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ORIGINAL ARTICLE

Novel Class 1 Integrons in Multi-drug Resistant Isolates from Eastern China

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Abstract Integrons are mobile genetic elements able to capture, express and excise resistance genes, playing an important role in the spread of bacterial resistance. The present study was to investigate the occurrence and diversity of integrons in 120 clinical multi-drug resistant Gram-negative isolates from eastern China. Screening of integrons was performed by PCR and gene cassettes were further characterized by PCR–RFLP and sequencing. Class 1 integrons were detected in 70.8 % of isolates and no class 2 and class 3 integrons were detected in any isolates. A total of 19 resistant gene cassettes were identified, four representative of novel gene cassettes: an *aacA3* variant (*aacA3c*), an aacA4 variant (*aacA4′-17*), a bla_{OXA} variant ($bla_{OXA-251}$), and a *catB8* gene cassette interrupted by an insertion sequence *IS10* (*catB8*::IS10). In addition, 14 cassette arrays were detected, including three novel integrons: *gcuD1-aacA4′-17-gcu38B-catB8*::*IS10* (In712), *aacA3c-aadA13-*$bla_{OXA-251}$ (In713) and *dfrA1-gcu37-aadA5* (In714). The presence of novel integron structures in clinical isolates suggests hospital environments may favor the formation of novel combination of gene cassettes. Moreover, the high prevalence of integrons in multi-drug resistant isolates highlights the urgent need to employ effective means to avoid dissemination of drug-resistant bacteria.

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Introduction

The spread of resistance genes through mobile genetic elements is now an increasing global concern, as it can lead to the rapid emergence of multi-drug resistant (MDR) bacteria [1, 2]. Integron is such a genetic element characterized by its ability to capture, express and excise resistance genes and its location often linked to plasmids and/or transposons, thus contributing to bacterial resistance [3]. Based on the encoded sequence of integrase, three classes of integron have so far been found associated with the mobilization of resistance genes [2]. Class 1 integrons are the most important and clinically relevant, with a wide distribution among bacteria species [4], whereas class 2 integrons are less common, and class 3 integrons few reported in clinical isolates [5].

Typically the structure of integron comprises a 5′conserved segment (5′CS), a 3′conserved segment (3′CS), and an internal variable region. The 5′CS contains an integrase gene coding for site-specific integrase (*intI*) and a recombination site (*attI*) involving gene cassette site-specific recombination [6]. The 3′CS includes the truncated *qacE* and *sul* genes that encode resistance to quaternary ammonium compounds and sulfonamides, respectively [7]. The variable region consists of one or more gene cassettes captured by integron through site-specific recombination mechanism [6].

In China, resistance to various antibiotics is common in clinical isolates, often more so than in western countries, especially among the most common causes of hospital-acquired opportunistic pathogens such as *Enterobacteriaceae*,

Pseudomonas aeruginosa and *Acinetobacter baumannii* [8, 9]. Over the past decades, much work has been done on the epidemiology of integrons and their important roles in bacterial resistance in both clinical and natural environments [2, 4, 8–11]. Surveillance of integrons therefore is essential for the investigation of MDR bacteria evolution. Thus, our study was to investigate the proportion and the diversity of integrons in a collection of 120 MDR Gram-negative isolates of clinical origin from eastern China.

Materials and Methods

Bacteria Strains

During a period from August 2010 to December 2010, a total of 120 non-duplicated Gram-negative isolates resistant to three key antibiotics including β-lactams, aminoglycosides and quinolones were randomly collected from five hospitals in Shandong, Anhui, and Jiangsu provinces of China. These isolates comprised 30 *Enterobacteriaceae*, 48 *P. aeruginosa*, and 42 *A. baumannii*. All these isolates were identified using API kits (bioMeriuex, France) and the antimicrobial susceptibility testing was performed by the disk diffusion method on Mueller–Hinton agar (Oxoid Ltd., Cambridge, UK) according to the guideline of the Clinical and Laboratory Standards Institute (CLSI) [12]. A pan of 14 antimicrobial agents were used, including piperacillin, piperacillin-tazobactam, ceftazidime, cefotaxime, cefepime, imipenem, meropenem, aztreonam, chloramphenicol, levofloxacin, gentamicin, tobramycin trimethoprim–sulfamethoxazole, and amikacin. Control strains such as *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used.

DNA Preparation and PCR Amplification of *intI* Gene

DNA template was prepared using boiling method [13]. Briefly, overnight bacteria culture (200 μL) was mixed with 800 μL sterile water and then boiled for 10 min. The resulting solution was centrifuged at 12,000×*g* for 2 min and the supernatants were stored at −20 °C, which were used as DNA templates. The specific primers for detecting *intI* genes and variable regions were shown in Table 1 [13–15]. To determine whether class 1 integrons contained the entire 3′-CS, the primers qacEΔ1-F and sul1-R were used [16]. PCR for amplification of all the genes was performed as previously described [13, 15, 16]. Allowing amplification of longer products than *Taq* DNA polymerase, we used *EX Taq* DNA polymerase (*TaKaRa*, Japan) for amplification of the variable region. *E.coli* ZJ2010038 that was confirmed to carry class 1 integron in our laboratory was used as PCR positive control. *P. aeruginosa* 09083 and *P. aeruginosa* 10028 provided by the Affiliated People Hospital of Jiangsu University were used as PCR positive control for *intI2* and *intI3* detection, respectively [15].

Table 1 Primers used in this study

No.	Primer	Primers sequence	Size (bp)
1	IntI1F	ACGAGCGCAAGGTTTCGGT	565
2	IntI1R	GAAAGGTCTGGTCATACATG	
3	IntI2F	GTGCAACGCATTTTGCAGG	403
4	IntI2R	CAACGGAGTCATGCAGATG	
5	IntI3F	CATTTGTGTTGTGGACGGC	717
6	IntI3R	GACAGATACGTGTTTGGCAA	
7	5′-CS	GGCATCCAAGCAGCAAG	Variable
8	3′-CS	AAGCAGACTTGACCTGAT	
9	qacEΔ1-F	AAGTAATCGCAACATCCG	878
10	sul1-R	GGGTTTCCGAGAAGGTGATTGC	

Restriction Fragments Length Polymorphism and DNA Sequencing of the Variable Region

The PCR products of the variable region were digested with *Rsa*I and *Hinf*I (*TaKaRa*, Japan). Products showing the same restriction pattern were considered to be the same and one representative product of each distinct RFLP was purified and sequenced by both strands. Sequence alignment and analysis were performed online using the BLAST program (www.ncbi.nlm.nih.gov).

Nucleotide Sequence Accession Numbers and Integron Number

The nucleotide sequences of three novel integrons were submitted to INTEGRALL database (http://integrall.bio.ua.pt) for integron (In) number assignment and to the GenBank, which had the assigned accession numbers: JN118547(In712), JN118546(In713) and JN157818(In714), respectively.

Results and Discussion

MDR bacteria have emerged as a serious threat to public health due to their high impacts on clinical therapy. In this study, we collected 120 MDR bacteria from five hospitals in eastern China, focusing on those where the occurrence of integrons had never been investigated before. Meanwhile, all these bacteria obtained were initially tested to be resistant to three main classes of antibiotics against Gram-negative bacteria, including β-lactams, aminoglycosides and quinolones.

Among these bacteria examined, 85 (70.8 %) carried *intI1* gene. *IntI1* genes were presented in 76.7 % (23/30) of *Enterobacteriaceae* isolates, 66.7 % (32/48) of *P.*

Table 2 Sizes, numbers, order and regional distribution of gene cassette arrays in class 1 integrons

Organism and species	Gene cassette(s) and order	Approximate length (bp)	Number	Regional distribution
Enterobacteriaceae	*aadA2*	1,000	2	Shandong
	dfrA17-aadA5	1,500	5	Jiangsu, Anhui
	dfrA1-aadA1	1,500	1	Shandong
	dfrA17-orf-aadA2	1,800	2	Shandong
	drfA12-orf-aadA2	1,800	9	Shandong, Jiangsu, Anhui
	gcuD1-aacA4′-17-gcu38B-catB8::IS10	3,200	1	Shandong
P. aeruginosa	blaP1	900	2	Jiangsu
	aacA7	1,000	3	Jiangsu
	aadB-aadA1	1,400	1	Shandong
	drfA12-orf-aadA2	1,800	6	Shandong, Jiangsu, Anhui
	aacA3C-aadA13-bla$_{OXA-251}$	2,700	5	Jiangsu
	aacA3-aadA13–cmlA8-bla$_{OXA-10a}$	4,200	9	Shandong, Jiangsu, Anhui
	blaP1 + *drfA12-orf-aadA2*		2	Jiangsu
A. baumannii	*dfrA17-aadA5*	1,500	5	Shandong, Jiangsu
	dfrA1-gcu37-aadA5	2,000	3	Anhui
	aacA4-catB8-aadA1	2,500	12	Shandong, Jiangsu, Anhui
	aacC1-orfP-orfQ-aadA1	3,000	10	Shandong, Jiangsu, Anhui

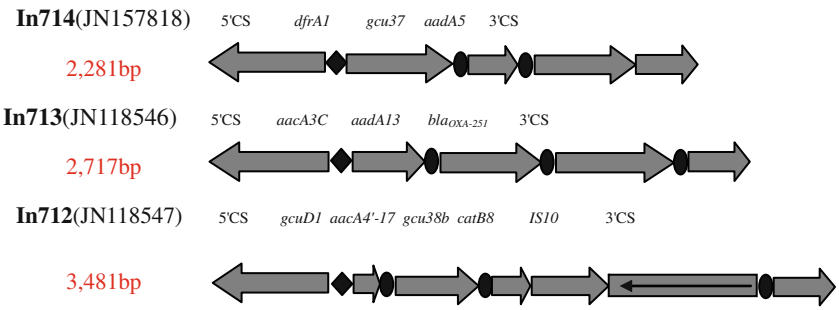

Fig. 1 Schematic representation of three novel integrons detected in this study. *Arrows* indicate direction of translation. *AttI* sites are represented as *black squares*, and the *attC* sites as *black ovals*. In714: 2,281 bp integron; In713: 2,717 bp integron; In712: 3,481 bp integron

aeruginosa isolates, and 71.4 % (30/42) of *A. baumannii* isolates, respectively. No class 2 and class 3 integrons were detected in any isolates.

Of the 85 *intI1* gene positive isolates, 78 showed individual amplicons that differed in size between ~1 and ~4 kb. No amplicon was obtained from the remaining seven isolates, which was probably due to the presence of atypical class 1 integrons or the larger variable region that could not be amplified [11, 15]. Restriction fragments length polymorphism and sequencing analysis of these amplicons identified nineteen different resistant gene cassettes and fourteen different arrays. Table 2 shows an overview of these gene cassettes and cassette arrays identified in the present study.

The most common gene cassettes were *aadA* family, conferring resistance to streptomycin and spectinomycin. In fact, the *aadA* gene cassettes are the most often reported cassettes in bacteria isolates and seem to persist in environments regardless of the limited use of such antibiotics at present [10, 17]. Other gene cassettes involved in antibiotic resistance included *aacA3C*, *aacA3*, *aacA4′-17*, *aacA7*, *aadB*, *drfA1*, *drfA12*, *drfA17*, *catB8*, *cmlA8*, *blaP1, bla*$_{OXA-10a}$, and *bla*$_{OXA-251}$. If these gene cassettes were full expressed, they may determine resistance to aminoglycosides, trimethoprim, chloramphenicol, and β-lactams antibiotics, respectively. Although the findings presented in our study showed that strains contained integrons responsible for multi-drug resistance, we also found that gene cassettes within integrons could not satisfied all the resistance phenotypes. This result implied that there may be other mechanisms responsible for bacterial resistance. Resistance to antibiotics could be due to chromosome mutation, lack of drug penetration, or resistance genes that were located in the chromosome or related to other mobile genetic elements such as insertion sequences, transposons and plasmids [18, 19].

Four novel resistant gene cassettes were firstly detected in the present study, including two *aacA* variants (named

aacA3C and *aacA4′-17*), a bla_{OXA} variant (named $bla_{OXA-251}$), and a *catB8* gene cassette interrupted by an insertion sequence of *IS10* (*catB8*:*IS10*). The *aacA3c* gene differed from *aacA3* gene (GenBank: M29695) by an amino acid mutation (Asp85Gly) while the *aacA4′-17* gene displayed Leu102Ser and Arg156Lys amino acid mutations compared to the sequence of *aacA4* gene (GenBank: U59183). The bla_{OXA} variant differed from its nearest match (Genbank: U37105) by three amino acid mutations Gly128Asp, Lys137Asn and Ile187Thr making it a new variant, named $bla_{OXA-251}$. Previous reports have showed the amino acid mutations of OXA enzyme may greatly affect its efficiency, leading to the change of antibiotics susceptibility [20, 21]. In this respect, whether OXA-251 was associated with changes in antibiotics susceptibility, requiring further study.

Novel gene cassette arrays were also identified, including *gcuD1-aacA4′-17-gcu38B-catB8*::*IS10*, *aacA3C-aadA13-*$bla_{OXA-251}$ and *dfrA1-gcu37-aadA5*. The first two arrays, to the best of our knowledge, have never been reported in any species before and have been submitted to INTEGRALL database (http://integrall.bio.ua.pt), with the assigned integron (In) number: In712, In713, respectively. As showed in Fig. 1, the In712 integron identified in a *K. pneumoniae* isolate had a sequenced size of 3,481 bp and its structure contained a *gcuD1*,an *aacA4′-17*,*a gcu38b* and a *catB8*::*IS10* gene. The *gcu38B* was not a typical gene cassette as it lacked 59 bp element. The potential ancestor of this integron may be the integron *gcuD1-aacA4′-17-gcu38B-catB8*, recently found in *E. coli* (GenBank: HM485586), *K. pneumoniae* (GenBank: GU129909) and *E. aerogenes* (GenBank: GU944729). The In713 integron possessed a sequenced size of 2,717 bp, which was presented in five clinical isolates of *P. aeruginosa* resistant to all the β-lactamase antibiotics examined. The In714 integrons found in three *A. baumannii* isolates had a sequenced size of 2,281 bp, and carried a *dfrA1*, *a gcu37* and an *aadA5* gene. The *aadA5* gene cassette was truncated due to its lack of a majority of *attC* site. Although our work firstly reported this novel array in *A. baumannii* and had an assigned integron (In) number, the same sequence has already been found in *K. pneumoniae* (Genbank: GU129910) and *E. cloacae* (GenBank: GU944728) in southern China, suggesting horizontal transfer of this array has occurred across bacteria species in China.

In conclusion, this study not only showed the wide distribution of class 1 integrons among clinical Gram-negative bacteria but also illustrated the genetic diversity and flexibility of integron's structure involved in antibiotic resistance. It seemed that hospital environments may favor the formation of novel combinations of gene cassettes. In view of the high prevalence of integrons in MDR bacteria, urgent work is needed to employ effective means to avoid dissemination of such bacteria.

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