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Molecular characterisations of integrons in clinical isolates of *Klebsiella pneumoniae* in a Chinese tertiary hospital

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ABSTRACT

Background: Integrons are mobile genetic elements that play an important role in the distribution of antibiotic-resistance genes among bacteria. This study aimed to investigate the distribution of integrons in clinical isolates of *Klebsiella pneumoniae* and explore the molecular mechanism of integron-mediated multiple-drug resistance in *K. pneumoniae.*

Methods: Class 1, 2, and 3 integrases were identified by polymerase chain reaction (PCR) among 178 *K. pneumoniae* clinical isolates. Antibiotic susceptibility was examined by disk-diffusion method. Conjugation experiments were conducted to evaluate the horizontal-transfer capability, and multilocus sequence typing (MLST) assays were conducted to explore the genetic relationships among the isolates. Highly virulent serotypes were identified by PCR from the 44 integron-positive isolates with variable regions.

Results: Class1 and 2 integrons were detected in 60.1% and 1.7% of isolates, respectively. One isolate carried both class 1 and 2 integrons. Class 3 integrons were not detected in all 178 isolates. Among the 44 integrons containing variable regions, 39 were located in conjugative plasmids. Dihydrofolate reductase (*dfrA*) and aminoglycoside adenyltransferase (*aad*) were found to be the most common in class 1 and 2 integrons. These gene cassettes encoded resistance to trimethoprim and aminoglycosides. Moreover, the association between integron carriage and antibiotic resistance was most significant for aminoglycosides, phenicols, and fluoroquinolones. Among the 44 integron-positive isolates with variable regions, 9 were classified as highly virulent serotypes (k1, k2, k20, and k54). In addition, MLST analysis detected 13 sequence types (STs), with the predominant ones being ST11 and ST15. The eBURST analysis revalued the existence of 11 singleton STs and one group, which is comprised of ST11 and ST437.

Conclusions: The wide diversity of detected integrons suggested that the horizontal transfer by mobile genetic elements played a major role in the distribution of antimicrobial resistance genes, thereby indicating the urgent need to use effective means of avoiding the spread of drug-resistant bacteria.

1. Introduction

Klebsiella pneumoniae is an opportunistic pathogen that is associated with both community-acquired and nosocomial infections. It is responsible for a wide spectrum of infections, such as respiratory, blood, and urinary tract infections [1] [2], and [3]. With the extensive use of antibiotics, the increasing resistance of *K. pneumoniae* to antimicrobial agents is becoming a global concern. Many of the antibiotic-resistance genes are contained in discrete genetic elements known as integrons, which can capture and exchange mobile gene cassettes by a site-specific recombination system [4]. Integrons are classified as mobile integrons when they are linked to mobile DNA elements such as insertion sequences, transposons and conjugative plasmids, and as chromosome integrons when they are located in bacterial chromosomes according to their genomic context [5]. Based on the encoding sequence of integrase, three classes of integrons associated with drug resistance have been found [4]. Class 1 integrons are the most common and wide-spread, with wide distribution among bacteria species. This class mainly carries antibiotic-resistance genes and is highly disseminated because of its close association with

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transposons, often embedded in conjugative plasmids [6]. Class 2 integrons are less common [7], and class 3 integrons only have few reports of detection in *K. pneumoniae* [8].

As described in previous research, some capsule serotypes such as k1, k2, k20, k54, and k57 are associated with serious infections and designated as hyper-virulent *K. pneumoniae* (hvKP) [9] and [10]. Currently, hvKP strains are still present in mainland China, and the frequency of antimicrobial resistance among their isolates is increasing over time [9]. Thus, combined with the risk of horizontal gene transfer of integrons, multidrug-resistant hvKP is bound to be a tough problem in clinical studies.

In the present study, 178 *K. pneumoniae* clinical isolates were collected from a tertiary hospital in Chongqing from 2005 to March 2016. Antimicrobial resistance patterns, integrons, and highly virulent serotypes were identified and analysed. In addition, conjugation experiments and multilocus sequence typing (MLST) assays were conducted to evaluate the horizontal transfer capability of integrons and to characterize the genetic relationship among isolates.

2. Materials and methods

2.1. Bacterial isolates

A total of 178 consecutive and unduplicated clinical isolates of *K. pneumoniae* were collected from a tertiary hospital in Chongqing from 2005 to March 2016. Majority of the isolates were from sputum (n = 81; 45.5%), urine (n = 43; 24.2%), blood (n = 18; 10.11%), pus (n = 11; 6.18%), bile (n = 10; 5.62%), abdominal drainage (n=9; 5.06%) and cerebrospinal fluid (n = 6; 3.37%). The standard strain of *Escherichia coli* (ATCC25922) was used as a susceptibility control. Rifampin-resistant strain *E. coli*600 and azide-resistant strain *E. coli*J53 were used as the recipient strains in the conjugation experiments.

2.2. Antimicrobial susceptibility

Antibiotic susceptibility was examined by disk-diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2015) guidelines [11]. The antimicrobial discs were as follows: ampicillin (AMP), piperacillin (PRL), piperacillin/tazobactam (TZP), cefepime (FEP), cefotaxime (CTX), cefoxitin (FOX), ceftazidime (CAZ), imipenem (IPM), meropenem (MEM), amikacin (AMK), gentamincin (GEN), streptomycin (STR), ciprofloxacin (CIP), and chloramphenicol (CLO). Isolates which were resistant to at least three different classes of antimicrobial agents were determined to be multidrug resistance (MDR) phenotypes.

2.3. Detection of integrons and variable regions by polymerase chain reaction (PCR)

The isolates were grown overnight (18 h) in Luria-Bertani (LB) broth at 37 °C, and the DNA template was prepared using boiling method [12]. The PCR mixture was prepared with a final volume of 25 μl, containing 1.5 μl of template DNA, 0.1 mM each dNTP, 150 μM $MgCl_2$, 1.5 U of Taq DNA polymerase, and 0.2 μM each primer. The specific primers for detecting integrase genes and variable regions are shown in Table 1. The amplified PCR products were separated by electrophoresis in 1.2% agarose gels and visualised after staining with Goldview dye.

2.4. Sequencing and analysing of the variable region of integrons

Sequencing analysis was performed on PCR products. Positive products of variable regions were sent to Sangon Biological Engineering Technology Inc for sequencing. The nucleotide sequences were analysed with the BLAST program at the NCBI homepage (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Conjugation experiments

Conjugation experiments were performed between strains with variable regions as donors and rifampin-resistant *E. coli 600* as recipient strain in solid media at 37 °C. Azide-resistant strains *E. coli J53* were used as receptors for strains containing rifampin-resistant gene cassette. In addition to rifampin (150 μg/ml) and azide (100 μg/ml), the selection of other antibiotics was according to the gene cassette carried by integron-positive isolates. The transconjugants were tested for presence of *intI*1, size of the variable region and corresponding resistance genes. The PCR products were preformed sequencing and analyzed with the BLAST program.

2.6. Detection of highly virulent serotypes

Five serotypes (k1, k2, k20, k54, and k57) related to the highly virulent strains were screened in a collection of 44 strains with variable regions of *K. pneumoniae* by PCR. The PCR mixtures were prepared with a final volume of 25 μl, containing 1.5 μl of template DNA, 0.1 mM of each dNTP, 150 μM $MgCl_2$, 1.5 U of Taq DNA polymerase, and 0.2 μM of each primer. The specific primers are shown in Table 1. PCR conditions were as follows: initial denaturation of 5 min at 94 °C, followed by 30 cycles of denaturation (94 °C, 40 s), annealing (58 °C, 30 s), and extension (72 °C, 1 min), with a final extension of 10 min at 72 °C. The amplified PCR products were separated by electrophoresis in 1.2% agarose gels and visualised after staining with Goldview dye.

2.7. Multilocus sequence typing

Molecular typing of 44 strains with variable region was performed in accordance with the international *K. pneumonia* MLST scheme (http://bigsdb.web.pasteur.fr/), including seven housekeeping genes as previously described [13]. Alleles and STs were assigned by using the MLST database (http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public). The eBURST method (http://eburst.mlst.net) was used to analyse the MLST findings.

2.8. Statistical analysis

Statistical analysis was performed by Fisher's exact test using SPSS (version 22.0), and a P-value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Prevalence of integrons and antibiotic susceptibility

Among the total 178 isolates of *K. pneumoniae*, 109(60.1%) and 2 (1.7%) were classified as class 1 (*intI*1) and 2 (*intI*2) integrons, respectively. One isolate carried both class 1 and 2 integrons, and none harbored class 3 integrons. The antibiotic susceptibility results are shown in Table 2. The isolates showed high resistance toward ampicillin (AMP) (96.6%), piperacillin (PRL) (70.8%), cefepime (FEP) (38.8%), cefotaxime (CTX) (67.4%), ceftazidime (CAZ) (32.6%), gentamincin (GEN) (58.4%), streptomycin (STR) (42.7%), ciprofloxacin (CIP) (39.9%), and chloramphenicol (CLO) (38.2%). According to the antibiotic susceptibility results, 126 strains were considered as MDR. Comparison of MDR phenotypes in the two groups of isolates showed that integron-positive strains had much