Research Article

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Epidemiological study of carbapenem-resistant *Klebsiella pneumoniae*

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Abstract: Background: This research is aimed to study the resistance and molecular epidemiological characteristics of carbapenem-resistant *Klebsiella pneumoniae* (CRKP).

Methodology: 38 isolated CRKP strains were collected from clinical specimens.

Results: The resistance rates were more than 70.0%. Ampicillin had the highest rates among them (100.0%). 34 strains (89.5%) among the 38 CRKP strains carried bla_{KPC-2} gene, and 3 strains (7.9%) carried bla_{IMP-4} gene. 36 strains (94.7%) among the 38 CRKP strains carried bla_{SHV} gene, 29 strains (76.3%) carried bla_{TEM} gene, and 26 strains (68.4%) carried bla_{CT-M} gene. 7 strains (18.4%) among the 38 CRKP strains carried bla_{DHA-1} gene. 15 strains (39.5%) in 38 CRKP strains lost two fenestra proteins, ompK35 and ompK36, and the rest 23 strains carried ompK36 genes. 38 CRKP strains were divided into five kinds of ST types, with ST11 type as the most (86.8%, 33/38). The rest of the ST types included 2 strains of ST23 (5.3%, 2/38), one strain of ST15, ST1373 and ST1415 (2.6%, 1/38).

Conclusions: CRKP resistance is severe, and the mechanism of drug resistance has become increasingly complex. Various ST types and resistance genes are related to CRKP. The clinical prevention and control work is imminent.

Keywords: K*lebsiella pneumoniae*; carbapenem; resistance

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1 Introduction

Klebsiella pneumoniae is a significant enterobacteriaceae in hospitals and community acquired infection that can lead to a variety of infections, such as the respiratory tract infection, urinary tract infection and blood infection. With the frequent use of antibiotics, infection caused by multiple drug resistance of gram-negative bacilli (MDRGN) is becoming an increasingly serious global problem. Meanwhile, with the increasing isolating rate of carbapenem-resistant *Klebsiella pneumoniae*, infections caused by carbapenem-resistant *Klebsiella pneumoniae* (CRKP) have drawn much more attention. The resistance mechanism of CRKP is complex, and is possibly related to the production of carbapenemases, excessive expression of plasmid type AmpC enzyme and loss of fenestra proteins, quantity descent, loss or decreased affinity of carbapenem high affinity site PBP2, etc. [1, 2]. In recent years, CRKP was reported in many countries. Molecular epidemiology data based on multiple locus sequence typing (MLST) showed the variety of *Klebsiella pneumoniae* ST type [3]. Various reports suggested that ST type was related to drug resistance of strains. Such as, *Klebsiella pneumoniae* ST258 type is the most common carbapenem resistant ST type in United States and Greece [4, 5]. Recently, a Russia study also showed that ST340 type was related to the *Klebsiella pneumoniae* that produces NDM-1 carbapenem [6].

In China, CHINET resistance monitoring showed that the number of carbapenem-resistant enterobacteriaceae (CRE) increased year by year, of which CRKP occupied the most important proportion (64.2%) [7]. A report in 2007 discovered carbapenem that produced KPC-type 2 enzyme in Zhejiang province [8]. Since after, strains produce KPC-type 2 enzyme have been continuously detected and reported [1, 9, 10]. To understand the epidemiological characteristics of CRKP infections in our hospital, 38 CRKP strains isolated from our hospital in 2015 was investigated to verify its resistant properties and molecular epidemiological characteristics.

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2 Methods

2.1 Strains

38 isolated CRKP strains were collected from clinical specimens in our hospital in 2015 without repeated isolates, including 28 strains from the intensive care unit, 5 strains from surgery department, 5 strains from medicine department; 22 strains isolated from sputum specimens, 10 strains from urine, 3 strains from blood, 2 strains from pyogenic fluid and 1 strain from ascites; 4 strains from patients aged 0-44, 12 strains from 45-59, and 22 strains from over 60. CRKP was defined when the strain was resistant to either meropenem or imipenem. Quality control strains were *Escherichia coli* ATCC 25922 (obtained from national center for clinical laboratories), produce KPC-2 enzyme *Klebsiella pneumoniae* (provided by professor Zhang Rong, Ph.D. of second affiliated hospital of Zhejiang university), produce IMI-1 enzyme *Enterobacter cloacae* (provided by professor Me-deiros, Ph.D. of American AA), *Klebsiella pneumoniae* ATCC 700603 and *Klebsiella pneumoniae* produce IMP-4 enzyme, SHV-3 enzyme and TEM-5 enzyme (provided by Jcoby, Ph.D. of GA, USA).

2.2 Reagents and instruments

Blood bacteria enrichment medium, blood agar plate, M-H agar and M-H broth were purchased for Hangzhou Tianhe Microorganism Reagents Company. Tested antimicrobial agents included cefazolin, cefepime, cefotaxime, cefoxitin, ampicillin, piperacillin/tazobactam, amoxicillin clavulanate, meropenem, imipenem, amikacin, tetracycline, ciprofloxacin, trimethoprim–sulfamethoxazole, tigecycline (Oxoid, British). The PCR detection kits and DNA marker were from Beijing Tiangen Company. Ethylenediaminetetraacetic acid (EDTA), Tris, ethidium bromide and agarose were for Shanghai Shenggong Company. MicroScanWalkAway 40 Plus Microbiology System (Siemens, Newark, DE, USA). SP1000 PCR amplification instrument (Bio-Rad Company, USA), DYY-III electrophoresis apparatus (Beijing Liuyi Instrument Factory), Tanon1600 gel imaging system (Shanghai Tianneng Company).

2.3 Bacteria identification and drug susceptibility test

Strains were identified at species level by the Siemens MicroScan WalkAway 40 Plus Microbiology System. Antimicrobial susceptibility was determined by Kirby-Bauer disk diffusion test. Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) 2015 guidelines [11]. Results of tigecycline were interpreted according to the breakpoint approved by the US Food and Drug Administration (FDA) [12].

2.4 DNA template preparation

Chromosome DNA of experimental strains was extracted using thermal cracking method. Experimental strains were inoculated to 5 ml nutrient broth, and cultured in 37°C for 6 hours, then centrifuged at 10000 RPM for 5 min and the supernatant was discarded. 1 ml saline was added and vibrated to suspend bacteria, then centrifuged at 10000 RPM for 5 min and the supernatant was discarded. 100 ml sterile distilled water was added to the precipitation and vibrated to suspend bacteria. After 10°C water bathing for 10 minutes and 10000 RPM centrifuging for 5 minutes, the supernatant was the template DNA for PCR amplification.

2.5 Resistant gene detection

According to references [13-16], resistance genes related to carbapenemases class A (GES, IMI, KPC, NMC and SME), class B (IMP, VIM, GIM, SPM, SIM and NDM) and class D (OXA), ESBLs (SHV, TEM and CTX - M), AmpC β-lactamases (LAT, ACT, CMY and DHA) and fenestra proteins (ompK35 and ompK36) of the bacterial strains were detected. Primers were synthesized by Shanghai Shenggong Biological Engineering Technology Service co., LTD. PCR amplification system: upstream and downstream primers each 2 μl, 2xTaq DNA Master Mix 20μl, ddH2O 14μl, template 2 μl, total reaction volume 40 μl. PCR amplification parameters: 95°C pre degenerated for 5 min; then 35 cycles of 9°C degenerated for 10 s, 50-62°C annealed for 20s, followed by 72°C extended for 20 s; at last, 72°C extended for 40s. Anneal temperature of each primer was determined according to the melting temperature (Tm, Tm+ 5°C). Amplification products were electrophoresed using 1.5% agarose gel with ethidium bromide (0.5 μg/ml), and ultraviolet light was used to observe results. Products of PCR amplification positive strains were sent to Shanghai

Shenggong Biological Engineering Technology Service co., LTD., for sequencing after purification. Nucleotide sequence analysis was performed by BLAST to identify genes.

2.6 MLST analysis

Seven housekeeping genes (rpoB, gapA, MDH, pgi, phoE, infB, tonB) , PCR amplification primer sequences and the PCR reaction parameters were from website (http://www.pasteur.fr/mlst/). Total PCR reaction volume was 50μl, including upstream and downstream primers each 2 μl, 2x Taq DNA Master Mix 25 μl, template 2 μl and sterile ddH_2O 19 μl. Amplification products were electrophoresed using 20 g/L agarose gel with ethidium bromide (0.5 μg/ml), and ultraviolet light was used to observe results. Products of PCR amplification positive strains were sent to Shanghai Shenggong Biological Engineering Technology Service co., LTD., for sequencing. Sequencing results were compared using the database (http://www.pasteur.fr/mlst/). Alleles of each strain were obtained to determine its MLST type. After setting the 6 genes of the 7 housekeeping genes as the same clone group, eBURST version 3.0 software was used for correlation analysis for each strain sequence type (ST) in strain clones complex (CC).

2.7 Data process and statistical analysis

After data were derived, EXCEL2007 was used for sorting. SPSS software (version 19.0) was used for statistics. Chi-square or Fisher's accurate inspection was used to compare the difference between the groups. $P < 0.05$ was considered statistically significant.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee.

Informed consent: Informed consent has been obtained from all individuals included in this study.

3 Results

3.1 Antimicrobial susceptibilities of CRKP strains

All 38 experimental strains were resistant to β-lactam antibiotics, β-lactam antibiotics/β-lactamase inhibitors and ciprofloxacin. The resistance rate was more than 70.0%.Ampicillin was the highest among them (100.0%). 37 strains of 38 experimental strains were resistant to imipenem, the rest 1 strain was sensitive to imipenem, but resistant to meropenem, so all 38 strains in the study were CRKP strains. All 38 experimental strains showed little resistance to tetracycline, amikacin and compound sulfanilamide, the rates of which were between 31.6~55.3%, and they were not resistant to tigecycline at all. Resistance of experiment strains is shown in Table 1.

3.2 Resistance genes test

Results of CRKP resistant gene detection showed that 34 strains (89.5%) among the 38 CRKP strains carried bla_{KPC-2} gene, and 3 strains (7.9%) carried bla_{IMP-4} gene. No resistant gene was found in the rest CRKP strains. 36 strains (94.7%) among the 38 CRKP strains carried carbapenem resistant gene, and there was one in the 36 carried both bla_{KPC-2} gene and bla_{IMP-4} gene. Results of ESBLs showed that 36 strains (94.7%) among the 38 CRKP strains carried bla_{SHV} gene, and there were 32 strains in the 36 carried bla_{SHV-11} gene, 3 strains carried $bla_{SHV-142}$ gene, 1 strain carried bla_{SHV-1} gene; 29 strains (76.3%) carried bla_{TEM} gene, and there were 15 strains of the 29 carried bla_{TEM-1} gene, 9 strains carried $bla_{TEM-135}$ gene, 5 strains carried $bla_{TEM-198}$ gene; and 26 strains (68.4%) carried bla_{CT-M} gene, there were 14 strains of the 26 carried $bla_{CTX-M-14}$ gene, 8 strains carried $bla_{CTX-M-65}$ gene, 2 strains carried both $bla_{CTX-M-15}$ gene and $bla_{CTX-M-3}$ gene. No ESBLs resistant gene was found in the rest CRKP strains. Results of AmpC enzyme showed that 7 strains (18.4%) among the 38 CRKP strains carried bla_{DHA-1} gene, and no AmpC enzyme resistant gene was found in the rest CRKP strains. 15 strains (39.5%) in 38 CRKP strains lost two fenestra proteins, ompK35 and ompK36, and the rest 23 strains carried ompK36 genes. In addition, 13 strains (34.2%) carried carbapenem gene and two fenestra proteins lost. And no fenestra protein lost was found in the non-carbapenem-resistant strains.

Table 1: Antimicrobial resistance of 38 carbapenem-resistant Klebsiella pneumoniaee (CRKP) strains

Antibiotics	**Department**				**Specimens**				**Age**				**Total (%) (n=38)**
	ICU (n=28)	**Surgery (n=5)**	**Medicine (n=5)**	**P Value**	**Sputum (n=22)**	**Urine (n=10)**	**Others (n=6)**	**P Value**	**0-44 (n=4)**	**45-59 (n=12)**	**>60 (n=22)**	**P Value**	
Tigecycline	0	0	0	-	0	0	0	-	0	0	0	-	0 (0)
Tetracycline	6	3	3	0.079	5	5	2	0.301	0	4	8	0.519	12 (31.6)
Compound sulfonamides	8	3	2	0.403	7	4	2	0.894	0	5	8	0.415	13 (34.2)
Amikacin	15	1	5	0.055	11	6	4	0.734	2	5	14	0.454	21 (55.3)
Cefoxitin	18	4	5	0.385	13	9	5	0.175	3	7	17	0.530	27 (71.1)
Ciprofloxacin	22	5	5	0.459	17	10	5	0.265	3	8	21	0.044	32 (84.2)
Piperacillin/Tazobactam	27	3	3	0.012	20	7	6	0.251	4	10	19	1.000	33 (86.8)
Meropenem	27	3	5	0.081	21	8	6	0.223	4	10	21	0.489	35 (92.1)
Cefazolin	27	4	4	0.164	21	8	6	0.223	4	11	20	1.000	35 (92.1)
Cefotaxime	26	4	5	0.612	20	9	6	1.000	4	10	21	0.489	35 (92.1)
Amoxicillin/Clavulanic acid	28	5	4	0.263	22	9	6	0.421	4	12	21	1.000	37 (97.4)
Cefepime	27	5	5	1.000	21	10	6	1.000	4	11	22	0.421	37 (97.4)
Imipenem	28	5	4	0.263	22	9	6	0.421	4	12	21	1.000	37 (97.4)
Ampicillin	28	5	5	-	22	10	6	-	4	12	22	-	38 (100.0)

Note: other specimens include ascites, fester and blood.

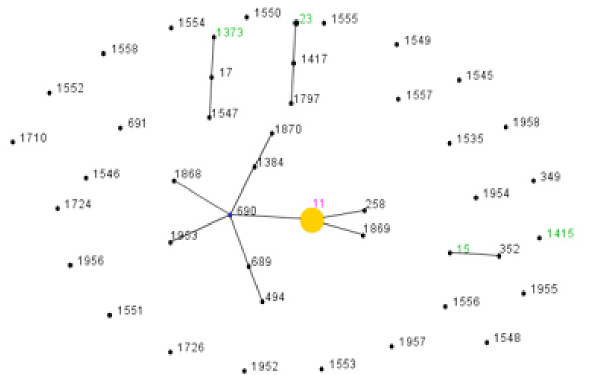

Figure 1: The cluster analysis full view diagram of 38 CRKP strains comparing with ST classification of *Klebsiella pneumoniae* in Zhejiang province.

The blue circle denotes the primary founder. The yellow circle denotes the subgroup founder. The pink number denotes the ST type exists in both experimental strains and strains in Zhejiang province. The green numbers denote the ST type only exists in the experimental strains, and black numbers are the ST types of *Klebsiella pneumoniae* exist in Zhejiang province.

3.3 MLST analysis results

38 CRKP strains were analyzed by MLST, and five ST types were detected.ST11(86.8%, 33/38)was the most frequent ST type. The rest of the ST types included 2 strains of ST23 (5.3%, 2/38), 1 strain of ST15, ST1373 and ST1415 (2.6%, 1/38). Among 33 strains of ST11, 31 strains (93.9%) carried $bla_{KPC\text{-}2}$ gene. eBURST version 3.0 software was used to compare each strain sequence type (ST) with the information in database (http://www.pasteur.fr/mlst/). The cluster analysis full view diagram of 38 CRKP strains comparing with ST classification of *Klebsiella pneumoniae* in Zhejiang province is shown in Figure 1.

4 Discussion

Carbapenems is the first choice for the treatment of multi-drug resistant *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Enterobacteriaceae* that produce ultra broad-spectrum β-lactamase (ESBLs) and AmpC enzyme. At times, it may be the only available antibiotic. The emergence of CRE limits the application of carbapenem in some patients with severe infections, which may lead to the raise of the fatality rate of infection. In recent 10 years, infections caused by CRE increased significantly. There are reports of CRE in many countries, such as Israel, Greece, Turkey, Egypt, Lebanon and Saudi Arabia [17-19]. Therefore, CRE has been transformed from the original scattered status into the international popular drug resistant strains. CHINET bacterial drug resistance monitoring network in China showed that clinical separated CRE strains from enterobacteriaceae previously were Klebsiella species, mainly the *Klebsiella pneumoniae* [20]. *Klebsiella pneumoniae* is one of the most common clinical hospital infection bacteria. The emergence of CRKP, with its complex mechanism, caused wide spread, and which has been one of the most difficult problems in clinical infection therapy. Therefore, this study isolated CRKP in our hospital in the latest year for resistance and molecular epidemiological study, which provides the basis for clinical treatment, prevention and control.

Results of this study showed that 38 CRKP strains were mainly from the intensive care unit, mainly older patients, and mostly isolated from sputum specimens. This may be related to the immunity state of patients, the long-time hospital treatment and some invasive operations. Susceptibility test results of 38 CRKP strains to 14 antimicrobial drugs showed that those strains were sensitive to tigecycline, tetracycline, amikacin and compound sulfanilamide, while they were highly resistant to most of the clinical commonly used antibiotics, especially the β-lactam antibiotics, except forcefoxitin. The resistant rates were above 90.0%.

This result was consistent with CHINET bacterial drug resistance monitoring network data in our country [20]. Besides, several studies have demonstrated that the polymyxin B, polymixin E, tigecycline and aminoglycoside antibiotics are sensitive to CRKP and thought to be the antibiotics of choice for CRKP, which are the last defense line to CRKP [21]. In this study, resistance rates of 38 experimental strains to tetracycline, amikacin and compound sulfanilamide were low, and those strains were not resistant to tigecycline at all. Therefore, in clinical treatment of CRKP, these antibiotics should be readily accessible, to avoid unnecessary new resistance by paying attention to identifying the pathogenic bacteria. It is worth noting that CRKP were not resistant to tigecycline, a glycyl tetracycline, which undoubtedly brought good news for clinical treatment. However, the urinary tract permeability of tigecycline was poor. After tigecycline enters the bloodstream, it quickly distributes. So the tigecycline application value of blood infection and urinary tract infection is questionable [22]. Considering the limitation of the treatment of urinary tract infection with tigecycline, more attention should be paid to the prevention, isolation and clinical medication treatment.

Results of CRKP resistant gene detection showed that 36 strains (94.7%) among the 38 CRKP strains carried

carbapenem resistant gene, and there was one in the 36 carried both bla_{KPC-2} gene and bla_{IMP-4} gene. Thus, as previously reported, carbapenem resistant gene is the main cause of *Klebsiella pneumoniae* resistant to carbapenem antibiotics [23]. 15 strains (39.5%) in 38 CRKP strains lost two fenestra proteins, ompK35 and ompK36, and two strains without carbapenem resistant gene lost all fenestra proteins. The lack of either OmpK35 or OmpK36 only caused reduced susceptibility to cefoxitin and cefotaxime, but was still sensitive to carbapenem. Only lack of both can lead to lower sensitivity to imipenem and meropenem [24]. Because of this phenomenon, the two strains without carbapenem resistant gene were still resistant to carbapenem. It is worth noting that in this study, 13 strains (34.2%) carried carbapenem resistant gene and two fenestra proteins lost, suggesting that resistance is generally widespread. Meanwhile, 38 CRKP strains all had ESBLs resistant gene, suggesting that resistance mechanism is increasingly complex, thus clinical prevention and control work is imminent.

According to the eBURST clustering analysis diagram, ST11 type *Klebsiella pneumoniae* is one of the clone strains of *Klebsiella pneumoniae* in Zhejiang province. At the same time, some studies showed that in Asian countries, ST11 type had become the main clone strain that carried bla_{KPC} and bla_{NDM}, and also related to carbapenem antibiotic resistance [25-29]. Results of MLST classification of 38 CRKP strains showed that ST11 type strain was the most (86.8%, 33/38). Among them, 31 strains (93.9%) carry bla_{KPC-2} gene. These statistics are similar in Asia. In addition, only one strain was with ST15, ST23, ST1373 and ST1415 in this research. According to the clustering analysis diagram, ST15, ST23 and ST1373, as well as ST352, ST1417 and ST17 were regarded as one clone, while ST1373 was an independent clone strain, suggesting that the carbapenem resistance related ST type could be evolved by existing strains, and could also be produced by new clone strains.

The abuse of antibiotics is the basis of appearance and popularity of CRKP. Quick detection method of CRE is limited in clinical currently, thus the relevant prevention and control work are not timely. Effective drugs used to treat infections caused by CRKP are limited, causing the epidemic spread of drug resistant strains. Various CRKP ST types are related to resistance gene, which provides a reliable basis for the prevention and control work in clinical treatment. Effective intervention and control measures should be taken urgently, to curb the spread caused by drug resistant strains, and reduce the currently high resistance rate [20].

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