized at the Affiliated People's Hospital of Jiangsu University, a hospital with in excess of 900 beds. Identification of isolates was carried out using the VITEK32 GNI card (bioMérieux, Hazelwood, MO, USA). Bacterial isolates were stored as suspensions in a 10% (wt/vol) sterilized milk solution containing 10% (vol/vol) glycerol at −20 °C until tests were performed.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined according to the Clinical and Laboratory Standards Institute (CLSI) using the Kirby–Bauer disk diffusion assay on Mueller–Hinton agar (Oxoid, UK).[8] The susceptibility profiles were determined for 13 antibiotics: piperacillin (PIP, 100 μg), piperacillin–tazobactam (TZP, 100/10 μg), ceftriaxone (CRO, 30 μg), cefepime (FEP, 30 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), gentamicin (GEN, 10 μg), amikacin (AMK, 30 μg), tobramycin (TOB, 10 μg), levofloxacin (LVX, 5 μg), ciprofloxacin (CIP, 5 μg), imipenem (IPM, 10 μg), and trimethoprim–sulfamethoxazole (SXT, 12.5/23.7 μg). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as controls.

DNA extraction

Genomic DNA used as template was obtained from bacterial suspensions grown overnight in Luria broth with shaking, suspended in 100 μl of sterile water, and boiled for 10 min.[9]

PCR amplifications and sequencing analysis

PCR was used to amplify the class 1 integrons, and the primers were designed as follows: forward primer: 5′-GGCATCCAAGCAGCAAG-3′, and reverse primer: 5′-AAGCAGACTTGACCTGA-3′.[10] Primers were synthesized by Shanghai GeneCore BioTechnologies Co., Ltd. A Mastercycler instrument (Eppendorf, Hamburg, Germany) was used with the following reaction conditions: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 2 min, and finally, 72 °C for 10 min. The amplicons were separated on 1% agarose gel prepared in TAE buffer and visualized using ultraviolet light after staining with ethidium bromide. PCR positive products were directly sequenced on both strands by the primer walking sequence strategy using the BigDye Terminator Cycle Sequencing Kit on an ABI PRISM 3730 automated DNA sequencer. The sequences were aligned in GenBank (http://www.ncbi.nlm.nih.gov/BLAST).

PCR-restriction fragment length polymorphism (RFLP)

To determine whether different isolates carried identical integrons, the amplicons of similar sizes were compared by RFLP typing using the restriction endonucleases HinfI and EcoRV. If the amplicons from two strains yielded the same RFLP pattern, the two integrons were considered to be identical. If the PCR products contained a different RFLP pattern, the new product was sequenced as well.

Pulsed-field gel electrophoresis (PFGE)

P. aeruginosa isolates carrying class 1 integrons were genotyped by PFGE with the restriction enzyme SpeI. DNA-PFGE marker (Amersham) was used as the size marker. The DNA fragments were separated on 1.0% agarose gels in 0.5× Tris–borate–EDTA buffer with a CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA, USA) at 6 V/cm for 20 h. Clonal relatedness based on the PFGE patterns was interpreted according to the criteria proposed by Tenover et al.[11]

Nucleotide sequence accession numbers

GenBank accession numbers are as follows: *aadB–aac6-II–pse-1*, **DQ266447**; *dfrA17–aadA5*, **DQ838665**; *aac6-II*, **EU723083**.

Results

Antimicrobial susceptibility of *P. aeruginosa* isolates

Susceptibility testing of the 71 *P. aeruginosa* isolates showed that all the isolates were resistant to at least one of the

antibiotics, and 64 (90.1%) isolates were resistant to three or more antimicrobials. Resistance was most often observed to cefotaxime (90.1%), followed by ceftriaxone (88.7%), trimethoprim–sulfamethoxazole (84.5%), and piperacillin (69.0%), and to a lesser extent tobramycin (35.2%), amikacin (31.0%), ceftazidime (29.6%), and imipenem (29.6%).

Integrons and characterization of gene cassettes

PCR-based examination for the presence of the class 1 integrons was carried out on the entire collection. Of 71 isolates, 27 (38.0%) carried detectable class 1-related integrons, showing the existence of amplicons of 2500 bp in three, 1600 bp in 22, and 750 bp in two of the class 1 integron-positive isolates. Amplicons of the same size gave identical restriction patterns with HinfI and EcoRV (Figure 1), which is indicative of their structural homogeneity. Sequence analysis of the class 1 integron variable region revealed that the 2500 bp amplicon harbored gene cassettes *aadB*, *aac6-II*, and *blaPSE-1*, among which *aadB* and *aac6-II* gene cassettes conferred resistance to aminoglycosides;[12,13] the *blaPSE-1* gene cassette contained the *blaPSE-1* beta-lactamase gene, conferring resistance to ampicillin, carbenicillin, cefoxitin, cefamandole, and piperacillin.[14] The 1600 bp amplicon harbored genes *dfrA17* and *aadA5*, conferring resistance to trimethoprim, as well as to streptomycin and spectinomycin,[15] and the 750 bp amplicon contained the *aac6-II* gene, conferring resistance as described previously (Figure 2). In this study, the most frequent variable region length among the class 1 integron-positive *P. aeruginosa* isolates was 1600 bp.

Susceptibility data from integron-positive and integron-negative *P. aeruginosa* isolates

Compared with the 44 integron-negative isolates, the 27 integron-positive clinical isolates were statistically more often resistant to certain antibiotics, including cefepime, ceftazidime, gentamicin, amikacin, tobramycin, levofloxacin, and ciprofloxacin ($p < 0.05$). Although there was no statistically significant difference ($p > 0.05$), the percentage of isolates susceptible to piperacillin, piperacillin–tazobactam, and trimethoprim–sulfamethoxazole was also lower among the integron-positive isolates (Table 1).

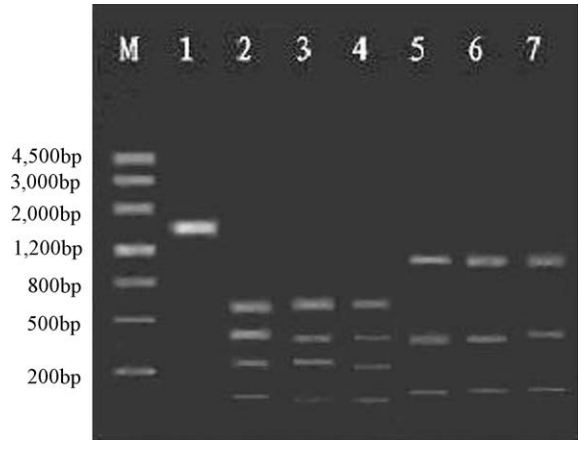

Figure 1 Schematic representation of the 1.6-kb integron digested with HinfI and EcoRV. M, DNA marker; 1, 1.6-kb integron PCR amplicon; 2–4, *ZJ070*, *ZJ199*, and *ZJ374* digested with HinfI; 5–7, *ZJ070*, *ZJ199*, and *ZJ374* digested with EcoRV.

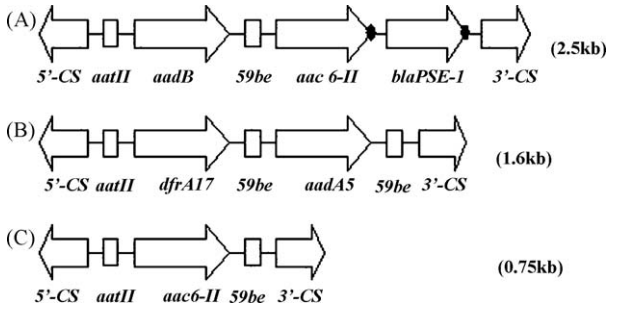

Figure 2 Schematic representation of the class 1 integrons from *P. aeruginosa* isolates. These integrons had approximate sizes of (A) 2.5 kb, (B) 1.6 kb, and (C) 0.75 kb. Arrows indicate the direction of transcription. The core is represented by black circles.

PFGE

To assess whether the high frequency of 1.6 kb-integrons in our isolates from different sources was caused by the spread of a specific clone, 22 isolates of 1.6 kb integron-positive *P. aeruginosa* were analyzed by PFGE profile. The PFGE profiles

Table 1 Antibiotic susceptibility of integron-positive and integron-negative strains of *P. aeruginosa*

Antibiotic	Integron-positive ($N = 27$)		Integron-negative ($N = 44$)		*p*-Value
	%S	%R	%S	%R	
PIP	18.5	81.5	38.6	61.4	NS
TZP	29.6	70.4	52.3	47.7	NS
CRO	11.1	88.9	11.4	88.6	NS
FEP	37.0	63.0	75.0	25.0	<0.01
CTX	7.4	92.6	11.4	88.6	NS
CAZ	51.9	48.1	81.8	18.2	<0.05
GEN	7.4	92.6	56.8	43.2	<0.01
AMK	33.3	66.7	90.9	9.1	<0.01
TOB	33.3	66.7	84.1	15.9	<0.01
LVX	29.6	70.4	79.5	20.5	<0.01
CIP	40.7	59.3	70.5	29.5	<0.05
IPM	74.1	25.9	68.2	31.8	NS
SXT	7.4	92.6	20.5	79.5	NS

%S, percentage susceptible; %R, percentage resistant; PIP, piperacillin; TZP, piperacillin–tazobactam; CRO, ceftriaxone; FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime; GEN, gentamicin; AMK, amikacin; TOB, tobramycin; LVX, levofloxacin; CIP, ciprofloxacin; IPM, imipenem; SXT, trimethoprim–sulfamethoxazole; NS, not statistically significant.
Statistical significance (p) was calculated using the Pearson Chi-square test in terms of the number of resistant strains and susceptible strains in the integron-positive and integron-negative groups. Intermediate isolates were considered as non-susceptible isolates.

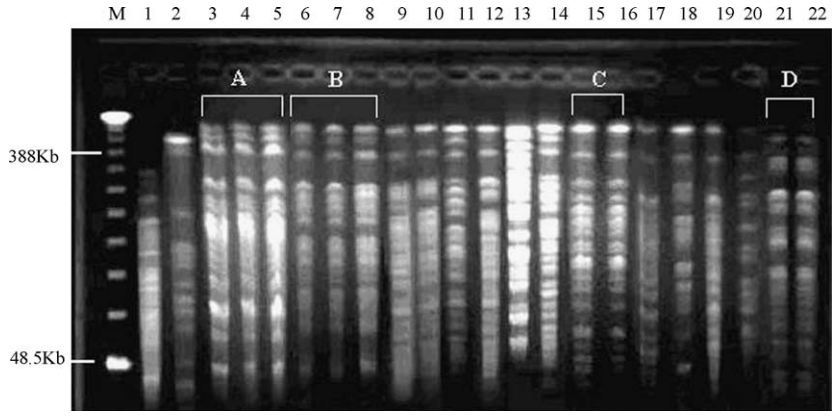

Figure 3 PFGE of SpeI-digested chromosomal DNA. M, molecular size marker; lanes 1—22, *P. aeruginosa* isolates from different patients. PFGE patterns A, B, C, and D of the isolates are shown.

obtained after SpeI digestion showed 15 to 20 DNA fragments. Ten strains (3—5, 6—8, 15 and 16, 21 and 22) were divided into four groups (namely groups A, B, C, and D) and each group had an identical DNA fingerprinting pattern; the others had different restriction patterns (Figure 3). The six isolates in clones A and B were obtained from the intensive care unit (ICU), and the four isolates in clones C and D came from the critical care unit (CCU), which indicates the spread of particular clones among the patients.

Discussion

Susceptibility testing of 71 *P. aeruginosa* isolates showed that all isolates were resistant to at least one of the antibiotics, and most of the isolates were multi-resistant. Sixty-four isolates (90.1%) were resistant to three or more antimicrobials. Resistance to cefotaxime, ceftriaxone, trimethoprim—sulfamethoxazole, and piperacillin was often detected.

The multiple resistance mechanism of *P. aeruginosa* is extremely complicated, and mainly involves plasmids and transposons, changes of outer membrane permeability, biofilm formation, and up-regulation of multi-drug efflux pumps, as well as integrons and gene cassette-mediated resistance.[16] In this study, we examined 71 *P. aeruginosa* isolates from patients in Zhenjiang and found that 38.0% contained class 1 integrons. This proportion is comparable to that found in other studies.[17,18] Integrons are known to be associated with multi-drug resistance, especially class 1 integrons, which are widely distributed among Gram-negative bacteria; our study also shows that multiple resistance often appears in isolates containing integrons. The *dfrA17—aadA5* array was detected in 22 *P. aeruginosa* strains and confers resistance to trimethoprim, as well as to streptomycin and spectinomycin. This array has been reported in other bacteria and in other regions, for example in China,[19,20] Korea,[21] Australia,[15] Norway,[22] and Hungary.[23] However, it was the first to be reported in *P. aeruginosa* isolates, which may indicate that the *dfrA17—aadA5* array can disseminate by self-transferable plasmids in humans or animals and in different areas of the world.[24] Therefore, the increase in clinically resistant isolates may be explained by antibiotic selective pressure and the widespread presence of integrons.[16] In addition, we also identified two aminoglycoside genes within the integrons, *aadB* and *aac6-II*, and one beta-lactamase gene, *blaPSE-1*, coding for aminoglycoside resistance and beta-lactamase resistance, respectively. Many isolates also had other antibiotic resistances not encoded by integron-associated resistance gene cassettes. These resistances may have resulted from chromosomal mutation, plasmid acquisition, or the presence of other integrons besides class 1 types.

From 22 isolates carrying the 1.6-kb integron, 10 were genetically related (from the ICU and CCU). These results show that there were four drug-resistant strains disseminated in our hospital through different patients and clinical wards, indicating that an infection control omission was occurring at the hospital. PFGE demonstrated that inter-hospital transmission might have occurred prior to our study. A number of factors might have contributed to the dissemination of clonal isolates in the ICU and CCU, including the use of suboptimal aseptic techniques and infection control practices by healthcare workers, inadequate cleaning and disinfection of the environment and medical equipment, and understaffing. Moreover, the infected patients in the ICU and CCU were often exchanged. We have no information to support any of these possibilities at this time.

In conclusion, integrons were prevalent and played an important role in multidrug-resistant *P. aeruginosa*, which may provide some important surveillance information reflecting the antibiotic selective pressure in this specific region. It is necessary to focus on tracing the source of the infections to lessen the occurrence of multi-resistant strains.

Acknowledgments

We thank Shihe Shao (Jiangsu University) for helpful suggestions on this work.

Funding: This work was supported by the Foundation of Social Development Item of Jiangsu Province (grant No. BS2007041) and the National Nature Science Foundation of China (grant Nos 30471627 and 30300169).

Conflict of interest: No conflict of interest to declare. We received no financial support or benefits with the exception of those noted under the heading 'funding'.

Ethical approval: Ethical approval for this study and agreement to participate from all patients were obtained from the Affiliated People's Hospital of Jiangsu University,

which abides by the Helsinki Declaration on ethical principles for medical research involving human subjects.

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