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Original Article

Molecular epidemiology of aminoglycosides resistance on *Klebsiella pneumonia* in a hospital in China

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Abstract: To investigate the molecular epidemiology of aminoglycosides resistance among *Klebsiella pneumonia* in hospitals in China, the antibiotics resistance and the possession of extended-spectrum β-lactamases (ESBLs) from 162 isolates were examined using Kirby-Bauer disk diffusion and PCR sequencing. Overall, 47.5% (77/162) of strains showed an ESBL phenotype. According to antibiotics resistance, ESBLs-positive *K. pneumoniae* showed significantly higher resistance to most antibiotics than ESBLs-negative strains (P<0.05). Moreover, 162 strains harboured aminoglycoside-modifying enzymes genes (AMEs) including *aac (3)-II* (n = 49), *aac (6')-Ib (n = 32), ant (3")-I (n = 22) and ant (2")-I* (n = 7). Overall, 11.1% (18/162) and 6.2% (10/162) of isolates carried 16S rRNA methylase genes (*arm*A and *rmt*B), in which the aminoglycoside MIC was more than 256 μg/ml. In conclusion, our study characterised aminoglycosides resistance among *K. pneumoniae* strains in China hospitals and revealed antibiotic resistance and the *increased* presence of AMEs and 16S rRNA methylase genes in *K. pneumonia*, enabling the prevalence of aminoglycosides resistance of *K. pneumoniae* to be tracked from patients.

Keywords: Aminoglycosides resistance, AMEs, 16S rRNA methylase, *Klebsiella pneumonia*

Introduction

Recently, *Klebsiella pneumonia* has become one of the most important conditional pathogenic bacteria in nosocomial infections. Accordingly, antimicrobial therapy continues to be a widely available tool for the prevention and control of this infection. However, owing to the overuse of antimicrobials, especially β-lactams or aminoglycosides, resistance has become increasingly prevalent, thus compromising their therapeutic efficacy. Indeed, the emergence and prevalence of antimicrobial-resistant *K. pneumoniae* strains has been described in many countries [1-3]. The underlying mechanism of β-lactam resistance is dominated by the expression of extended-spectrum β-lactamase (ESBLs) [1]. Moreover, the mechanisms of resistance to aminoglycosides also include enzymatic modification of this drug, modification of the ribosomal target and decreased intracellular antibiotic accumulation by alterations of the outer membrane permeability, decreased inner membrane transport or active efflux [4]. Among them, the production of aminoglycoside-modifying enzymes is the most common mechanism of resistance to aminoglycosides. Modification of 16S rRNA by these enzymes reduces binding to aminoglycosides, leading to high-level resistance to aminoglycosides, including arbekacin, amikacin and, kanamycin [5, 6]. Currently, seven 16S rRNA methylase genes have been identified (*arm*A, *rmt*A, *rmt*B, *rmt*C, *rmt*D, *rmt*E and *npm*A) [1-3].

The goals of the present study were to investigate the state of antibiotic resistance and the prevalence of ESBLs and the aminoglycoside resistance genes in *K. pneumoniae* strains, in order to assess which resistance mechanisms might contribute to the observed aminoglycosides resistance in *K. pneumoniae.*

Materials and methods

Bacterial isolates

Between October 2009 and December 2010, a total of 162 *K. pneumoniae* field strains were isolated from patients in the Third Affiliated Hospital of Sun Yat-sen University. These isolates were identified by conventional biochemi-

Table 1. The distribution of specimens of *K. pneumoniae* (*n* = 162)

Specimens	Strains (n)	Rate (%)
Sputum	80	49.4
Blood	26	16.0
Bile	16	9.9
Urine	13	8.0
Cutaneous mucous secretions	12	7.4
Throat swab	6	3.7
Hydrothorax and ascites	5	3.1
Others	4	2.5

Table 2. The distribution of *K. pneumoniae* in clinical departments (n = 162)

Department	Strains (n)	Rate (%)
Intensive care unit	37	22.8
Hepatobiliary Surgery	24	14.8
Neurosurgery	19	11.7
Respiratory Department	13	8.0
Haematology Department	13	8.0
Tumour Department	11	6.8
Cardiothoracic Surgery	10	6.2
Neurology Department	8	4.9
Rehabilitation Department	7	4.3
Outpatient and Emergency	7	4.3
Others	13	8.0

cal methods and the MicroScan Wa1kAway-40 automatic bacteria identification system.

Antimicrobial susceptibility testing

The antibiotic susceptibility of K. pneumoniae to 22 common antibiotics was determined using K-B disk diffusion method according to CLSI [7]. Twenty-two common antibiotics were used, including: Ampicillin (AMP, 10 μg), Ampicillin/sulbactam (SAM, 10 μg/10 μg), Piperacillin (PIP, 100 μg), Piperacillin/tazobactam (TZP, 100 μg/10 μg), Amoxycillin/clavulanic acid (AMC, 20 μg/10 μg), Ticarcillin/clavulanic acid (TLC, 75 μg/10 μg), Cefazolin (CZL, 30 μg), Cefotaxime (CTX, 30 μg), Ceftriaxone (CRO, 30 μg), Ceftazidime (CAZ, 30 μg), Cefepime (FEP, 30 μg), Cefoxitin (FOX, 30 μg), Aztreonam (ATM, 30 μg), Imipenem (IMP, 10 μg), Ciprofloxacin (CIP, 5 μg), Levofloxacin (LEV, 5 μg), Sulphamethoxazole/trimethoprim (SXT, 1.25 μg/23.75 μg), Amikacin (AMK, 30 μg), Gentamycin (GM, 10 μg), Tobramycin (TOB, 10 μg), Cefotaxime/clavulanic (CTX/CA, 30 μg/10 μg) and, Ceftazidime/clavulanic acid (CAZ/CA, 30 μg/10 μg). Generally, the breakpoints for the antimicrobial agents for *K. pneumoniae* were according to CLSI [7].

Moreover, minimal inhibitory concentrations (MICs) of amikacin, gentamycin and tobramycin to *K. pneumoniae* were detected. The reference strains *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218 and *K. pneumoniae* ATCC 700603 served as quality control strains for the determination of antibiotics susceptibility.

The ESBLs phenotypic confirmation

Confirmatory tests for ESBLs of 162 *K. pneumoniae* clinical isolates were performed by adopting the Kirby-Bauer diffusions method, according to CLSI [7].

Screening for aminoglycosides resistance genes

The isolates were selected for further molecular characterisation of aminoglycosides resistance by polymerase chain reaction (PCR) using *ExTaq* DNA polymerase (TAKARA, Dalian, China), and specific oligonucleotide primers, as previously described [1, 8-10]. The aminoglycosides resistance genes included *aac (3)-II, aac (6')-Ib, ant (3")-I, ant (2")-I, aac (3)-I, aac (6')-II, aac (6')-Iad* and 16S rRNA methylase genes (*arm*A, *rmt*A, *rmt*B, *rmt*C, *rmt*D, *npm*A). The DNA templates of all 162 *K. pneumoniae* strains were prepared using the standard boiling method [11]. The PCR amplicons were cloned into pMD-19T vectors (TaKaRa Inc., China) and sequenced by the Applied Biosystems 3730 sequence analyser (Applied Biosystems Inc., USA).

Statistical analysis

We used the SPSS13.0 statistics software package for analysis. The data was described as $\bar{x} \pm s$. The differences between groups were compared by the chi-square test ($P<0.05$ for statistical significance).

Results and discussion

Bacterial isolates

A total of 162 clinical isolates of *K. pneumoniae* were isolated from sputum (49.4%), plasma (16.0%) and bile (9.9%) sample (**Table 1**). For the sources of the samples, the top three

Table 3. Comparison of antimicrobial-resistance between ESBLs-positive and ESBLs-negative *K. pneumoniae*

Antibiotics	ESBLs (+) (n = 77)			ESBLs (-) (n = 85)			x^2	*P*
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)		
Ampicillin	98.7	0.0	1.3	87.0	11.8	1.2	7.984	0.005
Piperacillin	97.4	0.0	2.6	24.7	15.3	60.0	88.438	0.000
Ampicillin/sulbactam	83.1	11.7	5.2	12.9	11.8	75.3	80.024	0.000
Amoxycillin/clavulanic acid	26.0	27.3	46.7	3.5	5.9	90.6	16.707	0.000
Piperacillin/tazobactam	23.4	5.2	71.4	1.2	1.2	97.6	19.233	0.000
Ticarcillin/clavulanic acid	31.2	32.5	36.4	3.5	2.4	94.1	22.222	0.000
Cefazolin	93.5	1.3	5.2	7.1	1.2	91.8	120.936	0.000
Cefotaxime	84.4	9.1	6.5	1.2	1.2	97.6	115.948	0.000
Ceftazidime	48.0	23.4	28.6	0.0	0.0	100.0	52.934	0.000
Ceftriaxone	85.7	5.2	9.1	1.2	1.2	97.6	119.050	0.000
Cefepime	72.7	15.6	11.7	1.2	0.0	98.8	90.696	0.000
Cefoxitin	35.1	5.2	59.7	5.9	1.2	92.9	21.706	0.000
Aztreonam	61.0	10.4	28.6	1.2	0.0	98.8	69.437	0.000
Imipenem	1.3	0.0	98.7	1.2	0.0	98.8	0.005	0.944
Ciprofloxacin	45.5	11.7	42.8	16.5	2.4	81.1	16.087	0.000
Levofloxacin	37.7	5.2	57.1	8.2	3.5	88.3	20.242	0.000
Amikacin	22.1	2.6	75.3	12.9	1.2	85.9	2.648	0.104
Gentamycin	59.7	2.6	37.7	17.6	1.2	81.2	28.631	0.000
Tobramycin	44.2	14.3	41.5	10.6	1.2	88.2	23.348	0.000
Sulphamethoxazole/trimethoprim	68.8	0.0	31.2	21.2	1.2	77.6	37.268	0.000

P = comparison of *Klebsiella pneumoniae* between ESBLs (+) and ESBLs (-).

departments were the intensive care unit (ICU) (22.8%), hepatic surgery (14.8%) and the neurosurgery department (11.7%) (**Table 2**). Over the past 10 years, a progressive increase has been seen on a worldwide scale [12, 13]. In the USA, this phenomenon in *K. pneumoniae* was first described in North Carolina in 1996 [12], and the new emerging nosocomial pathogen is probably best known for an outbreak in Israel that began around 2006 within the healthcare system there [13].

Antibiotics resistance in K. pneumoniae

Antimicrobial susceptibility and the comparison of antimicrobial-resistance between ESBLs-positive and ESBLs-negative *K. pneumoniae* are shown in **Table 3**. The results of confirmatory tests showed that the rate of ESBLs-producing *K. pneumoniae* was 47.5% (77/162). Antibiotic susceptibility tests showed that ESBLs-producing *K. pneumoniae* was most sensitive to imipenem with a rate of 98.7%, followed by 75.3% for amikacin, and 71.4% for piperacillin/tazobactam. The resistance rate of ESBLs-negative *K. pneumoniae* to ampicillin was 87.0%, but was below 25% for the other antibiotics. Except for imipenem and amikacin, resistance rates of ESBLs-producing strains were significantly higher than those of ESBLs-negative strains (P<0.05), which may have been caused by other resistance mechanisms in those ESBLs-producing *K. pneumoniae* isolates [1, 14].

Prevalence of AMEs genes and 16S rRNA methylase genes

Molecular identification of the 162 isolates obtained from the hospital showed that the positive rates of AMEs genes, such as *aac (3)-II*, *aac (6')-Ib*, *ant (3")-I* and *ant (2")-I*, were 30.2%, 19.8%, 13.6% and 4.3%, respectively. Also 16S rRNA methylase genes were also identified with positive rates of armA and rmtB of 11.1% and 6.2%, respectively. All sequences of the detected amplicons were aligned and it was shown that there was over 99% identity with the reported target genes accessed from NCBI.

The distribution of AMEs and 16S rRNA methylase gene in *K. pneumoniae* is shown in **Table 4**. Among them, 28 strains carried both AMEs and 16S rRNA methylase genes. A total of 62 strains

Table 4. The distribution of AMEs and 16S rRNA methylase gene in *K. pneumoniae* (n = 162)

Gene Types	Strains Num.	Rate (%)
aac (3)-II	7	11.3
aac (6')-Ib	5	8.1
ant (3")-I	3	4.8
ant (2")-I	1	1.6
armA+aac (3)-II	5	8.1
armA+ant (3")-I	4	6.5
rmtB+aac (3)-II	5	8.1
aac (3)-II+aac (6')-Ib	8	12.9
aac (3)-II+ant (3")-I	4	6.5
armA+aac (3)-II+aac (6')-Ib	3	4.8
armA+aac (3)-II+ant (3")-I	1	1.6
rmtB+aac (3)-II+aac (6')-Ib	4	6.5
aac (3)-II+aac (6')-Ib+ant (3")-I	2	3.2
aac (3)-II+aac (6')-Ib+ant (2")-I	2	3.2
armA+aac (3)-II+aac (6')-Ib+ant (3"")-I	3	4.8
rmtB+aac (3)-II+aac (6')-Ib+ant (3")-I	1	1.6
aac (3)-II+aac (6')-Ib+ant (3")-I+ant (2")-I	2	3.2
armA+aac (3)-II+aac (6')-Ib+ant (3")-I+ant (2")-I	2	3.2

carrying resistance genes included 16 strains with 1 genotype, 26 strains with 2 genotypes, 12 strains with 3 genotypes, 6 strains with 4 genotypes and 2 strains with 5 genotypes. The most common genotype was *aac (3)-II+aac (6')-Ib*, and the positive rate was 12.9% (8/62); this was followed by *aac (3)-II* with the a positive rate of 11.3% (7/62). It was reported that 16S rRNA methylases first appeared in *K. pneumoniae* in 2003 [15]. *RmtB* was first identified in *S. marcescens* from Japan in 2004, and was subsequently found in *K. pneumoniae* and *E. coli* isolates from Taiwan, Korea and Belgium [1, 8, 16, 17]. To date, the 16S rRNA methylase genes were prevalent globally [1]. In this study, AMEs genes of *aac (3)-II, aac (6')-Ib, ant (3")-I and ant (2")-I* and 16S rRNA methylase genes of *armA* and *rmtB* were all prevalent in *K. pneumoniae* in China.

Conclusions

The present study reported the prevalence of the *K. pneumoniae* infection, the antimicrobial resistance and, the characterisation of ESBL, and underlined the importance of the prudent use of antimicrobials and routine monitoring of susceptibility patterns to minimise the spread of antibiotic resistance. Of note, these findings also showed that the emergence of *arm*A, *rmt*B, *aac (3)*-II, *aac (6')-Ib, ant (3")-I*, and *ant (2")-I* in *K. pneumoniae* in China, is related to aminoglycosides antimicrobial resistance. Moreover, the exact role and the spread mechanisms of these resistance genes in *K.* pneumoniae still await further studies.

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Disclosure of conflict of interest

None.

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