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Prevalence of Carbapenem-Resistant *Klebsiella Pneumoniae* (CRKP) and the Distribution of Class 1 Integron in Their Strains Isolated from a Hospital in Central China

Qin Ou¹, Wenfang Li², Bei Li^{1*}, and Chunfang Yu¹

¹Department of Medical Microbiology, ²Laboratory of Infectious Diseases, Taihe Hospital, Hubei University of Medicine, Shiyan, Hubei 442000, China

Key words: carbapenem resistance; *klebsiella pneumoniae*; integron; gene cassette

Objective The aim of our study is to investigate the prevalence of Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) and the genetic characteristics of the class 1 integron in CRKP on multi-drug resistance.

Methods Clinical *Klebsiella pneumoniae* strains were collected from multiple departments of a hospital in central China. CRKP strains were identified among the isolates, and antibiotics susceptibility of CRKP strains was analyzed. The polymerase chain reaction (PCR) was adopted to amplify the class 1 integron variable area. The integron genetic structure was analyzed with enzyme digestion and DNA sequencing technology. The relation between class 1 integron and drug resistance was analyzed statistically.

Results Totally 955 strains of *Klebsiella pneumoniae* were isolated from varied sites of the hospital, and 117(12.3%) of them were identified as CRKP, with a separation rate of 8.9% (26/292) in 2013, 11.3% (38/336) in 2014 and 16.2% (53/327) in 2015, which shows an increasing trend by year. 44.4% (52/117) of CRKP strains were separated from specimen of ICU, and 61.5% (72/117) were from sputum. Over 95% CRKP strains were resistant to ampicillin/sulbactam, aztreonam, imipenem, meropenem, ceftazidime, cefotaxime, cefepime, and piperacillin, while relatively low resistant rates were found in tigecycline (12.8%) and colistin (35.9%). The class 1 integron was detected in 77.8% (91/117) of CRKP strains. Class 1 integron of CRKP was significantly correlated with the antibiotic resistance to the tobramycin, gentamicin and amikacin (all $P<0.01$). The gene cassette analysis of variable area of class 1 integron showed that *aadA2* accounts for 64.8% (59/91), *aacA4-catB8-aadA1* 23.1% (21/91), and *aadA2-dfrA25* 12.1% (11/91).

Conclusions CRKP has an increasing trend in a clinical setting in China, and most of them were resistant to multiple antibiotics. Class 1 integron in CRKP has strong ability to capture the genes resistant to aminoglycosides antibiotics from environment, with the *aadA2* gene as the most popular one.

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KLEBSIELLA pneumoniae (Kp) is a kind of gram-negative pathogenic bacteria which commonly exists in the upper respiratory tract, urinary tract and intestinal tract of human.¹ It is widely recognized that Kp could cause multiple infections including liver abscess, urinary tract infection and surgical incision infection. Kp is also a potential nosocomial multidrug resistant bacterium.² Carbapenem-resistant Klebsiella pneumoniae (CRKP) is resistant to many structurally unrelated antibiotics because of its dominant array of chromosome and plasmid-mediated antibiotic resistance factors.³ It also could capture many resistance genes from other bacteria. CRKP, as well as other resistant nosocomial pathogens, has been associated with serious infections and posed a serious challenge for treatments.

The multidrug resistant Kp acquires drug resistance through mechanisms mainly mediated by integrons, transposons and plasmids.⁴ These mobile genes usually encode multiple drug-related proteins, and play an important role in the dissemination of drug resistance among bacteria. Integrons are genetic elements that capture and integrate gene cassettes by site-specific recombination and express various antibiotics resistance related enzymes.⁵ Integrons are closely related to Kp's resistance to multiple drugs, such as aminoglycosides, beta-lactams and other antibiotics, by disseminating drug resistance genes.⁶ Through constantly integrating and expressing resistance genes, integrons play important roles in resistance of Kp to antibiotics.

CRKP was frequently reported to be identified in the hospital;⁶ however, only a few studies on the association of multidrug resistance of CRKP with integrons mediated mobile genetic elements have been published in our region. The present study was performed to investigate the integron-associated antibiotics resistance of CRKP in central China, aiming at characterizing the gene cassettes of integron on multidrug resistance.

MATERIALS AND METHODS

Samples collection and bacteria isolation

Clinical specimens were collected from a variety of departments such as ICU, Burn, and Geriatrics between March 2013 and September 2015 in an affiliated hospital of Hubei University of Medicine in central China. The specimens collected, including sputum, blood, and drainage of all kinds of catheters, were frozen in -80°C . CRKP strains were isolated using the VITEK2 bacterial identification instrument compact (Bio Merieux, France), which identifies the bacteria and tests antibiotics sensitivity in parallel.

CRKP separation rate was defined as the percentage of CRKP strains in the isolated Kp strains.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by the disk diffusion method on Mueller-Hinton agar (Oxoid, UK) according to the Clinical and Laboratory Standards Institute guidelines.⁷ Antimicrobial susceptibility testing was performed for 13 antimicrobial agents: piperacillin (PRL, 100 μg), Ampicillin/Sulbactam (AS, 100 μg), ceftazidime (CAZ, 30 μg), cefotaxime (CTX, 30 μg), colistin (CST, 10 μg), cefepime (CFP, 30 μg), aztreonam (CAR, 100 μg), gentamicin (GEN, 10 μg), tobramycin (TOB, 10 μg), amikacin (AMK, 30 μg), tetracycline (TET, 30 μg), imipenem (IPM, 10 μg), and meropenem (MPM, 10 μg).

Integron detection

Class 1, 2 and 3 integrons were initially detected by PCR using degenerate primer set hep35 (5'-TGCGGGTYAARGA-TBTKGATT T-3') and hep36 (5'-CARCAGATGCGTRTARAT-3') (Shen Gong Bio, Shanghai, China), which hybridized to conserve regions of integron-encoded integrase genes *intI1*, *intI2*, and *intI3*.⁸ The class of integron was determined by analyzing integrase PCR products through enzyme digestion according to the molecular weight of the product.

Characterization of class 1 integron cassette arrays and DNA sequencing

Class 1 integron cassette regions were amplified using hep58 and hep59 primers as described previously.⁹ PCR adopted the following modified conditions: initial denaturation at 94°C for 5 min, 35 cycles of 94°C with 30 s for each cycle, 55°C for 45 s, extension at 72°C for 45 s, and final extension at 72°C for 10min. Amplified gene cassette of the same fragment sizes were further sequenced to determine the cassette array. The amplicons were purified and sequenced at a sequencing facility (Taihe Biotech, Beijing, China).

Statistical analysis

The statistical analysis was performed using SPSS (version 17.0) Software. Chi-square test was used to analyze the relationship between class 1 integron and drug resistance of CRKP, and Fisher exact test was adopted when appropriate. A *P* value of >0.05 was considered significant.

RESULTS

Pathogenic bacteria and their distribution

Totally 955 strains of *Klebsiella pneumoniae* were isolated from multiple sites of the hospital, and 117 strains of CRKP were successfully separated. Among them, 26, 38 and 53 strains of CRKP were separated from the isolated Kp strains in the year of 2013 (292 stains), 2014 (336 strains), and 2015 (327 stains), with the separation rate of 8.9% (26/292), 11.3% (38/336), and 16.2% (53/327), respectively.

CRKP distribution among the departments and sources of specimens

The separated CRKP strains mainly distributed in the ICU (44.4%, 52/117), geriatrics (9.4%, 11/117), burn (11.1%, 13/117), and emergency department (7.7%, 9/117). The details were shown in Table 1. Among 117 strains of CRKP, 61.5% (72/117) were from sputum, 21.4% (25/117) from blood, and 17.1% (20/117) from catheters.

The drug susceptibility of CRKP strains

Susceptibility tests showed that over 95% of 117 CRKP strains were resistant to Ampicillin/Sulbactam, Aztreonam, Imipenem, Meropenem, Ceftazidime, Cefotaxime, Cefepime, and Piperacillin, while only 12.8% and 35.9% of strains were resistant to Tigecycline and Colistin respectively. Details of drug susceptibility were shown in Table 2.

Integron variable area detection and the sequencing results

The positive rate of class 1 integron variable range was 77.8% (91/117), none of them carried class 2 or class 3 integron. The PCR products of class 1 integron variable range had three size models that are 2200 bp (21/91, 23.1%), 1700 bp (11/91, 12.1%) and 1000 bp (59/91, 64.8%). The 2200 bp, 1700 bp and 1000 bp fragments contained aacA4-catB8-aadA1, aadA2-dfrA25 and aadA2, respectively.

Table 1. Distribution of isolated CRKP strains in departments of a hospital in Shiyan, central China ($n=117$)

Department	ICU	Burn	Geriatrics	Emergency	Oncology	General medicine	General surgery	Brain Surgery
Strains (n)	52	13	11	9	7	5	4	5
Percentage(%)	44.4	11.1	9.4	7.7	6.0	4.3	3.4	4.3
Department	Orthopaedics	Neurology	Gastroenterology	Respiratory	Cardiology	Hematology	Rheumatology	Thoracic
Strains (n)	2	1	1	1	1	1	2	2
Percentage(%)	1.7	0.9	0.9	0.9	0.9	0.9	1.7	1.7

Table 2. Distribution of antibiotics resistance in 117 strains of CRKP isolated from a hospital in Shiyan, central China [n , (%)]

Susceptibility	Antibiotics						
	Ampicillin /Sulbactam	Amikacin	Aztreonam	Tigecycline	Colistin	Imipenem	Meropenem
Sensitive	0	9 (7.7)	3(2.6)	69(59.0)	63(53.8)	1(0.9)	2(1.7)
Medium sensitive	0	3(2.6)	2(1.7)	33(28.2)	12(10.3)	2(1.7)	3(2.6)
Resistant	117(100)	105(89.7)	112(95.7)	15(12.8)	42(35.9)	114(97.4)	112(95.7)
Susceptibility	Antibiotics						
	Tobramycin	Ceftazidime	Gentamicin	Cefotaxime	Cefepime	Piperacillin	
Sensitive	7(6.0)	0	8(6.8)	0	1(0.9)	0	
Medium sensitive	6(5.1)	3(2.6)	3(2.6)	0	3(2.6)	2(1.7)	
Resistant	104(88.9)	114(97.4)	106(90.6)	117(100)	113(96.5)	115(98.3)	

Relation between class 1 integron and drug resistance

Among 13 antibiotics, significant correlation between antibiotics resistance and class 1 integron was only found in tobramycin, gentamicin and amikacin (Table 3). In the Tobramycin, Gentamicin and Amikacin resistance strains,

high class 1 integron expression was found. The resistance rate of tigecycline and Colistin in class 1 integron positive strains was 66.7% (10/15) and 73.8% (31/42) respectively, which was higher than that in class 1 integron negative strains [33.3% (5/15), and 26.2% (11/42), respectively].

Table 3. Association between class 1 integron and antimicrobial resistant phenotypes in 117 strains of CRKP [*n*, (%)]

Class 1 integron	Strains resistant to antibiotics						
	Ampicillin/ Sulbactam (<i>n</i> =117)	Amikacin (<i>n</i> =105)	Aztreonam (<i>n</i> =112)	Tigecycline (<i>n</i> =15)	Colistin (<i>n</i> =42)	Imipenem (<i>n</i> =114)	Meropenem (<i>n</i> =112)
Negative (<i>n</i> =26)	26 (100)	18 (69.2)	24 (92.3)	5 (19.2)	11 (42.3)	25 (96.2)	25 (96.2)
Positive (<i>n</i> =91)	91 (100)	87 (95.6)	88 (96.7)	10 (10.9)	31 (34.1)	89 (97.8)	87 (95.6)
<i>P</i> -value	1	0.001	0.328	0.268*	0.440†	0.220	0.903

Class 1 integron	Strains resistant to antibiotics					
	Piperacillin (<i>n</i> =115)	Cefotaxime (<i>n</i> =117)	Cefepime (<i>n</i> =113)	Tobramycin (<i>n</i> =103)	Ceftazidime (<i>n</i> =114)	Gentamicin (<i>n</i> =106)
Negative (<i>n</i> =26)	25 (100)	26 (69.2)	25 (96.2)	17 (65.3)	25 (96.2)	18 (69.2)
positive (<i>n</i> =91)	90 (98.9)	91 (95.6)	88 (96.7)	86 (94.5)	89 (97.8)	88 (96.7)
<i>P</i> -value	1	1	1	0.000	1	0.000

* $\chi^2=1.299$ by Chi-square test; † $\chi^2=0.597$ by Chi-square test.

DISCUSSION

Since the first CRKP strain was reported by Mac Kenzie in 1997, it has been widely reported all over the world.¹⁰ The mechanism for drug resistance of CRKP is mainly related to two aspects: the excessive expression of Amp Combined with pore protein loss, and the production of carbapenem enzymes.¹¹ In addition to carbapenem and beta lactam resistance, CRKP was found frequently to carry resistant genes to other antibiotics, and easily develop into multiple-drug resistance bacterium.¹² Although reported CRKP detection rate was variable, it has shown a rapid growth trend worldwide. In this study the CRKP separation rates in 2013, 2014 and 2015 were 8.9%, 11.31% and 16.21% respectively, indicating an elevation over time, which is consistent with the general understanding.

Our results showed that compared to other departments in the hospital, ICU is the one where most CRKP was isolated from (44.4%). This might be because that patients

in ICU are usually in critical condition with severe diseases.¹³ Burn patients were also a susceptible population, because loss of body fluid decreases resistant ability to pathogens. In our study, CRKP strains derived from the burn department accounted for 11.1% in all isolated strains, which is consistent with the literature from other countries.¹⁴ There were as high as 61.5% of CRKP strains separated from sputum in this study, which suggested that the respiratory tract was the main planting area of CRKP. Physicians should be alert to CRKP infection for patients with severe respiratory disease. Bacteriology examination should be performed in order to identify CRKP infection in time.

In this study, drug susceptibility examinations showed that 95.0% of CRKP strains were resistant to abroad spectrum of penicillin and beta-lactam antibiotics. However, the drug resistance rates for tigecycline (12.8%) and colistin (35.9%) were relatively low. The reason might be that penicillin and beta-lactam class are the first-line antibiotics, and they have been widely used for a long time, so that

drug-resistant strains have gradually increased; while clinical application of tigecycline and colistin is new, therefore resistant strains have not been well developed.¹⁵ However, clinical colistin resistant gram-negative Enterobacteriaceae bacteria has shown a gradually increasing trend Recently.¹⁶ Tigecycline also showed a good safety and tolerance profile for ICU patients with CRKP infections after orthotopic liver transplantation.¹⁷ In another report, tigecycline resistance rate in susceptible Kp was reported as 16%,¹⁸ which was higher than that in our study. These results highlight the importance of a rational utilization of antibiotics in clinical practice; otherwise, the effectiveness of treatment for infectious diseases would be overwhelmed by the newly developed resistant bacteria strains.

There are three main well-characterized classes of antibiotic resistance integrons, categorized as class 1, 2 and 3. The class 1 integron is the most common one, which could be found in majority of the Enterobacteriaceae family.¹⁹ However, class 2 and class 3 integrons among these nosocomial pathogens were rarely reported.²⁰ In this study, the positive expression rate of class 1 integron variable area was 77.78% in 117 strains of CRKP, which was consistent with results in other reports.²¹ This suggests that class 1 integron is strongly associated with multiple drug resistance among clinical isolates of Kp.

Gene cassette analysis showed that these integrons carried multiple aminoglycoside antibiotics drug resistance genes.²² In our study, integron-mediated resistance genes were obviously associated with aminoglycoside resistance. The gene cassettes that were detected in the class 1 integron belong to aadA family, which confer the resistance to streptomycin and spectinomycin.²³ The aadA2 accounted for 64.84% (59/91) in the gene cassette analysis. Tobramycin, gentamicin and amikacin are all aminoglycoside. Resistant phenotype analysis showed that Integrin 1 was closely related to the aminoglycosides antibiotic resistance. These suggested that class 1 integron is involved in the aminoglycosides antibiotic resistance of CRKP.

On the whole, the present study analyzed the CRKP epidemic situation in a hospital which is located in the central mainland of China. We found that high integron 1 substructure existed in the CRKP, and the strongly harbored aminoglycoside antibiotics resistant genes could easily disseminate themselves to the neighboring bacteria, resulting in a rapid spread of antibiotic resistance. The dissemination of the class 1 integron and associated gene cassettes in CRKP and other clinically important pathogens would increase the difficulty in controlling infectious diseases in the hospital.

Conflict of Interest Statement

The authors have no conflicts of interest to disclose.

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