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ORIGINAL RESEARCH

High Prevalence of 16S rRNA Methyltransferase Genes in Carbapenem-Resistant *Klebsiella pneumoniae* Clinical Isolates Associated with Bloodstream Infections in 11 Chinese Teaching Hospitals

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Objective: The 16S rRNA methylase-mediated high-level resistance to aminoglycosides has become a great concern. The purpose of the study was to investigate the occurrence of 16S rRNA methyltransferase (RMTase) genes in carbapenem-resistant *Klebsiella pneumoniae* (CRKP) clinical isolates associated with bloodstream infections (BSIs) in China.

Methods: From July 2015 to December 2018, a total of 137 unique CRKP clinical isolates associated with BSIs were collected from 11 Chinese teaching hospitals. PCR and DNA sequencing were used to identify 16S RMTase genes. Whole-genome sequencing (WGS) was performed on all CRKP clinical isolates. Relevant information was extracted from WGS data (antibiotic resistance determinants, K-type and wzi allelic types). All 16S RMTase-producing CRKP clinical isolates were characterized by antimicrobial susceptibility testing, multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE).

Results: In this study, 137 CRKPs were found to harbor at least one carbapenemase gene. Among 137 CRKPs, 78 (56.9%, 78/137) were positive for 16S RMTase genes (5 for armA, 70 for rmtB, 3 for both armA and rmtB) and highly resistant to gentamicin and amikacin (MICs ≥256 mg/L). Seventy-five isolates harboring 16S RMTase genes also produced ESBLs. In this study, 5 sequence types (STs) and 6 capsule serotypes were found among 78 isolates positive for 16S RMTases genes, while 14 STs and 6 capsule serotypes were found among 59 isolates negative for 16S RMTases genes. Compared with the isolates negative for 16S RMTases genes, the STs and capsular serotypes of 16S RMTases-positive strains are more concentrated. Among 78 16S RMTases-positive strains, the most prevalent clone type is ST11-PFGE-B-KL64-wzi64 (62.8%, 49/78), which mainly carries the rmtB and bla_{KPC} genes and is distributed in 7 provinces in China.

Conclusion: A high prevalence of 16S RMTase genes was found among CRKP clinical isolates associated with BSIs from Chinese teaching hospitals, which was attributed to the dissemination of the ST11-PFGE-B-KL64-wzi64 clone.

Keywords: CRKP, BSIs, 16S RMTase genes, aminoglycosides, molecular characteristics

Introduction

Aminoglycosides are broad-spectrum and highly effective antibiotics, often used in combination with β-lactam antibiotics to treat bacterial infections. In the USA, the most commonly used prescription aminoglycosides for the treatment of serious

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infections include amikacin, gentamicin and tobramycin.[1] Although the nephrotoxicity and ototoxicity of aminoglycosides have been confirmed,[2,3] they play a key role in antibacterial therapy, especially against multidrug resistance (MDR) Gram-negative bacilli.[4] However, increasing resistance to aminoglycosides is becoming an extremely serious clinical problem.[5–7]

Among the resistance mechanisms against aminoglycosides, the most common in *Enterobacteriaceae* are aminoglycoside modifying enzymes (AMEs).[8,9] These enzymes include acetyltransferase (AAC), phosphotransferase (APH) and nucleotide transferase (ANT), which inactivate aminoglycosides by covalently modifying specific amino or hydroxyl groups.[9] In addition to AMEs, 16S RMTases have been shown to confer high-level resistance to all clinically relevant aminoglycosides (MICs≥256 mg/L).[10] Since the first 16S RMTases gene, armA, was identified in 2003.[10] Ten 16S RMTase-encoding genes, including armA, rmtA, rmtB, rmtC, rmtD, rmtE, rmtF, rmtG, rmtH and npmA, have been found in clinical isolates of Gram-negative bacilli from multiple geographic locations.[11] Most of the genes encoding 16S RMTases are typically located on plasmids along with those for extended-spectrum beta-lactamases (ESBLs) and carbapenemases resistance determinants.[6,10,12] This may limit the choice of antimicrobials used to treat multi-drug resistant Gram-negative infections. The 16S RMTase-producing isolates have become a concern to clinical treatment in the world.[11] The reports about the prevalence of 16S RMTase genes among carbapenem-resistant *Klebsiella pneumoniae* (CRKP) clinical isolates associated with bloodstream infections (BSIs) in China are unknown. The aim of the present study was to investigate the occurrence of 16S RMTase genes in CRKP clinical isolates associated with BSIs in several Chinese teaching hospitals.

Materials and Methods

Collection and Identification of CRKP Isolates

From July 2015 to December 2018, a total of 137 unique (one isolate per patient) CRKP isolates were collected from 11 tertiary teaching hospitals in eight provinces of China, including Jiangxi (n = 40), Shandong (n = 19), Shanghai (n = 18), Henan (n = 15), Zhejiang (n = 18), Hubei (n = 11), Fujian (n = 9), and Hunan (n = 7). All isolates were isolated from patients with BSIs. All isolates were identified as *K. pneumoniae* by Gram staining and a VITEK-2 automated platform (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's protocol. *Escherichia coli* ATCC25,922, *Pseudomonas aeruginosa* ATCC27853 and *Staphylococcus aureus* ATCC25923 were used as control strains for the species identification. In this study, carbapenem resistance was defined as resistance to meropenem or imipenem based on the 2018 Clinical and Laboratory Standards Institute (CLSI) guidelines.[13] Clinical data were extracted from medical records and oral informed consent was obtained from the relevant patients.

Detection of 16S RMTase Genes

The 16S RMTase genes, including armA, rmtA, rmtB, rmtC, rmtD, rmtE, rmtF, rmtG, rmtH and nmpA, were detected using polymerase chain reaction (PCR) as described previously.[6,14,15] After PCR, PCR products were sequenced by Sanger sequencing. The obtained DNA sequences were compared with the DNA sequence in GenBank using the BLAST program.

Phenotype Confirmation of Carbapenemase

A modified carbapenem inactivation test (mCIM) for 137 CRKP isolates to detect carbapenemases according to CLSI 2018 standards.[13] All tested CRKP isolates were incubated with meropenem disk (BIO-KONT, Wenzhou, China) in 2 mL TSB at 37°C for 4 hours. The *E. coli* ATCC25922 was used as an Indicator bacteria. A suspension of *E. coli* ATCC 25922 was adjusted to 0.5 McFarland standard with sterile physiological saline solution. The *E. coli* ATCC25922 suspension was evenly spread on MH agar plates. After drying for 3–10 minutes, the meropenem disk (10 μg) was placed on the surface of the agar plate. All treated plates were incubated at 37°C for 18–24 hours. Following incubation, measure the zones of inhibition.

Whole-Genome Sequencing (WGS) and Analysis

DNA extraction was carried out using the Ezup Column Bacteria Genomic DNA Purification Kits (Sangon Biotech, shanghai, China) according to the manufacturer's instructions. Genomic sequencing libraries were prepared using the Nextera XT kit and sequenced using the Illumina Miseq platform (paired end reads of 150 bp) according to the manufacturer's protocol. Trimmed reads were de novo assembled using SPAdes 3.9.1.[16] Resistance genes carried

by all isolates were evaluated using Resfinder program provided by the Center for Genome Epidemiology (https://cge.cbs.dtu.dk/services/).[17] Multi-locus Sequence Typing (MLST) was also determined from the assembled genome. The serotypes (K-types and wzi allelic types) of all *K. pneumoniae* isolates were analyzed using the *Klebsiella* WGS data online platform tool Kaptive-web (http://kaptive.holtlab.net/).

Antimicrobial Susceptibility Testing

The minimum inhibitory concentration (MIC) of various antibacterial drugs for CRKP isolates tested was determined by the broth micro-dilution method and interpreted according to CLSI criteria as described previously.[13,18] The MIC was defined as the lowest concentration of antibiotic completely inhibiting visible growth. A total of 17 antimicrobials including carbapenems (imipenem and meropenem), aminoglycosides (amikacin and gentamicin), β-lactams/β-lactamase inhibitor complexes (ceftazidime-avibactam and piperacillin-tazobactam), cephalosporin (ceftazidime, cefepime, cefotaxime and cefoxitin), fluoroquinolones (ciprofloxacin), folate metabolic pathway inhibitors (sulfamethoxazole), polymyxin B, tetracyclines (tigecycline, minocycline and tetracycline) and monocyclic β-lactam (aztreonam) were tested. The *E. coli* ATCC25922 was used as control strain for determining the antimicrobial susceptibility for *K. pneumoniae* clinical isolates.

Multilocus Sequence Typing (MLST)

Multilocus sequence typing (MLST) was used to investigate the genetic relationship of the isolates positive for 16S RMTase genes. Amplification of seven standard housekeeping loci by PCR, including gapA, infB, mdh, pgi, phoE, rpoB and tonB, followed by Sanger sequencing as described previously.[19] The alignment analysis was performed using an online database on the Pasteur Institute MLST website (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) to obtain the assigned sequence type (ST) of the isolates.

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE analysis was performed on 78 clinical isolates of CRKP positive for 16S RMTase genes. The *K. pneumoniae* cells were embedded in agar plates and digested with XbaI for 3.5 hours at 37°C to prepare genomic DNA. Electrophoresis was carried out for 19 hours using the Bio-Rad CHEF III system with parameters set to 14°C, 120° angle, 6 V/cm, and switching times of 6 and 36 seconds. The *Salmonella enterica* serotype Braenderup strain H9812 was chosen as the control standard strain and universal molecular weight marker. Electrophoresis results are interpreted according to the criteria proposed by Tenover et al.[20] Analysis of PFGE results using Bioices software (Applied Maths, Sint-Martens-Latem, Belgium) with the Dice similarity coefficients. Clinical isolates with more than 80% homology are defined as the same PFGE cluster.

Statistical Analysis

SPSS statistical software (version 23, IBM SPSS Statistics) was used for statistical analysis. The $\chi 2$ or Fisher's exact tests were used for categorical variables. $P < 0.05$ was considered statistically significant.

Results

Detection of 16S RMTase Genes

Among 137 tested CRKP isolates, 78 (78/137, 56.9%) were positive for 16S RMTase genes determined by PCR and Sanger sequencing. Of 78 isolates with 16S RMTase genes, 70 were positive for rmtB gene and were distributed in 8 provinces, including Jiangxi (n = 22), Shandong (n = 11), Hubei (n = 9), Henan (n = 8), Shanghai (n = 7), Zhejiang (n = 5), Fujian (n = 5) and Hunan (n = 3); 5 were positive for armA gene (3 in Shanghai and 2 in Henan); 3 were found to carry both rmtB and armA (2 in Shanghai and 1 in Zhejiang). Each of the 137 CRKP isolates carries at least one carbapenem-resistant gene, which contains five different carbapenemase genes, including bla_{KPC-2} (n = 110), bla_{NDM-5} (n = 18), bla_{NDM-1} (n = 16), bla_{IMP-4} (n = 1) and bla_{IMP-30} (n=2). Among them, 6 isolates possessed both bla_{NDM-1} and bla_{KPC-2}, 3 isolates harbored bla_{NDM-5} and bla_{KPC-2}, and 1 isolate carried bla_{IMP-4} and bla_{KPC-2}. Seventy-five (75/78, 96.2%) of 78 isolates harboring 16S rRNA methylase genes were extended-spectrum β-lactamase (ESBL) producers. Seventy and Thirty-six isolates with 16S rRNA methylase genes were positive for bla_{CTX-M} and bla_{SHV} genes, respectively. Among the 78 isolates, it was confirmed that 3 strains carried $bla_{CTX-M-3}$, 1 strain carried $bla_{CTX-M-14}$, 3 strains carried $bla_{CTX-M-15}$, 1 strain carried $bla_{CTX-M-27}$, 66 strains carried $bla_{CTX-M-65}$ and 36 strains carried bla_{SHV-12}. In this study, compared to 16S RMTases-negative strains, 16S RMTases-positive strains harbored fewer bla_{NDM} genes, but carried more bla_{KPC} genes (Table 1). Carbapenemase genes and ESBL genes were extracted from WGS data.

Aminoglycoside Modification Enzyme (AME) Genes

Besides the 16S RMTase genes, other aminoglycoside resistance genes, including AME genes and streptomycin-related resistance genes, were identified by analyzing the WGS data of 78 CRKP isolates. Various aminoglycoside modifying enzyme genes were identified, including aac3-IId (23/78, 29.5%), aph3-Ia (5/78, 6.4%), aadA2 (65/78, 83.3%), aadA1-pm (2/78, 2.6%), aadA16 (3/78, 3.8%) and aadB (6/78, 7.7%). In addition, 24 isolates were also found to carry streptomycin-related resistance genes strA and strB.

Phenotypical Identification of All CRKP Isolates

All 137 CRKP isolates were found to produce carbapenemases determined by the mCIM assay.

Antimicrobial Resistance Among 16S RMTases-Positive and -Negative CRKP Isolates

Among tested 137 CRKP isolates, 94 (68.6%) and 78 (56.9%) isolates were resistant to gentamicin and amikacin. All 78 amikacin-resistant isolates were concomitantly resistant to gentamicin and positive for 16S rRNA methylase genes. Relative to 16S RMTases-negative isolates, 16S RMTases-positive isolates were more sensitive to ceftazidime/avibactam, tetracycline, minocycline and sulfamethoxazole, but had higher resistance rates to aztreonam, gentamicin, amikacin and ciprofloxacin. All 78 isolates positive for 16S RMTases genes were resistant to clinically often used antimicrobial agents, including imipenem, meropenem, cefoxitin, cefotaxime, cefepime, ceftazidime, aztreonam, gentamicin and amikacin, but more sensitive to ceftazidime/avibactam, polymyxin B and tigecycline. All 16S rRNA methylase gene-positive isolates had high levels of resistance to gentamicin and amikacin (MICs ≥256 μg/mL) (Table 2).

Capsular Serotyping of 16S RMTases-Positive and -Negative CRKP Isolates

Of 78 16S RMTases-positive isolates, 6 capsular serotypes were identified, including KL64-wzi64 (70.5%, 55/78), KL47-wzi209 (20.5%, 16/78), KL19-wzi19 (5.1%, 4/78), KL3-wzi59 (1.3%, 1/78), KL27-wzi27 (1.3%, 1/78), and KL24-wzi101 (1.3%, 1/78). A total of 11 capsular serotypes were found in 59 16S RMTases-negative strains, including KL64-wzi64 (35.6%, 21/59), KL24-wzi101 (11.9%, 7/59), and KL21-wzi21 (10.2%, 6/59), KL47-wzi209 (10.2%, 6/59), KL48-wzi151 (5.1%, 3/59), KL102-wzi173 (5.1%, 3/59), KL19-wzi19 (3.4%, 2/59), KL67-wzi198 (3.4%, 2/59), KL2-wzi72 (1.7%, 1/59), KL37-wzi37 (1.7%, 1/59), and KL74-wzi174 (1.7%, 1/59). Six 16S RMTases-negative isolates (10.2,6/59) were not typed successfully. The results showed that compared with 16S RMTases-negative strains, 16S RMTases-positive strains had fewer types of capsular serotypes and more concentrated distribution. In all 16S RMTases-positive and -negative CRKP strains, the major capsular serotype were KL64-wzi64. However, more 16S RMTases-positive isolates (70.5%) belonged to KL64-wzi64 relative to 16S RMTases-negative isolates (35.6%).

Molecular Characteristics for 16S RMTases-Positive and -Negative CRKP Isolates

Multi-locus sequencing typing (MLST) was performed on all 16S RMTases-positive and -negative CRKP isolates. Among 78 16S RMTases-positive isolates tested, 5 STs were identified. ST11 was the most prevalent ST (92.3%, 72/78), followed by ST15 (2.6%, 2/78) and ST2237 (2.6%, 2/78). ST45

Table 1 Antibiotic Resistance Gene Profiles in 16S RMTases-Positive and -Negative Strains

CRKPs	Carbapenem Resistance Genes, n (%)					ESBL Genes, n (%)		
	bla_{NDM}	bla_{KPC}	bla_{IMP}	bla_{NDM}+bla_{KPC}	bla_{IMP}+bla_{KPC}	bla_{CTX-M}	bla_{SHV}	bla_{CTX-M}+bla_{SHV}
16S+(n=78)	1 (1.3)	69 (88.5)	0	7 (8.9)	1 (1.3)	38 (48.7)	5 (6.4)	31 (39.7)
16S-(n=59)	24 (40.7)	31 (52.5)	2 (3.4)	2 (3.4)	0	24 (40.7)	4 (6.8)	16 (27.1)
P-values	0.00	0.00	0.184	0.299	1.00	0.349	1.00	0.123

Notes: 16S+: Represents the 16S RMTases-positive CRKP strains. 16S-: Represents the 16S RMTases-negative CRKP strains. +: Represents one CRKP strain harboring two antibiotic resistance genes simultaneously. P <0.05 was considered statistically significant.

Table 2 The Antimicrobial Resistance Profiling of 16S RMTases-Positive and -Negative Strains

Antimicrobials	CRKPs (n=137)				P-values	Breakpoints (μg/mL)		
	16s+ (n=78)	%	16s- (n=59)	%		S	I	R
Imipenem	78	100.0	56	94.9	0.078	≤1	2	≥4
Meropenem	78	100.0	59	100	–	≤1	2	≥4
Cefoxitin	78	100.0	57	96.6	0.184	≤8	16	≥32
Cefotaxime	78	100.0	59	100	–	≤1	2	≥4
Cefepime	78	100.0	58	98.3	0.431	≤2	–	≥16
Ceftazidime	78	100.0	58	98.3	0.431	≤4	8	≥16
Aztreonam	78	100.0	49	83.1	0.000	≤4	8	≥16
Gentamicin	78	100.0	16	27.1	0.000	≤4	8	≥16
Amikacin	78	100.0	0	0	0.000	≤16	32	≥64
Ceftazidime/avibactam	9	11.5	27	45.8	0.000	≤8/4	–	≥16/4
Polymyxin B	5	6.4	1	1.7	0.236	–	≤2	≥4
Tigecycline	2	2.6	6	10.2	0.075	≤1	–	≥2
Piperacillin/tazobactam	77	98.7	56	94.9	0.315	≤16/4	32/464/4	≥128/4
Ciprofloxacin	74	94.9	39	66.1	0.000	≤0.06	0.12–0.5	≥1
Tetracycline	34	43.6	36	61	0.016	≤4	8	≥16
Minocycline	25	32.1	29	49.2	0.043	≤4	8	≥16
Sulfamethoxazole	38	48.7	39	66.1	0.042	≤2/38	–	≥4/76

Notes: 16S+: Represents the 16S RMTases-positive CRKP strains. 16S-: Represents the 16S RMTases-negative CRKP strains. P <0.05 was considered statistically significant.
Abbreviations: S, susceptible; I, intermediate; R, resistant.

and ST395 were found among only one isolate. Among 59 16S RMTases-negative strains, a total of 14 sequence types (STs) were identified, including ST11 (45.8%, 27/59), ST45 (11.9%, 7/59), ST290 (11.9%, 7/59), and ST15. (8.5%, 5/59), ST438 (3.4%, 2/59), ST1319 (3.4%, 2/59), ST307 (3.4%, 2/59), ST1692 (1.7% 1/59), ST375 (1.7% 1/59), ST35 (1.7% 1/59), ST107 (1.7% 1/59), ST485 (1.7% 1/59), ST2390 (1.7% 1/59), and ST2236 (1.7% 1/59). The results showed that 16S RMTases-positive strains had fewer types of STs and more concentrated distribution relative to the isolates negative for RMTases genes. In all 16S RMTases-positive and -negative CRKP strains, the predominant ST was ST11. However, more 16S RMTases-positive isolates (92.3%) belonged to ST11 clonal strains relative to 16S RMTases-negative isolates (45.8%).

PFGE results revealed seven distinct clusters (cluster A to cluster G). Among them, 72 ST11 isolates were divided into four different PFGE clusters, including A clusters (5/78, 6.4%), B clusters (62/78, 79.5%), C clusters (3/78, 3.8%) and D clusters (2/78, 2.6%). Two ST2237 and two ST15 isolates belong to the same cluster (cluster F). Each of the remaining two isolates formed a singleton (cluster E and cluster G). (Figure 1)

Characteristics of Different Clonetypes in 16S RMTases-Positive Strains

Among 78 16S RMTases-positive strains, 11 different clonal strains were identified, including ST11-PFGE-A-KL64-wzi64 (3/78, 3.8%), ST11-PFGE-A-KL47-wzi209 (2/78, 2.6%), ST11-PFGE-B-KL64-wzi64 (49/78, 62.8%), ST11-PFGE-B-KL47-wzi209 (12/78, 15.4%), ST11-PFGE-B-KL27-wzi27 (1/78, 1.3%), ST11-PFGE- C-KL64-wzi64 (3/78, 3.8%), ST11-PFGE-D-KL47-wzi209 (2/78, 2.6%), ST15-PFGE-F-KL19-wzi19 (2/78, 2.6%), ST45-PFGE-G-KL24-wzi101 (1/78, 1.3%), ST395-PFGE-E-KL3-wzi59 (1/78, 1.3%), and ST2237-PFGE-F -KL19-wzi19 (2/78, 2.6%).

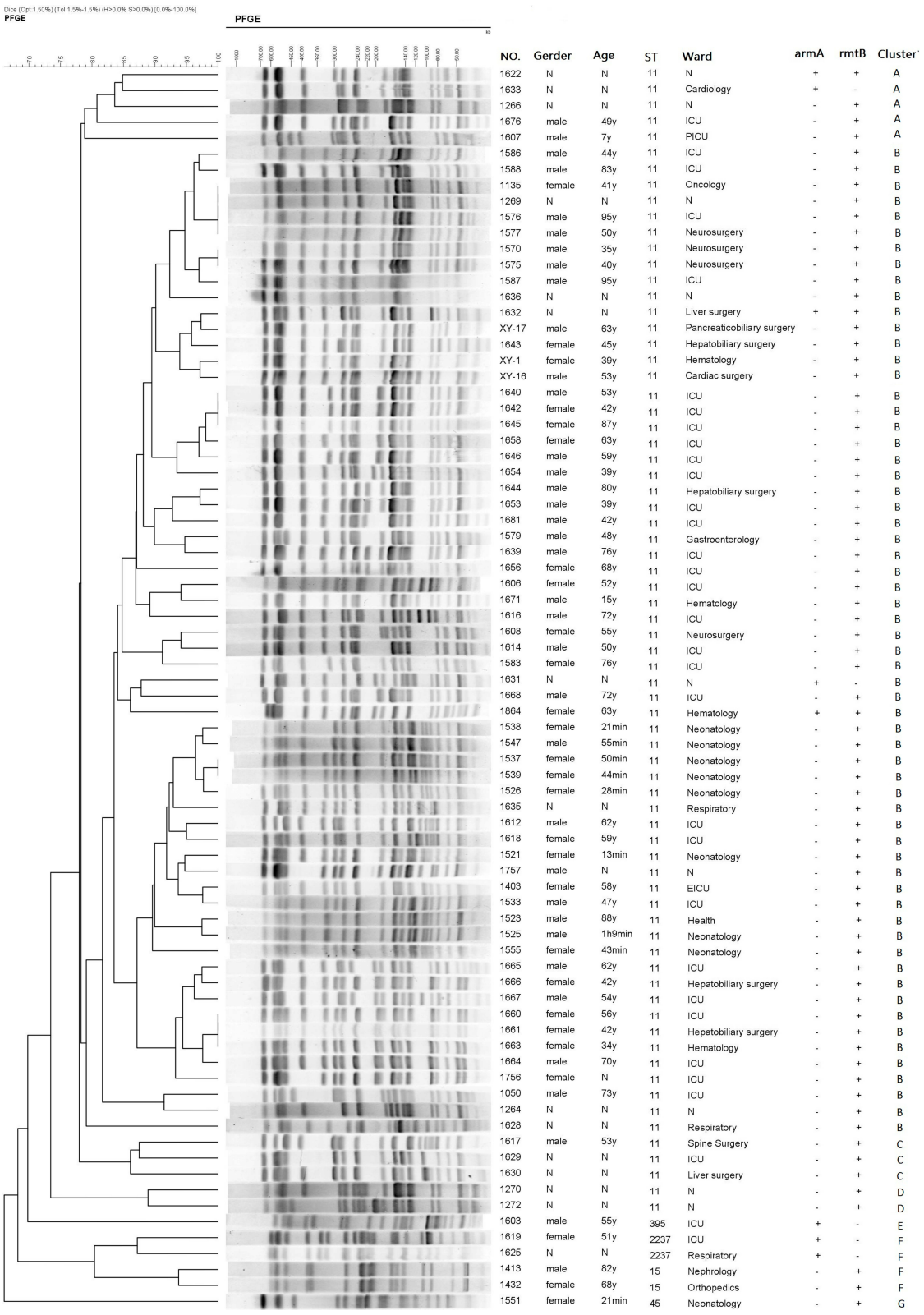

Figure 1 PFGE results for 78 CRKP isolates harboring 16S rRNA methyltransferase genes. The adjacent information shown on the right represents number of strains, gender, age, ST type, ward, armA and rmtB presence, and cluster, respectively.

Abbreviations: PFGE, pulsed-field gel electrophoresis; CRKP, carbapenem-resistant *Klebsiella pneumoniae*.

Table 3 Characteristics of Different Clonotypes in 16S RMTases-Positive Strains

Clonotypes (n=78)	16S rRNA methyltransferase genes			Distribution Areas
	armA	rmtB	armA +rmtB	
ST11-PFGE-A-KL64-wzi64 (n=3)	1	1	1	SH (n=2), JX (n=1)
ST11-PFGE-A-KL47-wzi209 (n=2)	0	2	0	FJ (n=1), HN (n=1)
ST11-PFGE-B-KL64-wzi64 (n=49)	1	48	0	JX (n=18), SD (n=10), HB (n=7), SH (n=6), HN (n=4), ZJ (n=2), FJ (n=2)
ST11-PFGE-B-KL47-wzi209 (n=12)	0	10	2	HN* (n=3), JX (n=3), HN (n=2), HB (n=2), SH (n=1), ZJ (n=1)
ST11-PFGE-B-KL27-wzi27 (n=1)	0	1	0	ZJ (n=1)
ST11-PFGE-C-KL64-wzi64 (n=3)	0	3	0	SH (n=2), HN (n=1)
ST11-PFGE-D-KL47-wzi209 (n=2)	0	2	0	FJ (n=2)
ST15-PFGE-F-KL19-wzi19 (n=2)	0	2	0	ZJ (n=2)
ST45-PFGE-G-KL24-wzi101 (n=1) ST395-PFGE-E-KL3-wzi59 (n=1)	0 1	1 0	0 0	SD (n=1) HN (n=1)
ST2237-PFGE-F-KL19-wzi19 (n=2)	2	0	0	SH (n=1), HN (n=1)

Note: [+]Represents one CRKP strain harboring two antibiotic resistance genes simultaneously.
Abbreviations: SH, Shanghai; JX, Jiangxi; FJ, Fujiang; HN, Henan; SD, Shandong; HB, Hubei; ZJ, Zhejiang; HN*, Hunan.

ST11-PFGE-B-KL64-wzi64 (62.8%) is the most prevalent clone strains, distributed in 7 provinces in China, including Jiangxi (n = 18), Zhejiang (n = 2), Fujian (n = 2), and Shandong (n = 10), Hubei (n = 7), Henan (n = 4), and Shanghai (n = 6). The main prevalent clonal strains of ST11-PFGE-B-KL64-wzi64, mainly carrying the rmtB (48/49, 98%) gene.(Table 3)

Discussion

The emergence of CRKPs poses an urgent threat to public health worldwide.[21,22] BSI caused by CRKP is a more serious problem due to ineffective antibiotics and high mortality.[23–26] Clinically, therapeutic options against CRKP infections are limited. Aminoglycosides are still important companion antibiotics in combination therapy.[27] However, 16S RMTases have been found to mediate high levels of resistance to all clinically relevant aminoglycosides, such as amikacin, tobramycin and gentamicin.[10] To the best of our knowledge, this is the first report of describing the prevalence of 16S RMTase genes in CRKP clinical isolates associated with BSIs in several Chinese teaching hospitals.

To date, three plasmid-encoded 16S RMTase, including ArmA, RmtB and RmtC, have been found in clinical isolates of Gram-negative bacilli in China.[28,29] The overall prevalence (56.9%) of 16S RMTase genes in CRKP Clinical isolates in the present study is much higher than what was found in Greece.[30] Surprisingly, the prevalence of 16S RMTase genes in CRKP isolates has reached 75% reported by a teaching hospital in northeast China in 2019.[31] The difference in the prevalence of 16S RMTase genes may be due to the type of specimens collected. This study shows that rmtB is the most prevalent gene among the 16S RMTase genes identified, which is consistent with previous studies.[31,32] In addition, among 78 isolates positive for 16S RMTase genes, 72 (72/78, 92.3%) also harbored one or more AME genes. Due to the coexistence of these genes, this will make it difficult to predict the aminoglycoside resistance profile based on the effects of a single gene. Most (96.2%; 75/78) of the 16S RMTase-producing isolates in our collection were extended-spectrum β-lactamase (ESBL) producers and each isolate harbored at least one carbapenemase gene. These results indicate that a strong association between these resistance mechanisms. Therefore, the treatment of 16S RMTase-producing CRKP isolates with aminoglycoside and beta-lactam antibiotic combinations may have lost its clinical significance. Among contemporary CRKP clinical isolates from China, where KPC-producing *K. pneumoniae* remain

predominant, followed by NDM-producing *K. pneumoniae*. The results are similar to those reported in previous studies of CRKP clinical isolates from different geographic regions in China.[33–35] In this study, 78 16S RMTases positive strains were identified with 5 STs and 6 capsule serotypes. Compared with 16S RMTases-negative strains, the STs and capsular serotypes of 16S RMTases-positive strains are more concentrated, which may be caused by the dissemination of clone strains. In all 16S RMTases-positive and -negative CRKP strains, the major clonotype was ST11-KL64-wzi64, which is in accordance with previous study. A tertiary hospital in China reported the dominant clone ST11 CRKPs has undergone subclonal shift, of which the previously prevalent capsule locus (KL) 47 has been replaced by KL64.[36] PFGE results showed that most (62/78, 79.5%) 16S RMTase-producing CRKP isolates belong to cluster B, indicating that horizontal gene transfer and clonal spread were responsible for the dissemination of armA and rmtB genes. Among 78 16S RMTases-positive strains, the most prevalent clone type is ST11-PFGE-B-KL64-wzi64 (62.8%, 49/78), which mainly carries the rmtB and bla_{KPC} genes and is distributed in 7 provinces in China. These results indicate that the ST11-PFGE-B-KL64-wzi64 CRKP clone strains may be spread in China, resulting in multiple resistance to aminoglycosides and other antibiotics.

In conclusion, a high prevalence of 16S RMTase genes was found among CRKP clinical isolates associated with BSIs in Chinese teaching hospitals. Dissemination of 16S RMTase genes among multidrug resistance (MDR) isolates is an unwelcome event. Therefore, intense molecular surveillance is thus imperative to inform strategies for containment.

Ethics Statement

Because the *Klebsiella pneumoniae* isolates in this study were part of the routine hospital laboratory procedure, the Ethics Committee of the first affiliated hospital of Wenzhou Medical University exempted this research for review.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

All authors declare that they have no conflicts of interest in this work.

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