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The research on mechanism of *Klebsiella pneumoniae* resistant to carbapenem antibiotics.

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Abstract

Background: To investigate the resistance mechanism of *Klebsiella pneumoniae* (Kpn) resistant to carbapenems.

Methods: K-B assay was used to determine the susceptibility of *Klebsiella pneumoniae* strains to antibiotics. Six isolated strains were identified and collected, which were resistant to carbapenems using modified Hodge test. The phenotypes of metal enzyme were detected with EDTA (Ethylene diamine tetraacetic acid) disk diffusion method. The genes of beta lactamases, included *KPC* gene were confirmed.

Results: The six carbapenems resistant strains of *Klebsiella pneumoniae* were resistant to imipenem, meropenem, aztreonam, etc., but sensitive to amikacin, fosfomycin, minocycline, and polymyxin. The metal enzyme could not be produced by these six pathogens, but all these six strains could produce carbapenemases. Moreover, the six strains partically carried with blaTEM or blaSHV, but all with blaKPC-2.

Conclusion: We thought that the key factor of the resistance mechanism to carbapenem for *Klebsiella pneumoniae* maybe these pathogens contain *blaKPC-2* gene.

Keywords: *Klebsiella pneumoniae*, β-lactamase, Carbapenemase, Resistance mechanism.

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Introduction

In recently, the resistance of some *Enterobacteriaceae* strains to extended-spectrum cephalosporins became a thorny issue in the world [1]. *Klebsiella pneumoniae* and *Escherichia coli* produce extended spectrum β-lactamases (ESBLs), which have led to the use of more carbapenems in the treatment of serious infections in hospitalized patients [2]. Later, carbapenemases were discovered, including class B metallo-β-lactamases VIM, IMP, SPM and GIM in the earliest report [3]. These enzymes were used for zinc as the enzyme's active site. These enzymes were mainly found in *Acinetobacter* and *Pseudomonas* species, and had a very low detection rate in *Escherichia coli* and *Klebsiella pneumoniae*.

In the late 1990s, *Klebsiella* strains that were less susceptible to carbapenem antibiotics, especially in New York City, that were reported in New York City, North Carolina and Maryland [4]. Many hospitals in New York City had reported strains that were less susceptible to carbapenem antibiotics. Initially, a few of researchers tried to detect metal beta-lactamases in these strains, but the MICs of these strains on carbapenem antibiotics were not inhibited by EDTA as expected, and the results confirmed that the bacteria produced KPC-type carbon penemzyme [5].

The clinical isolation rate of imipenem resistant *Enterobacteriaceae* (imipenem-resistant *Enterobacter*, IRE) continued to rise [6]. The emergence of carbapenem-resistant *Enterobacteriaceae* was great importance to clinical anti-infective therapy [7]. One of the main mechanisms of carbapenem antibiotic resistance was bacterial production of carbapenemase KPC (*Klebsiella pneumoniae* carbapenemases) [8]. The first KPC carbapenemase was reported in the United States in 2001 [9]. *Enterobacteriaceae* bacteria that subsequently produce KPC enzymes were continuously being separated in the United States [10]. Twenty-four states in the United States have reported production of KPC enzyme strains

that have caused epidemics in the states of New York and New Jersey in the eastern United States, and then clinical cases of infection with KPC-producing strains were reported in France, Colombia, Greece, the United Kingdom, Argentina, Brazil, Norway, Sweden and China [11]. Currently, the majority of *Klebsiella pneumoniae* producing KPC-2 or KPC-3 are mainly cloned [12]. KPC strains are easily undetected in the laboratory because many strains can be appearing to mediators or sensitive to imipenem or meropenem, with many similarities in the phenotypic characteristics of KPC-producing strains [13]. In addition, the ESBLs producing strains, its misjudgment was ESBLs. Ertapenem is a better indicator of KPC enzyme detection than other carbapenem antibiotics [14]. KPC genes are often located on larger plasmids, which in turn often carry other drug resistance genes, resulting in multiple drug resistance and increasing the difficulty of treatment [15].

In world, the resistance to extended-spectrum cephalosporins in *Enterobacteriaceae* strains became a thorny issue in recent years. Therefore, this work investigated the resistance mechanism of *Klebsiella pneumoniae* (Kpn) resistant to carbapenems.

Materials and Methods

The Klebsiella pneumoniae strains

The *Klebsiella pneumoniae* strains were collected in our hospital (Guangdong Second Provincial General Hospital, Southern Medical University) from January 2017 to September 2017. These *Klebsiella pneumoniae* strains were derived from sputum or airway secretions of different patients with lung infection. All the six specimens of *Klebsiella pneumoniae* Strain was resistant to carbapenem (imipenem, Meropenem). The age of these six patients was 70-90 y old, including 2 females. They were admitted with infectious diseases, and there were large area of shadow in their lungs. These patients had dyspnea, additionally with different degree of increasing white blood cell count and C-reactive protein (CRP). There were invasive procedures and the history of used high-dose broad-spectrum antibiotics before admission. Specimens were identified by the API method bacteria identification system from Merial France. The control strain is *Escherichia coli* ATCC25922.

The ethical approval and written consent was obtained from the local Ethics Committee (The Ethics Committee and Institutional Review Board of Southern Medical University) in our hospital (Guangdong Second Provincial General Hospital, Southern Medical University). Moreover, the Ethics Committee reference number is 2017-0358. The written consent was obtained from the patients or patients' parent.

Drug sensitive test strips and the main reagent

Drug sensitive papers were purchased from Beijing Tiantan Biological Products Co., Ltd. The main antibacterials were cefotaxime CTX, ceftazidime, cefuroxime, amoxicillin/ clavulanic acid (AMC), ampicillin (AM), ampicillin/sulbactam (GEN), cotrimoxazole (SXT), cefoperazone/sulbactam (SCF), piperacillin/azobactam TZP, ATM, cefepime (FEP) levofloxacin (LEV), meropenem (MEM), imipenem (IPM), polymyxin B (PO), fosfomycin (P), amikacin (AN) and minocycline).

DNA Plasmid Extraction Kit and DNA Gel Recovery Kit were purchased from Wiegers Biotech (Beijing) Co., Ltd. Taq enzyme was purchased from Shanghai Dingguo Biotechnology Co., Ltd.. T4 DNA ligase and IPTG were purchased from Promega. DNA Marker and restriction endonuclease were purchased from TaKaRa (Dalian). X-α-gal (Clontech, Japan), DNA ligase, pGEM-T, IPTG and X-β-gal (Promega, USA), EcoR I (Japan TaKaRa), and PCR reaction system were the remaining chemical reagents domestic analytical and biochemical reagents.

The phenotypic screening and phenotype confirmation

Specimen collection and isolation and culture in strict accordance with 'National Clinical Laboratory Procedures' (3rd edition). The resistance of CRKP in our hospital was analysed using WHONET 5.6 software. According to the 2009 Clinical and Laboratory Standards Institute (CLSI) descriptionand the 'NDM-l pan-resistant *Enterobacteriaceae* bacterial infection diagnosis and treatment guidelines' come from China's Ministry of Health and Chinese Medicine Administration [16], the clinical screening of 6 CRKP phenotype were performed, and then through the modified Hodge test were used for confirming carbapenemases, and the metalloenzyme phenotype were confirmed with EDTA inhibition test.

The extraction of bacterial DNA plasmid

The colonies cultured overnight on the plate were inoculated into 5 ml of LB broth and cultured at 37°C for 12-16 h with shaking at 12,000 r/min. The cells were pelleted by centrifugation for 2 min, and then the supernatant was completely discarded. Strictly according to the instructions for plasmid extraction.

PCR amplification and gene sequence analysis

The primers used for the amplification of the resistant carbapenemase-resistant and ESBLs-resistant genes as well as the size of the expected products were selected based on published in GenBank (http://www.ncbi.nlm.nih.gov). Various types of *bla* gene sequence design primers in Table 1. The above primers were synthesized by Invitrogen, USA.

All the PCR reaction system consisted of 25 μl each, including 2 μl of DNA template, 12.5 μl of Premix Taq DNA Polymerase, and a mixture of DNase, buffer, and dNTP at 2-fold concentrations for all PCRs were purchased from Dalian Bao Bioengineering Co., Ltd.), and the corresponding primers 1 μl.

PCR reaction conditions: 95°C 5 min, 35 cycles, 94°C 1 min, 56°C for 30 s, 72°C for 1 min and finally 72°C for 10 min. The

PCR amplification products were electrophoresed on a 1% agarose gel and stained with Gold View dye. The results were observed with a gel imaging system and photographed. In the long-wave UV lamp, the imaging strip was cut out of the DNA strip DNA gel recovery.

Connection and transformation

The DNA recovery products were connected with T vector using T4 ligase dubbed the system. After sealing film 16°C water bath overnight, the ligation products were transformed with *E. coli* competent cells. IPTG was used as an inducer and X-gal was used as a staining reagent. The plates were incubated overnight at 37°C. The blue and white screenings were conducted. The white colonies were picked to re-shake, and then the bacteria mentioned plasmid was obtained.

Digest

All PCR products were purified and sequenced by Beijing Jinweizhi Technology Co., Ltd. The sequencing results were confirmed by Blast sequence alignment software.

Statistical analysis

All experiments were repeated at least 5 times. Data were showed as mean ± standard deviation (SD). The GraphPad Prism program (GraphPad Software, USA) was used for statistical analyses. Treatment groups were compared by one-way analysis of variance (ANOVA) followed by Dunnett's test, with $P<0.05$ considered statistically significant.

Results

Drug susceptibility test

The results of drug susceptibility test suggested that 6 *Klebsiella pneumoniae* were tested *in vitro* resistant to cefotaxime, ceftazidime, cefuroxime, ampicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, gentamicin, cotrimoxazole, cefepime (FEP), piperacillin/tazobactam, levofloxacin (LEV) and aztreonam. Moreover, the inhibition zones of meropenem, imipenem and cefoperazone/sulbactam were small, and the inhibition zones of polymyxin, minocycline, amikacin, fosfomycin were larger. At the same time, the resistant strains were determined with MIC method (Table 2).

The improved Hodge test

The modified Hodge test showed that positive results of all strains, which confirmed the carbapenemase production from these strains. ATCCBAA-1705 was employed as the positive control, and ATCCBAA-1706 was used as a negative control. The positive result of strain 089 was showed in Figure 1, and the remaining five strains of bacteria had the same results.

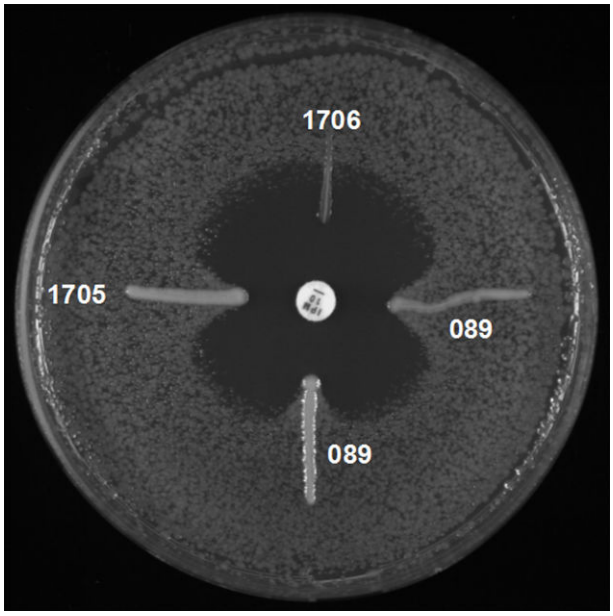

Figure 1. *The results of improved Hodge test. The positive result of modified Hodge test about strain 089 was showed in figure, ATCCBAA-1705 was employed as the positive control, and ATCCBAA-1706 was used as a negative control.*

EDTA inhibition test

Double paper synergy tests were performed in the direction of EDTA paper, but imipenem inhibition zone did not expand, so the results of EDTA inhibition test about strain 102 was negative. The other five strains all achieved the same result (Figure 2).

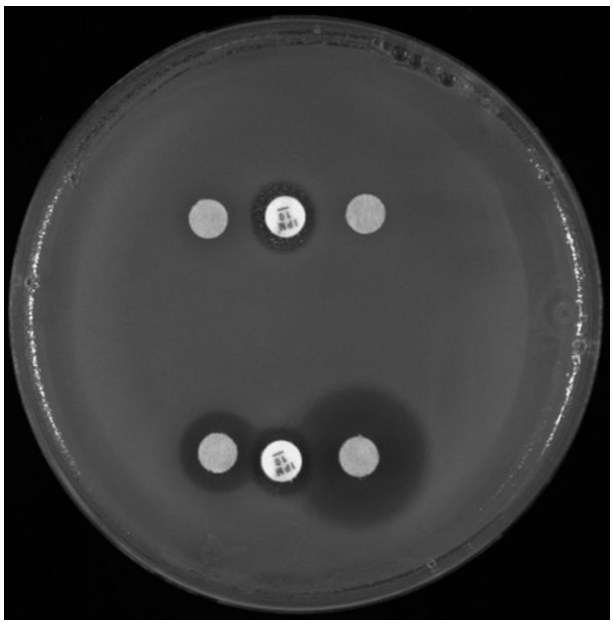

Figure 2. *The results of EDTA inhibition test. Double paper synergy tests were performed in the direction of EDTA paper, the EDTA inhibition test showed negative result.*

The results of MIC tests

Moreover, the results of MIC about strain 102 were showed in Figure 3, and the other five strains all achieved the same result.

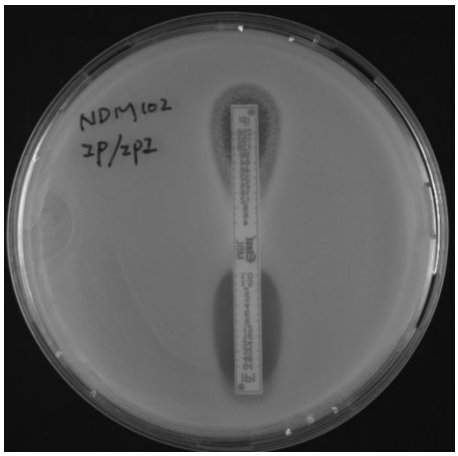

Figure 3. The result of MIC test. Moreover, the result of MIC about strain 102 were showed in figure, the other five strains all achieved the same result.

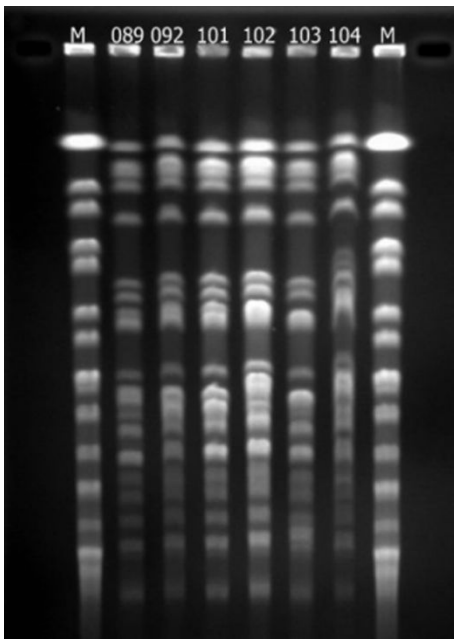

Figure 4. The result of PFGE maps. The results of PFGE (pulsed field gel electrophosresis) maps showed that the chromosome bands of the 6 strains of Klebsiella pneumoniae were basically same as other strains. (M: DNA marker; 089, 091, 101, 102, 103 and 104 were the six Klebsiella pneumoniae strains.

The maps of PFGE

The result of pulsed field gel electrophosresis (PFGE) was showed in Figure 4. PFGE maps showed that the chromosome bands of the six *Klebsiella pneumoniae* strains were basically same as other strains, and maybe originated from the same clone.

The confirmation of drug resistance gene

According to the above steps, the digested products were electrophoresised. The results of electrophoresis showed the successful banding plasmid sent to Beijing Jin Wei Zhi Technology Co., Ltd. for sequencing, and finally the sequencing results were blasted using Blast sequence alignment software for confirmation of drug resistance gene. Our results suggested that the six *Klebsiella pneumoniae* strains all carried drug resistance genes-carbapenemase genes, of which six strains all carried *KPC-2* gene, five strains carried *SHV* gene, and three strains carried *TEM* gene. Moreover, of which two strains also carried three kinds of resistance genes.

Table 1. The primers sequence of drug resistance genes, and the size of the amplified fragment.

Drug resistance gene	Primer5′ → 3′	Size of amplified fragment (bp)
blaKPC-2	GCTACACCTAGCTCCACCTTC	989
	ACAGTGGTTGGTAATCCATGC	
blaSHV	GGGTTATTCTTATTTGTCGC	927
	TTAGCGTTGCCAGTGCTC	
blaTEM	ATGAGTATTCAACATTTCCGTG	861
	TTACCAATGCTTAATCAGTGAG	
blaVEB	CGACTTCCATTTCCCGATGC	642
	GGACTCTGCAACAAATACGC	
blaPER	TGACGATCTGGAACCTTT	850
	AACTGCATAACCTACTCC	
blaCTX-M-1	ATGGTTAAAAAATCACTGCGC	944
	TCCCGACGGCTTTCCGCCTT	
blaCTX-M-2	ATGATGACTCAGAGCATTCG	900
	TCCCGACGGCTTTCCGCCTT	
blaCTX-M-9	CGGCCTGTATTTCGCTGTTG	877
	TCCCGACGGCTTTCCGCCTT	
blaOXA-1	CTGTTGTTTGGGTTTCGCAAG	720
	CTTGGCTTTTATGCTTGATG	
blaOXA-10	GTCTTTCAAGTACGGCATTA	720
	GATTTTCTTAGCGGCAACTTA	

Table 2. The results of diameter of inhibition zone and breakpoints or MIC of these six drug resistance Klebsiella pneumoniae strains.

	Diameter of inhibition zone (mm)	Breakpoints (K-B) (mm)	MIC (mm)	Breakpoints (MIC) (mm)

Cefotaxime	6	22~26	-	-
Ceftazidime	6	17~21	≥ 64	4~16
Cefuroxime	6	14~23	≥ 64	8~32
Ampicillin	6	13~17	≥ 32	8~32
Amoxicillin/clavulanic acid	6	13~18	-	-
Ampicillin/sulbactam	6	11~15	≥ 32	8/4~32/16
Gentamicin	6	12~15	≥ 16	4~16
Cotrimoxazole	6	10~16	≥ 320	2/38~4/76
Piperacillin/tazobactam	6	17~21	≥ 128	16/4~128/4
Aztreonam	6	17~21	≥ 64	4~16
Cefepime (FEP)	6	14~18	≥ 64	8~32
Levofloxacin (LEV)	6	13~17	≥ 8	2~8
Meropenem	8	13~16	≥ 16	4~16
Imipenem	9	13~16	≥ 16	4~16
Cefoperazone/sulbactam	16	15~21	-	-
Polymyxin	18	-	-	-
Minocycline	20	12~16	-	-
Amikacin	24	14~17	≤ 2	16~64
Fosfomycin	15	12~16	-	-

Discussion

Carbapenem antibiotics play an important role in the treatment of clinically serious infections, especially *Enterobacteriaceae* [17]. However, with the increasing use of clinical carbapenem antibiotics, there are resistant strains, mainly against *Pseudomonas aeruginosa* and *Acinetobacter*. In recent years, imipenem-resistant *Enterobacteriaceae* (imipenem-resistant *Enterobacter*, IRE) were reported increasing [18]. Bacteria for carbapenem antibiotic resistance mechanisms are mainly in the following areas: (1) high-yielding AmpC enzyme with loss porin or decreased expression levels, then induced the decreasing permeability of antibiotics from cell membrane; (2) the efflux pump decreased intracellular antibiotic concentration; (3) the change of target aimed by carbapenem antibiotic; (4) production of carbapenema [19].

In addition, the L1 enzyme of *Stenotrophomonas maltophilia*, Ambler classification of A, B, D are three kinds of hydrolysis of carbapenem antibiotics β-lactamase [20]. KPC (carbapenem carbapenemases) carbapenemases is a newly discovered carbapenemase in recent years, belonging to the Ambler class A, such carbapenemases can almost hydrolyze all β-lactams antibiotic [1,7]. A total of nine subtypes (KPC 1-9) have been identified. KPC-1 sequencing was confirmed to be incorrect (http://www.1ahey.org/Studies/) and is actually the same genotype as *KPC-2* [7]. KPC enzyme producing strain was first discovered in the United States, after which KPC enzyme producing strains were found in France, Colombia, Israel, China, Norway, Brazil, Britain, Greece and other regions, these strains were *Enterobacteriaceae* bacteria, including pneumonia grams *Salmonella, Salmonella, Escherichia coli, Enterobacter cloacae, Citrobacter freundii, Serratia marcescens, Proteus mirabilis*, and the like, were also found in *Pseudomonas aeruginosa* and *Pseudomonas putida* strain, which could produce KPC enzyme [8]. Since KPC-producing strains are resistant to carbapenem-based antibiotics but are also resistant to many other antibacterials based on transmission of plasmids, etc. Moreover, carbapenem-resistant *Enterobacter* section bacteria have become a major clinical challenge treatment [10].

Klebsiella pneumoniae, which produces KPC enzymes in hospitals, is found mainly in the contaminated medical devices, hands of medical staff or in the patient's gastrointestinal tract [10]. Bratu et al. had isolated *Klebsiella pneumoniae* strains that produced KPC enzymes in venous cannulas, bed railings, and sphygmomanometers in patients' rooms [21]. The spread of KPC-producing strains showed in the same hospital, between different hospitals and in different regions and even in different countries. All this indicated that environmental factors may be a way to get infected or colonize bacteria. PFGE results showed that the six strains of *Klebsiella pneumoniae* that we isolated belonged to the same clone. All the six patients were hospitalized from January 2017 to September 2017, and the cultures of patients who had lived in the same ward were isolated Strain. This indicated that *Klebsiella pneumoniae* producing KPC had a brief clonal spread in our ward during the above period. The reason for the

clonal spread may be a variety of medical interventions. Surveillance of nosocomial infections, detection of drug-resistant strains and the timely introduction of precautionary measures are prerequisites for preventing a wider spread.

Detection of KPC enzyme is an important part of the control of its dissemination, but also can provide the basis for clinical drug selection. However, the identification of KPC enzymes is more difficult, mainly in the following aspects: (1) many strains producing KPC enzymes are sensitive to the MIC of meropenem and imipenem; (2) the characteristics of KPC enzymes and ESBLs are similar, and easily misjudged as ESBLs; (3) the phenotypic identification of local resistance strains restricted by carbapenem-resistant mechanisms (for example, there are other carbapenemases such as metal β-lactamase, ESBLs-producing or AmpC combined with loss of membrane protein), culture media, strains, drugs and methods or other factors, there is no uniform standard. Our susceptibility results showed that the six strains of *Klebsiella pneumoniae* were resistant to aztreonam, piperacillin/tazobactam, cefotaxime, cefoperazone/sulbactam, and imipene. The sensitivity to meropenem and ertapenein decreased. The results of PCR showed that the six strains of *Klebsiella pneumoniae* contained genes of SHVβ-lactamase and TEM gene in addition to *KPC-2* gene.

Patient-susceptibility factors for isolates of KPC strains include seniors, intensive care unit hospitalization, and ventilator use. The six patients in our hospital are all over the age of 65 y and lived in the same ward, similar to those reported in the literature. In addition, all the six patients had underlying disease. Regarding the use of antibacterials in relation to the production of KPC enzymes, studies by Bratu et al. [21]. It demonstrated that only 20% of the 60 patients infected with KPC pathogens used carbapenem antibiotics before isolating the pathogens, and 60% of the patients have used beta-lactam antibiotics or antibiotics with beta-lactamase inhibitors, and 60% have used quinolone antibacterials. Bradford and other studies have shown that only three of thirteen patients were treated with carbapenem before isolating KPC strains. These data suggest that treatment with carbapenem antibiotics previously did not appear to be necessary for infecting or colonizing KPC pathogens. Most of the patients in this group have a history of using antibiotics and antifungal drugs. Confirmed by the PFGEE, the six strains of *Klebsiella pneumoniae* were the same clone, so infection-related pathogens such as hospital-acquired infections may be more important.

KPC strains for the production of less information on the treatment, our results of drug susceptibility test indicated that polymyxin, timentin, SMZ sensitive, combined with polymyxin B and tylinin in the treatment of KPC bacterial infections have been some institutions of conventional therapy, but this information needs further study. This treatment is difficult to implement due to difficult access to medicines in the country and none of effective result, then these patients died after treatment. The sensitive antibiotics requiring special medicines should be sought to strengthen the treatment of underlying diseases and to strengthen the supportive care to improve the patients' basis status, timely release of indwelling needle, intubation and other infection-related factors such as comprehensive treatment measures seem to be particularly important. More critical is the rational use of antibiotics to strengthen the monitoring of drug-resistant strains.

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