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Original Article

Antimicrobial Susceptibility and Molecular Epidemiology of Multidrug-Resistant *Klebsiella pneumoniae* in Central China

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SUMMARY: Extended-spectrum-beta-lactamase-producing and carbapenemase-producing *Klebsiella pneumoniae* strains have rapidly spread through clinical units worldwide. This study investigated the epidemiology and resistance profiles of *K. pneumoniae* strains isolated in central China between 2009 and 2014. Antimicrobial susceptibility testing and polymerase chain reaction were used to investigate the prevalence of extended-spectrum beta-lactamases (ESBL) and carbapenemase production by these *K. pneumoniae* strains, and the prevalence of *K. pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* was investigated by multilocus sequence typing. Carbapenem resistance has emerged as a major concern in *K. pneumoniae* infections, as phenotype testing has detected carbapenemases in nearly 20% of isolates. KPC-producing isolates in a local epidemic were clonally related, with ST11 being the reservoir for the $bla_{\text{KPC-2}}$ gene and ESBL genes. During the 6-year collection period, the prevalence of ESBLs was dynamic, and suggested that $bla_{\text{CTX-M-55}}$ might become prevalent in the future. Our findings demonstrate the high prevalence of carbapenemase- and ESBL-producing *K. pneumoniae* in central China and predict a future local epidemic of KPC-2 and CTX-M-55.

INTRODUCTION

Klebsiella pneumoniae has emerged as a clinically and epidemiologically important human pathogen due to its ability to persist in clinical environments and acquire antibiotic resistance. *K. pneumoniae* isolates can persist on environmental surfaces and on human skin as well as within the respiratory and urinary tracts, and it can be easily transferred between patients via clinical operations and examinations (1). *K. pneumoniae* has become one of the most frequent outbreaks in intensive care units (2), and carbapenem-resistant *K. pneumoniae*, which has spread in some areas of the world, is a major threat (3,4).

A common mechanism of beta-lactam resistance involves extended-spectrum beta-lactamase (ESBL) enzymes. CTX-M enzymes, or plasmid-mediated cefotaximases, are divided into 7 clusters based on their phylogeny. CTX-M enzymes have a significant clinical impact (5). The epidemic situation, antibiotic resistance, and genotype distribution of CTX-M type ESBL enzymes vary among countries, at different time periods, and especially according to bacterial species. Although large nationwide surveillance studies of ESBL-producing *Escherichia coli* were recently conducted in China (6–12), studies on *K. pneumoniae* have mainly focused on carbapenem resistance. However, one study showed that detection rates of CTX-M-producing *K. pneumoniae* isolates differ significantly among hospitals in the same region (13).

Carbapenems are used as a last resort in the treatment of infections caused by multi-resistant gram-negative bacteria, including ESBL-producing isolates. However, *K. pneumoniae* carbapenemase (KPC)-producing isolates are becoming increasingly prevalent. KPC-producing *K. pneumoniae* isolates have been widely detected in China, and an outbreak of bla_{KPC}-containing *K. pneumoniae* occurred in Zhejiang province in eastern China (14). Previous studies have demonstrated the presence of the bla_{KPC} gene in all of these isolates, and sequencing has revealed that they carry the same KPC-2 allele. After KPC-2, NDM-1 is the second most important carbapenemase in *K. pneumoniae*. In addition, IMP, as well as other metalloenzymes, have been detected sporadically (15).

The purpose of this study was to investigate the antibiotic resistance mechanism of multidrug-resistant *K. pneumoniae* in central China and to examine the antimicrobial susceptibility and epidemiologic properties of the isolates obtained over a 6-year period.

MATERIALS AND METHODS

Setting and study design: This research was conduct-

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ed at the First Affiliated Hospital of Zhengzhou University, one of the largest hospitals in central China, with approximately 7,000 beds. We performed a retrospective study of *K. pneumoniae*-infected patients from January 2009 to December 2014. The infectious isolates were mainly community acquired, and the infected patients resided in different cities or counties of central China. Only the isolates obtained from October to December in 2010, 2012, and 2014 were collected and stored for molecular experiments because the data for these 3 time periods were intact and sufficient. No repetitive isolates from a single patient were included. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Antimicrobial susceptibility testing: The antimicrobial susceptibility of all isolates was tested using Vitek II GNI+ cards, and the carbapenem-resistant isolates were re-screened using the Kirby Bauer disk diffusion method. The tested antibiotics were cefazolin (CFZ), cefuroxime (CXM), ceftriaxone (CRO), cefotaxime (CTX), piperacillin (PIP), sulfamethoxazole (SXT), amoxicillin, gentamicin (GEN), aztreonam (ATM), ceftazidime (CAZ), ciprofloxacin (CIP), cefoxitin, cefepime (FEP), minocycline, levofloxacin, amikacin (AMK), PIP-tazobactam, cefoperazone/sulbactam, ertapenem, imipenem (IPM), and meropenem (MEM). The MIC were determined according to the Clinical and Laboratory Standards Institute (CLSI) 2014 guidelines (16). The antibiotics were provided by AB Biodisk (Solna, Sweden), and *K. pneumoniae* strain ATCC 700603 was used as the control strain.

Screening for ESBL-producing strains and carbapenemase-producing strains: The disk diffusion method was used to identify ESBL-producing *K. pneumoniae* strains using CTX (30 μg) and CAZ (30 μg) as well as these 2 antibiotics in combination with clavulanic acid (10 μg) according to the CLSI guidelines (16). The suspected carbapenemase-producing *K. pneumoniae* isolates were examined with the Modified Hodge Test, and the results were interpreted according to the CLSI standards. Additional genotypes were confirmed by PCR.

E. coli strain ATCC 25922 was used as the susceptible control strain, *K. pneumoniae* strain ATCC 700603 was used as the ESBL-producing positive control strain, and *K. pneumoniae* strain A1500 was used as the carbapenemase-producing control strain.

Nucleic acid extraction: Genomic DNA was extracted from *K. pneumoniae* isolates with the Ezup Column Bacteria Genomic DNA Purification Kit (Sangon, Shanghai, China) according to the manufacturer's instructions, and the DNA solution was stored at −20°C.

PCR detection and sequencing of ESBL and carbapenemase genes: To determine the ESBL and carbapenemase genotypes of the isolates, we performed a polymerase chain reaction targeting the ESBL type genes bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-1}}$, and $bla_{\text{CTX-M}}$ and the carbapenemase genes bla_{NDM}, bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{GIM}, and $bla_{\text{OXA-48}}$.

The primers used are shown in Table 1. The amplified genes were sequenced by Sangon Biotech (Shanghai, China), and the DNA sequences were confirmed with BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/).

Multilocus sequence typing of KPC-2-producing *K. pneumoniae*: We performed multilocus sequence typing (MLST, http://www.mlst.net/) of all 34 KPC-2-producing *K. pneumoniae* isolates, including 2 strains detected in 2010, 13 strains detected in 2012, and 19 strains detected in 2014 (detected by previous PCR tests). Seven housekeeping genes were amplified and sequenced according to the protocol described on the *K. pneumoniae* MLST website.

RESULTS

Prevalence of ESBL-producing and carbapenemase-producing *K. pneumoniae*: From January 2009 to December 2014, a total of 74,729 isolates were collected and tested in the First Affiliated Hospital of Zhengzhou University, China. Of these, 8,203 isolates were classified as *K. pneumoniae* by the Vitek II compact system (bioMérieux, Marcy l'Étoile, France).

The isolates were obtained from the respiratory tract, blood, sputum, urine, cerebrospinal fluid, and so on, and the distribution of the *K. pneumoniae* strains is shown in Table 2. Throughout the study period, the detection rate of *K. pneumoniae* remained constant at 10%, whereas the detection rate of gram-negative strains decreased slightly over time. The prevalence of ESBL-producing *K. pneumoniae* exhibited a decreasing pattern, with a relatively low rate of 31.3% in 2014. Since the first detection of KPC-2-producing *K. pneumoniae* in 2011, the detection rate of carbapenemase-positive isolates has increased, and it was nearly 20% in 2014.

We collected 94 ESBL-producing isolates from October to December 2010, 76 ESBL-producing isolates and 11 carbapenemase-producing isolates from October to December 2012, and 72 ESBL-producing isolates and 19 carbapenemase-producing isolates from October to December 2014. All isolates were stored at −80°C for molecular experiments.

Antimicrobial susceptibility: All 8,203 *K. pneumoniae* isolates were tested for antimicrobial susceptibility, and the results are shown in Fig. 1. *K. pneumoniae* resistance to PIP, which was used as a representative of semi-synthetic penicillin, increased from 74% in 2009 to 99.5% in 2014. In contrast, *K. pneumoniae* susceptibility to first and second generation cephalosporins (CFZ and CXM) showed an obvious and consistent increase. *K. pneumoniae* susceptibility to third and fourth generation cephalosporins (CTX, CAZ, CRO, and FEP) increased from 2009 to 2011, decreased in 2012, and then increased dramatically in 2013 and 2014. Before 2012, the rate of *K. pneumoniae* resistance to IPM and MEM was less than 1%; however, resistance increased in successive years, which was accompanied by an increase in

Table 1. Primers of ESBL and carbapenemase type genes

Gene	Primer	sequence(5'→3')	Annealing temp(℃)	Fragment(bp)
$bla_{\text{CTX-M-1}}$	M-1F	CAGCGCTTTTGCCGTCTAAG	52	946
	M-1R	GGCCCATGGTTAAAAAATCACTGC		
$bla_{\text{CTX-M-2}}$	M-2F	ATGATGACTCAGAGCATTCG	54	866
	M-2R	TGGGTTACGATTTTCGCCGC		
$bla_{\text{CTX-M-8}}$	M-8F	ACTTCAGCCACACGGATTCA	52	878
	M-8R	CGAGTACGTCACGACGACTT		
$bla_{\text{CTX-M-9}}$	M-9F	ATGGTGACAAAGAGAGTGCA	54	870
	M-9R	CCCTTCGGCGATGATTCTC		
$bla_{\text{CTX-M-25}}$	M-25F	ATGATGAGAAAAAGCGT	55	876
	M-25R	TTAATAACCGTCGGTGAC		
bla_{TEM}	TEM-F	CATTTCCGTGTCGCCCTTATTC	56	800
	TEM-R	CGTTCATCCATAGTTGCCTGAC		
bla_{SHV}	SHV-F	AGCCGCTTGAGCAAATTAAAC	55	713
	SHV-R	ATCCCGCAGATAAATCACCAC		
$bla_{\text{OXA-1}}$	OXA-1F	GGCACCAGATTCAACTTTCAAG	55	564
	OXA-1R	GACCCCAAGTTTCCTGTAAGTG		
$bla_{\text{OXA-10}}$	OXA-10F	CCACCAAGAAGGTGCCATGA	55	835
	OXA-10R	GCGACCTTGAGCGACTTGTT		
bla_{NDM}	NDM-F	CACCTCATGTTTGAATTCGCC	58	984
	NDM-R	CTCTGTCACATCGAAATCGC		
bla_{KPC}	KPC-F	ATGTCACTGTATCGCCGTCT	55	893
	KPC-R	TTTTCAGAGCCTTACTGCCC		
bla_{VIM}	VIM-F	GTCTATTTGACCGCGTC	60	774
	VIM-R	CTACTCAACGACTGAGCG		
bla_{IMP}	IMP-F	TCACATTTCCATAGCGACAG	50	450
	IMP-R	AGTGGTACTTTTTTTGCTTTCAT		
bla_{SPM}	SPM-F	CTGCTTGGATTCATGGGCGC	55	784
	SPM-R	CCTTTTCCGCGACCTTGATC		
bla_{GIM}	GIM-F	TTGCCAGCTTTAGCTCAGGGTC	60	713
	GIM-R	TAATCAGCCGACGCTTCAGCGG		
$bla_{\text{OXA-48}}$	OXA-48F	ATGCGTGTATTAGCCTTATC	50	798
	OXA-48R	CTAGGGAATAATTTTTTCCT		

Table 2. The epidemiology of ESBL-producing and carbapenemase-producing. *K. pneumoniae* isolates from 2009 to 2014

	2009	2010	2011	2012	2013	2014
Total isolates						
n	6,986	6,892	16,340	14,480	15,575	14,456
Geam-negative isolates						
n_1	4,928	5,108	11,875	9,237	10,581	9,409
$(n_1/n)\%$	70.5	74.1	72.7	63.8	67.9	65.1
Klebsiella pneumoniae						
n_2	764	719	1,771	1,696	1,656	1,597
$(n_2/n)\%$	10.9	10.4	10.8	11.6	10.6	11.1
ESBL-producing *Klebsiella pneumoniae*						
n_3	366	318	708	618	568	500
$(n_3/n_2)\%$	47.9	44.2	39.9	36.4	34.3	31.3
Carbapenemase-producing *Klebsiella pneumoniae*						
n_4	0	2	7	90	143	316
$(n_4/n_2)\%$	0.0	0.3	0.4	5.0	8.6	19.8

carbapenemase production.

Molecular epidemiology of antibiotic resistance genes: As shown in Table 3, isolates with an ESBL or carbapenemase phenotype were tested for the presence of resistance genes. In 2014, 97% of the isolates harbored the bla_{SHV} gene, which seems to be the main resistance gene in this area. The second most common resistance gene was bla_{TEM} (71.6%). The detection rate of $bla_{\text{OXA-1}}$ increased from 6.4% in 2010 to 20.9% in 2014. There were 49 CTX-M ESBL-producing *K. pneumoniae* isolates (49/67, 73.1%) in 2014, and the percentages detected in 2010 and 2012 were higher at 89.4% and 85.5%, respectively. The prevalence of $bla_{\text{CTX-M-1}}$-positive isolates (31/67, 46.3%) exceeded that of

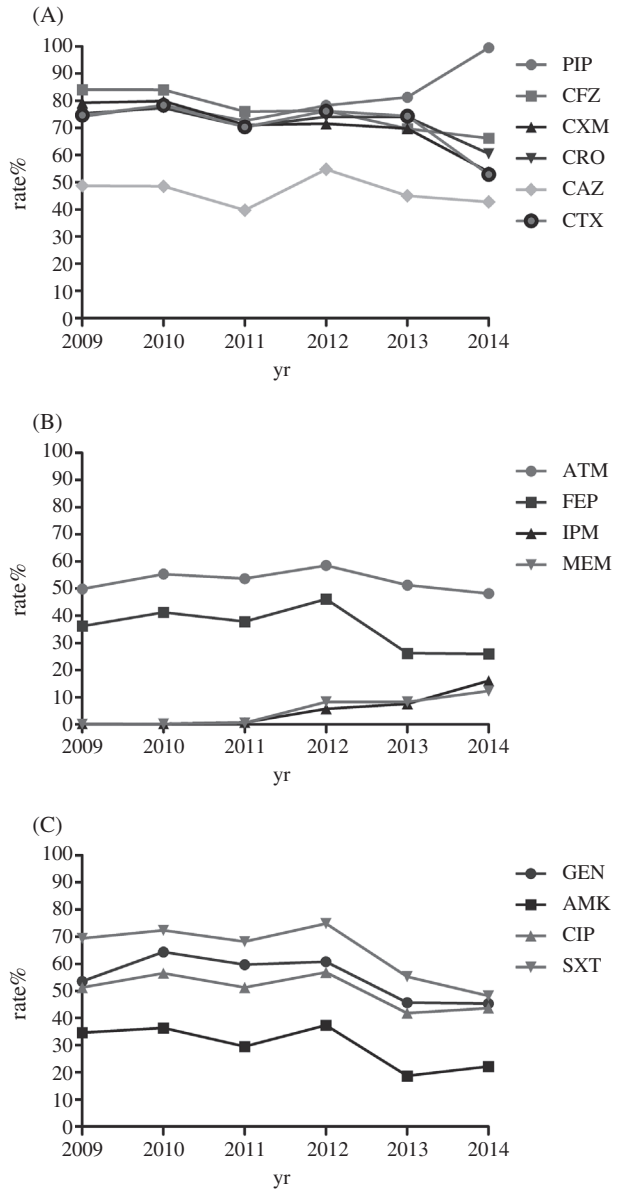

Fig. 1. Antimicrobial susceptibilities of *K. pneumoniae* isolates to commonly used antibiotics from 2009 to 2014. (A) PIP and commonly used cephalosporins (second and third generation antibiotics). (B) The fourth generation cephalosporins ATM and carbapenem. (C) Commonly used non-beta-lactam antimicrobials. Abbreviations for the antibiotics are as follows: piperacillin (PIP), cefazolin (CFZ), cefuroxime (CXM), ceftriaxone (CRO), ceftazidime (CAZ), cefotaxime (CTX), aztreonam (ATM), cefepime (FEP), imipenem (IPM), meropenem (MEM), gentamicin (GEN), amikacin (AMK), ciprofloxacin (CIP), and sulphamethoxazole (SXT).

the $bla_{\text{CTX-M-9}}$-positive isolates (24/67, 35.8%), and $bla_{\text{CTX-M-1}}$ was the most frequently detected $bla_{\text{CTX-M}}$ gene in 2014. In contrast, $bla_{\text{CTX-M-25}}$ was detected in 2010 (6.4%), but was not detected in 2012 or 2014. CTX-M-55, a variant of CTX-M-15 resulting from a single amino acid substitution (valine for alanine) at position 80, was detected for the first time in 2014, and it accounted for 4.5% of the CTX-M isolates detected, which was third most common after CTX-M-3 (32.8%) and CTX-M-15 (9.0%). Interestingly, CTX-M-65 was also first detected

Table 3. Molecular characterization of ESBL-and carbapenemases-producing *K. pneumoniae* isolates in 2010, 2012, and 2014

genotype	2010 (n = 94)		2012 (n = 76)		2014 (n = 67)	
	n	%	*n*	%	*n*	%
TEM	74	78.7	54	71.1	48	71.6
SHV	56	59.6	70	92.1	65	97.0
OXA-1	6	6.4	11	14.5	14	20.9
CTX-M-3	27	28.7	23	30.3	11	16.4
CTX-M-15	3	3.2	5	6.6	5	7.5
CTX-M-55	0	0.0	0	0.0	3	4.5
CTX-M-9	5	5.3	3	3.9	1	1.5
CTX-M-14	30	31.9	22	28.9	12	17.9
CTX-M-27	0	0.0	2	2.6	3	4.5
CTX-M-25	6	6.4	0	0.0	0	0.0
CTX-M-3+CTX-M-14	19	20.2	7	9.2	2	3.0
CTX-M-15+CTX-M-14	0	0.0	0	0.0	1	1.5
NDM-1	0	0.0	3	3.9	1	1.5
KPC-2	2	2.1	11	14.5	1	1.5
CTX-M-3+NDM-1	0	0.0	1	1.3	1	1.5
CTX-M-3+KPC-2	0	0.0	0	0.0	5	7.5
CTX-M-14+KPC-2	0	0.0	2	2.6	2	3.0
CTX-M-65+KPC-2	0	0.0	0	0.0	8	11.9
CTX-M-3+CTX-M-65+KPC-2	0	0.0	0	0.0	3	4.5

Table 4. Genotypic characteristics of KPC-2-producing *Klebsiella pneumoniae* isolates

Sequence type (ST)	yr	isolate	genotype
ST11	2012/2014		
		12	KPC-2
		4	CTX-M-3+KPC-2
		2	CTX-M-14+KPC-2
		5	CTX-M-65+KPC-2
		2	CTX-M-3+CTX-M-65+KPC-2
ST15	2012/2014		
		1	CTX-M-3+KPC-2
		2	CTX-M-14+KPC-2
		3	CTX-M-65+KPC-2
		1	CTX-M-3+CTX-M-65+KPC-2
ST438	2010		
		2	KPC-2

in 2014, and $bla_{\text{CTX-M-65}}$ was found to co-occur with $bla_{\text{KPC-2}}$. The detection rate for $bla_{\text{KPC-2}}$ was 2.1% in 2010 but increased dramatically to 28.4% in 2014. NDM-1 was another carbapenemase that was detected sporadically in *K. pneumoniae* isolates in central China. However, none of the isolates carried 2 carbapenemase genes.

MLST of KPC-producing *K. pneumoniae*: The MLST results for the 34 KPC-producing *K. pneumoniae* isolates are shown in Table 4. In 2010, 2 KPC-producing *K. pneumoniae* isolates were detected in Henan province, and these isolates were determined to be ST438 by MLST testing. No ESBL-type gene was detected in these 2 isolates. Nevertheless, ST11 replaced ST438 as the most widely detected sequence type in subsequent years, and no ST438-type *K. pneumoniae* isolates have been detected since 2010. ST11 and ST15, which ac-

count for nearly 90% of *K. pneumoniae* isolates, not only contained bla_{KPC-2} but also carried other ESBL-type genes.

DISCUSSION

Our study shows that the frequency of antimicrobial resistance in *K. pneumoniae* isolates is increasing at an alarming rate. In addition, 2 different trends in β-lactam antibiotic resistance were revealed: (i) *K. pneumoniae* resistance to PIP, IPM, and MEM has increased enormously over the study period. (ii) Clinical bacteria appear to have become more "sensitive" to the commonly used cephalosporins (especially second and third generation antibiotics) than in the past. This may seem counterintuitive to the general thought that the increasing incidence of KPC would be coupled with increased susceptibility to cephalosporins. One possible explanation is that KPC production severely reduces bacterial fitness in patients with non-carbapenem-resistant *K. pneumoniae*, making it very likely that this type of bacterium undergoes partial or full deletion of the sequences encoding antibiotic resistance enzymes after a few rounds of replication. However, it does not mean that clinical isolates are now more susceptible to cephalosporins, but it does suggest that there is a decreased proportion of cephalosporin-resistant *K. pneumoniae* isolates. However, owing to the lack of nationwide surveillance studies for *K. pneumoniae*, additional research is needed to detect the specific enzymes present in these isolates.

The emergence and dissemination of ESBL and carbapenemase genes in *K. pneumoniae* isolates worldwide poses a considerable threat to public health (17,18). The detection rate of ESBL-producing *K. pneumoniae* dropped from 47.9% in 2009 to 31.3% in 2014. This finding is identical to the results of the molecular tests; PCR showed a continuously decreasing trend in the presence of ESBL-coding genes (bla_{CTX-M} and bla_{TEM}). CTX-M-3 was found to be the major CTX-M-1 group ESBL, followed by CTX-M-15 and CTX-M-55. However, previous studies have shown a completely different distribution for CTX-M-1 group beta-lactamases, and CTX-M-55 has already replaced CTX-M-3 as the main concern with regard to antibiotic treatment options for infections in mainland China (7,11,19). The rising proportion of $bla_{CTX-M-55}$ in our study suggests 2 possibilities. One is the possible transmission of pathogens carrying $bla_{CTX-M-55}$ or the transmission of the gene itself from remote locations to local provinces. The other possibility is that the $bla_{CTX-M-55}$ allele may have evolved from $bla_{CTX-M-3}$ or $bla_{CTX-M-15}$ through an Ala-80-Val or Asp-242-Gly substitution, respectively, during transmission in Henan province (20–22). For the latter hypothesis, the low proportion of $bla_{CTX-M-55}$ implies a much slower evolutionary rate for $bla_{CTX-M-1}$ group genes in central China than in other parts of the country.

According to the MLST results, the first 2 KPC-2-producing *K. pneumoniae* isolates identified were ST438. ST11, currently the most prevalent KPC-producing *K. pneumoniae* strain in China (23), was rarely detected before 2010. Furthermore, bla_{CTX-M}, bla_{TEM}, and bla_{SHV} were detected in the ST11 and ST15 clones, but no resistance genes other than bla_{KPC} were detected in the ST438 clones. ST11 clones are more likely to carry several other resistance genes (e.g., ESBL or AmpC) and persist in a complicated clinical environment (23). Therefore, we speculated that the transmission of plasmids or the mobilization of genetic elements rather than the epidemic clone itself was key in the sporadic occurrence of bla_{KPC}. In contrast, the ST11 *K. pneumoniae* clone may serve as a reservoir for the dissemination of KPC-2 in a limited geographical area, such as Henan province. Another interesting finding regarding the co-occurrence of $bla_{CTX-M-65}$ and bla_{KPC-2} in *K. pneumoniae* is that the $bla_{CTX-M-65}$ gene was only detected in KPC-2-producing isolates. Further studies are needed to explore the genetic environment of bla_{KPC-2} and $bla_{CTX-M-65}$ and the mechanism of co-transmission.

In summary, the detection rate of carbapenemase-producing *K. pneumoniae* isolates in central China increased dramatically from 2009 to 2014. ST11 has become the most dominant KPC-producing clone in central China, and the $bla_{CTX-M-55}$ allele may become epidemic in the future. It is very important for public healthcare departments to monitor and report the changes in antimicrobial-resistant isolates, strictly control hospital epidemics, and guide doctors in prescribing the proper antibiotics in case of resistance gene evolution.

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Conflict of interest None to declare.

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