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ORIGINAL RESEARCH

Fecal Carriage and Epidemiology of Carbapenem-Resistant *Enterobacteriaceae* Among Hospitalized Patients in a University Hospital

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Purpose: To determine the prevalence and epidemiology of fecal carriage of carbapenem-resistant *Enterobacteriaceae* (CRE), antimicrobial susceptibility, carbapenemase phenotype/genotype, and the colistin-resistance gene *mcr-1* in a university hospital in China.

Methods: A comprehensive study of the fecal carriage of CRE in 704 patients was performed. Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) were applied to elucidate the molecular epidemiology of the isolates.

Results: In total, 60 CRE were detected in the 704 stool samples (8.5%), including 42 *Klebsiella pneumoniae*, 7 *Escherichia coli*, 3 *Citrobacter freundii*, 3 *Klebsiella oxytoca*, 3 *Enterobacter cloacae*, 1 *Enterobacter aerogenes*, and 1 *Raoultella planticola*. Fifty-five CRE isolates were positive for the carbapenemase phenotype, of which 39 were *Klebsiella pneumoniae* carbapenemase (KPC) producers. Thirty KPC-producing *K. pneumoniae* sequence type (ST) 11 isolates were identified and 28 were grouped into one cluster with a similarity of ≥85%, of predominately intensive care unit (ICU) strains. Three KPC-producing ST1889 strains were isolated from the pediatric ward, all indistinguishable and resistant to tigecycline. All CRE were susceptible to colistin and negative for *mcr-1*.

Conclusion: This study revealed a predominant fecal carriage of the KPC-producing *K. pneumoniae* ST11 clone, with several indistinguishable strain clusters, and the emergence of ST1889 in a Chinese university hospital. This evidence of cross-infection supports the urgent need for the implementation of infection control measures to prevent CRE dissemination.

Keywords: fecal carriage, CRE, *Klebsiella pneumoniae*, KPC, ST11

Introduction

The emergence of *Klebsiella pneumoniae* carbapenemase (KPC) in the USA and New Delhi metallo-β-lactamase (NDM) in India, and their rapid spread across the world, is well documented.[1,2] A European survey has shown distinctive patterns of carbapenem-resistant *Enterobacteriaceae* (CRE) genotypes in different countries.[3,4] However, a survey of clinical isolates across China showed that KPC in *Klebsiella pneumoniae* and NDM in *Escherichia coli* were the dominant genotypes.[5–7] In China, high rates of infection by CRE have been reported recently, mainly due to *K. pneumoniae*.[8] CRE infection has been found to be hard to treat with most antibiotics, except for colistin and tigecycline, which may result in significant mortality.[8] KPC-2-producing *K. pneumoniae* sequence type (ST) 11 has become endemic in some Chinese centers.[9,10]

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The gastrointestinal tract is the most important reservoir for CRE in hospital patients, resulting in cross-transmission and infections.[11] Fecal carriage of CRE has been reported as an important risk factor for developing CRE infection.[12–14] Recently, the emergence of colistin-resistance gene *mcr-1* in China has become a significant concern, especially considering colistin is a valid treatment option for CRE infections.[15] However, few studies of *mcr-1* have been undertaken for fecal CRE isolates. An understanding of the fecal carriage rates of CRE is essential to developing control measures.[16] The prevalence of fecal CRE carriage has been reported widely.[17] However, despite the size of China and the wide spread reports of CRE infections, there are only a few studies of fecal CRE carriage in this country.[12,18] Three hundred and three patients in 2011/2012 were screened in a Fujian hospital, where 8 carbapenemase-producing bacteria were found.[12] The aim of this study was to determine the prevalence of fecal carriage of CRE, antimicrobial susceptibility, carbapenemase phenotype/genotype, and the colistin-resistance gene *mcr-1* in a large Chinese university hospital. The molecular epidemiology of the predominant CRE species was investigated. As screening for CRE is very rarely undertaken in Chinese hospitals, we aimed to assess whether this was a significant reservoir for cross-infection.

Materials and Methods
Study Design
All stool samples submitted for routine analysis at Xiangya Hospital from December 2014 to February 2015 were included in this study. Xiangya Hospital is a large university hospital with 3500 beds (68 wards) in Hunan, China. Stool samples from patients hospitalized for at least 48 h were included, those from outpatients were excluded. For each patient, only the first sample was considered. This study was reviewed and approved by the Ethics Committee of Xiangya Hospital of Central South University in accordance with the Declaration of Helsinki and its amendments or comparable ethical standards. The patient informed consent was waived, because this study only focused on the characteristics of the bacterial isolates and no information on the patients was used.

Bacteria Isolates
CRE screening was performed by using MacConkey agar and 10μgmeropenem discs. Lactose-fermenting colonies from the MacConkey plate with an inhibition zone of ≤26mm were selected for identification (MALDI Biotyper, Bruker, Bremen, Germany) and antimicrobial-susceptibility testing.[19]

Antimicrobial Susceptibility
The minimum inhibitory concentrations (MICs) of antimicrobial agents were analyzed by using a Vitek 2 Compact instrument (bioMérieux, France) (except for colistin and meropenem); colistin-susceptibility analysis was performed by the broth microdilution method; meropenem susceptibility was analyzed by disc diffusion test (Oxoid). *E. coli* strain ATCC 25922 was used for quality control. Results were interpreted using the Clinical and Laboratory Standards Institute (CLSI)[20] breakpoints for all the antimicrobial agents except tigecycline and colistin, which were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[21] breakpoints.

Carbapenemase Phenotypeand Genotype
Confirmation of carbapenemase phenotype was performed by Carba NP test.[22,23] *K. pneumoniae* ATCC BAA-1705 was used as a positive control, and *K. pneumoniae* ATCC BAA-1706 was used as a negative control. Carbapenemase-encoding genes (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{OXA-48}) were identified by PCR, as described elsewhere.[18,24] The PCR primers were as follows: KPC-F (5′-CGTCTAGTTCTGCTGTCTTG-3′) and KPC-R(5′-GG TTTGGCGATCTGGTTTTC-3′); NDM-F(5′-GGTTTGGC GATCTGGTTTTC-3′) and NDM-R(5′-CGGAATGGCTCA TCACGATC-3′); IMP-F(5′-GGAATAGAGTGGCTTAAYT CTC-3′) and IMP-R(5′-CCAAACYACTASGTTATCT-3′); VIM-F(5′-GATGGTGTTTGGTCGCATA-3′) and VIM-R (5′- CGAATGCGCAGCACCAG-3′); OXA-48-F(5′-GCGT GGTTAAGGATGAACAC-3′) and OXA-48-R(5′- CATCA AGTTCAACCCAACCG-3′). Primers CLR5-F (5′-CGGTC AGTCCGTTTGTTC-3′) and CLR5-R (5′-CTTGGTCGGT CTGTA GGG-3′) were used for detecting the presence of colistin-resistance gene mcr-1.[15] PCR products were sequenced (Sangon, Shanghai, China) and then analyzed by using BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Clonal Relatedness of Carbapenemase-Producing *K. pneumoniae*
The clonal relatedness of carbapenemase-producing *K. pneumoniae* isolates was investigated by multilocus sequence typing (MLST) and pulsed-field gel electrophoresis

(PFGE). MLST was performed using the protocol on the Institute Pasteur MLST website (www.pasteur.fr/mlst). In brief, seven housekeeping genes of *K. pneumoniae* were *amplified* by PCR. The primers used are shown in Supplementary Table S1. PCR products were sequenced (Sangon, Shanghai, China) and the sequencing data were then compared to sequences in the database to obtain a ST. Sequences that could not be matched with those in the existing collection were submitted to the MLST database for a new name. PFGE was performed by using XbaI(Takara, Dalian, China) to digest genomic DNA samples from the isolates. The DNA fragments were separated with a CHEF-Mapper XA PFGE system (Bio-Rad, USA). Electrophoresis was performed for 20h at 14°C with pulse times ranging from 6 to 36s at 6 V/cm. The PFGE profiles were analyzed according to the criteria proposed by Tenover et al,[25] using BioNumerics software.

Results
Samples
Among 1156 consecutive stool samples submitted for routine analysis at Xiangya Hospital from December 2014 to February 2015, 107 samples from outpatients, 170 samples from patients hospitalized for less than 48 h, and 175 duplicate samples were ruled out. A total of 704 stool samples were screened for CRE. They originated from 65 of the 68 wards, and 60 stool samples were from the central intensive care unit (ICU), 37 from the traditional Chinese medicine ward, 29 from the pediatric ward, and the rest from other wards.

CRE Isolates
From the 704 stool samples screened, 60 CRE were isolated (8.5%), comprising 42 *K. pneumoniae*, 7 *E. coli*, 3 *Citrobacter freundii*, 3 *Klebsiella oxytoca*, 3 *Enterobacter cloacae*, 1 *Enterobacter aerogenes*, and1 *Raoultella planticola*, originating from 22 of the 65 wards. Among the 60 CRE, 15 CRE isolates were from patients of the central ICU, 6 were from patients of the traditional Chinese medicine ward, and 6 were from patients of the pediatric ward. The remaining 33 CRE isolates were from other wards.

Antimicrobial Susceptibility of CRE
According to CLSI and EUCAST breakpoints, all CRE isolates were resistant to ampicillin, ampicillin/sulbactam, cefazolin, ceftriaxone, and ertapenem, and most CRE were resistant to β-lactam and β-lactam/β-lactamase inhibitor

combinations, including piperacillin/tazobactam (93.4%), cefoxitin (98.3%), cefepime (98.3%),aztreonam (95.0%), meropenem (95.0%), and imipenem (90.0%). Nine (15.8%) isolates were resistant to tigecycline, and 7 were *K. pneumoniae*, of which 4 were isolated from a pediatric ward (Table 1). All CRE isolates were susceptible to colistin.

Phenotypic and Molecular Characterization
Among the 60 CRE, 55 were positive in a carbapenemase phenotype confirmatory CarbaNP test. Carbapenemase genes were detected in 54 CRE, while no carbapenemase gene was found in one *K. pneumoniae* with a carbapenemase phenotype. The 54 carbapenemase-producing CRE were resistant to all three tested carbapenems (imipenem, meropenem, and ertapenem), showing positive correlation between genotype and phenotype. The 6 CRE isolates not producing carbapenemase were only resistant to one or two of the tested carbapenems. KPC (72.2%, 39/54) was the most prevalent carbapenemase, detected in35 *K. pneumoniae*, 2 *E. coli*, 1 *C. freundii*, and 1 *K. oxytoca*; followed by NDM (22.2%, 12/54) and IMP (11.1%, 6/54), including 3 CRE coproducing two different carbapenemase genes. VIM, OXA-48, andMCR-1 encoding genes were not detected (Table 2).

Clonal Relatedness of Carbapenemase-Producing *K. pneumoniae*
MLST analysis resulted in 8 different sequence types (STs) being detected among the 40 KPC-producing *K. pneumoniae* isolates. ST11 was the dominant ST type (75%, 30/40), and all the ST11*K. pneumoniae* carried KPC. Three KPC-producing *K. pneumoniae* isolated in the pediatric ward had the same novel sequence type, designated ST1889 in the MLST database. ST309(n=2) was also detected from the pediatric ward. Other STs were ST1466(n=1), ST307 (n=1), ST414 (n=1), ST208 (n=1), and ST722 (n=1). Nine PFGE clusters were identified at a similarity index of 85% among 40 carbapenemase-producing *K. pneumoniae*. Twenty-eight ST11 *K. pneumoniae* were grouped into one cluster with a similarity of ≥85%, and 13 of them were identical. Three ST1889 *K. pneumoniae* had ≥90%similarity in their PFGE patterns (Figure 1).

Discussion
This study revealed a higher rate of fecal carriage of CRE (8.5%) in hospitalized patients than that reported in a previous study (6.6%) in Fujian, China.[12] In that study,

Table 1 Antimicrobial Susceptibility of Carbapenem-Resistant *Enterobacteriacae* Isolated from Fecal Samples

Antimicrobial	K. Pneumoniae, n=42			E. Coli, n=7			Other Species, n=11			Total, n=60		
	R,n (%)	I, n (%)	S, n (%)	R, n (%)	I, n (%)	S, n (%)	R,n (%)	I, n (%)	S, n (%)	R,n (%)	I, n (%)	S, n (%)
Ampicillin	100(42)	0(0)	0(0)	100(7)	0(0)	0(0)	100(11)	0(0)	0(0)	100(60)	0(0)	0(0)
Amoxicillin/clavulanic	100(42)	0(0)	0(0)	100(7)	0(0)	0(0)	100(11)	0(0)	0(0)	100(60)	0(0)	0(0)
Piperacillin/tazobactam	95.2(40)	2.4(1)	2.4(1)	100(7)	0(0)	0(0)	81.8(9)	9.1(1)	9.1(1)	93.4(56)	3.3(2)	3.3(2)
Cefazolin	100(42)	0(0)	0(0)	100(7)	0(0)	0(0)	100(11)	0(0)	0(0)	100(60)	0(0)	0(0)
Cefoxitin	100(42)	0(0)	0(0)	100(7)	0(0)	0(0)	90.9(10)	9.1(1)	0(0)	98.3(59)	1.7(1)	0(0)
Ceftriaxone	100(42)	0(0)	0(0)	100(7)	0(0)	0(0)	100(11)	0(0)	0(0)	100(60)	0(0)	0(0)
Cefepime	100(42)	0(0)	0(0)	100(7)	0(0)	0(0)	90.9(10)	0(0)	9.1(1)	98.3(59)	1.7(1)	0(0)
Aztreonam	95.2(40)	0(0)	4.8 (2)	100(7)	0(0)	0(0)	90.9(10)	0(0)	9.1(1)	95.0(57)	0(0)	5.0(3)
Ertapenem	100(42)	0(0)	0(0)	100(7)	0(0)	0(0)	100(11)	0(0)	0(0)	100(60)	0(0)	0(0)
Meropenem	100(42)	0(0)	0(0)	71.4(5)	14.3(1)	14.3(1)	90.9(10)	9.1(1)	0(0)	95.0(57)	3.3(2)	1.7(1)
Imipenem	95.2(40)	0(0)	4.8(2)	71.4(5)	0(0)	28.6(2)	81.8(9)	9.1(1)	9.1(1)	90.0(54)	1.7(1)	8.3(5)
Amikacin	71.4(30)	0(0)	28.6(12)	14.3(1)	14.3(1)	71.4 (5)	54.5(6)	0(0)	45.5(5)	61.6(37)	1.7(1)	36.7(22)
Gentamicin	83.3(35)	2.4(1)	14.3(6)	42.9(3)	0(0)	57.1(4)	81.8(9)	0(0)	18.2(2)	78.3(47)	1.7(1)	20.0(12)
Tobramycin	76.2(32)	9.5(4)	14.3(6)	42.8 (3)	28.6(2)	28.6(2)	54.5(6)	27.3(3)	18.2(2)	68.3(41)	15.0(9)	16.7(10)
Ciprofloxacin	80.9(34)	4.8(2)	14.3(6)	85.7(6)	0(0)	14.3(1)	81.8(9)	0(0)	18.2(2)	81.7(49)	3.3(2)	15.0(9)
Levofloxacin	81.0(34)	0(0)	19.0(8)	85.7(6)	0(0)	14.3(1)	45.4(5)	27.3(3)	27.3(3)	75.0(45)	5.0(3)	20.0(12)
Tigecycline	16.7(7)	–	83.3(35)	0(0)	–	100(7)	18.2(2)	–	81.8(9)	15.8(9)	–	85.0(51)
Notrofurantoin	90.5(38)	7.1(3)	2.4(1)	14.2(1)	42.9(3)	42.9(3)	36.4(4)	27.2(3)	36.4(4)	71.7(43)	15.0(9)	13.3(8)
Trimethoprim/ sulfamethoxazole	14.3(6)	0(0)	85.7(36)	57.1(4)	0(0)	42.9(3)	72.7(8)	0(0)	27.3(3)	30.0(18)	0(0)	70.0(42)
Colistin	0(0)	–	100(42)	0(0)	–	100(7)	0(0)	–	100(11)	0(0)	–	100(60)

Abbreviations: R, Resistant; I, Intermediate; S, Susceptible.

Table 2 Molecular Characteristics of Carbapenem-Resistant *Enterobacteriacae* Isolated from Fecal Samples

Isolates	Number (%)	Carbapenemase Phenotypic Confirmatory Test Positive (n)	Carbapenemase Gene						MCR-1
			KPC	NDM	IMP	VIM	OXA48	Other	
K. pneumoniae[a]	42(70)	41	35	1	5	–	–	1	–
E. coli	7(11.6)	5	2	3	–	–	–	–	–
K. oxytoca[b]	3(5.0)	3	1	2	1	–	–	–	–
C. freundii[c]	3(5.0)	3	1	3	–	–	–	–	–
E. cloacae	3(5.0)	2	–	2	–	–	–	–	–
E. aerogenes	1(1.7)	0	–	–	–	–	–	–	–
R. planticola	1(1.7)	1	–	1	–	–	–	–	–
Total	60(100)	55	39	12	6	0	0	1	0

Notes: [a]One *K. pneumoniae* coproducing KPC and IMP. [b]One *K.oxytoca* coproducing NDM and IMP. [c]One *C. freundii* coproducing NDM and KPC.

the authors screened 303 patients for fecal CRE without identifying the CRE species, and they did not investigate epidemiology. The study presented here demonstrated that *K. pneumoniae* was the main fecal CRE species (70%,42/60). In another recent study, the prevalence of carbapenem-resistant *K. pneumoniae* (CRKP) isolated from sputum and fecal samples was 20.8% in ICU patients in Zhejiang Province, China.[18] Worldwide, the reported rate of CRE fecal carriage in hospitalized patients has varied from 0.3 to 69.5%.[26–29] We included stool samples from patients who had been hospitalized for at least 48 h to ensure the prevalence of CRE fecal carriage reflected the hospitalized patients' situation. CRE rectal colonization in patients has been reported to be an important risk factor for CRE infection, especially for critically ill patients.[12–14]

In comparison to the isolates in a previous study in China,[12] the CRE isolates in our study exhibited high resistance rates to β-lactam antimicrobial agents, including penicillins, carbapenems, cephalosporins, and the monobactam aztreonam. In contrast, CRE isolates showed some sensitivity to non-β-lactam antibiotics. A substantial proportion of isolates showed susceptibility to Trimethoprim/
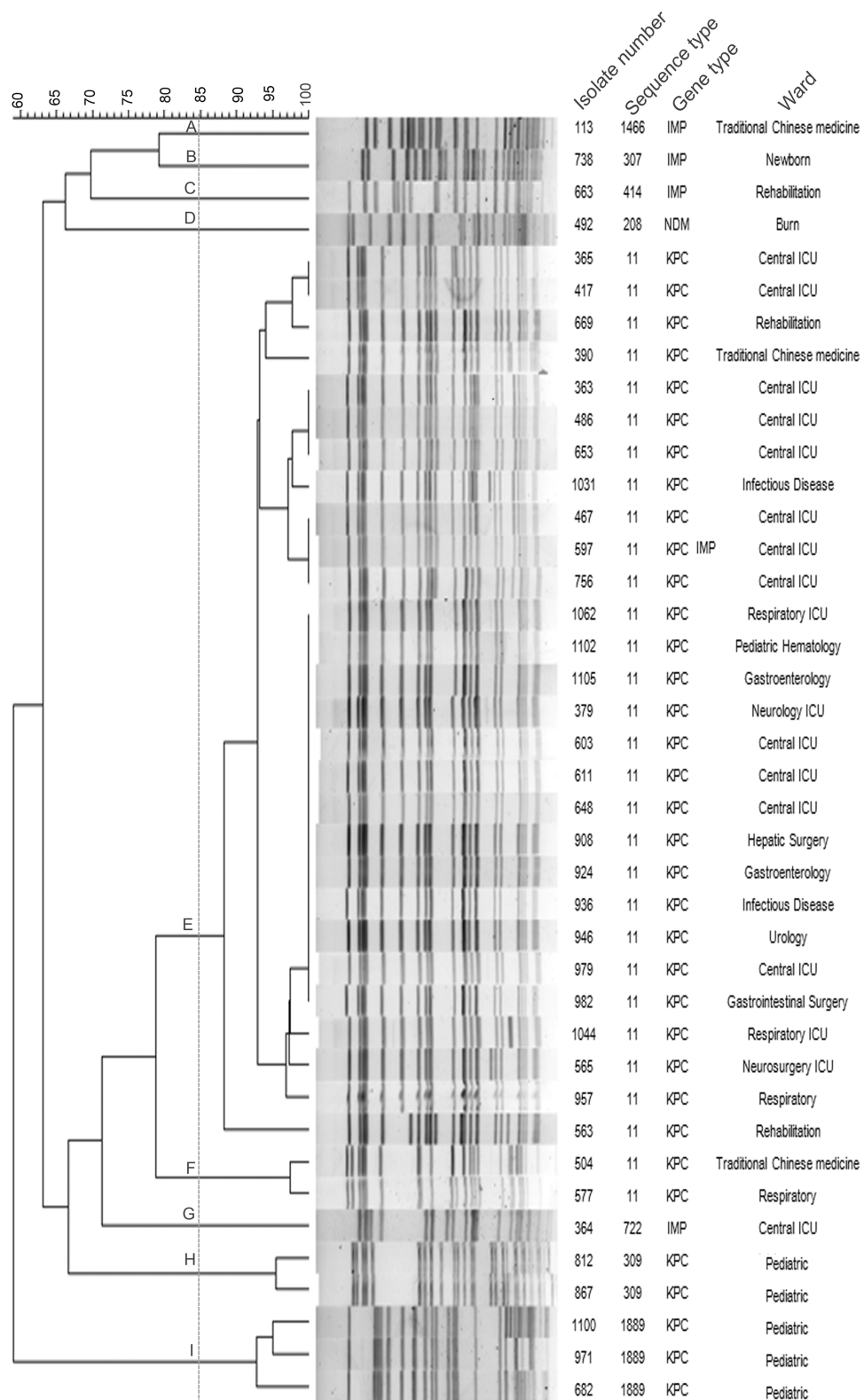
Figure 1 The XbaI PFGE-based dendrogram for the 40 carbapenemase-producing *Klebsiella penumoniae* isolates. A,B,C,D,E,F,G,H, and I represent nine different PFGE clusters identified at a similarity index of 85%.

sulfamethoxazole (70%), amikacin (36.7%), gentamicin (20%), levofloxacin (20%), and ciprofloxacin (15.0%). In addition, nonsusceptibility to tigecycline was 15.8%, but all isolates remained susceptible to colistin. Therefore, in some cases, the combination of colistin and/or tigecycline with these other antimicrobial agents may be considered for the treatment of complex infections caused by CRE.

In this study, our aim was to investigate colistin susceptibility and the *mcr-1* gene, because colistin is one of the few drugs that can be selected for CRE treatment. Despite the fact that colistin-resistant MCR-1-producing *E. coli* are very common in Chinese chicken arms,[30] we found neither any colistin-resistant strains among these fecal CRE isolates, nor the *mcr-1* gene. This is in accordance with previous studies of clinical isolates, which showed that MCR-1positivity occurred mostly in Gram-negative bacilli sensitive to carbapenems.[31,32]

KPC (72.2%, 39/54) was the dominant carbapenemase in our study, while only 4 KPC were detected in 8 carbapenemase-producing *Enterobacteriaceae* (CPE,50%, 4/8) in a previous study in Fujian, China.[12] A possible explanation for this was the geographic difference or the time variance between the two studies. We found one *K. pneumoniae* with a positive carbapenemase phenotype that did not carry any of the five carbapenemase genes tested, possibly because a different gene was present. Notably, we identified three CRE coproducing two different carbapenemases (Table 2), illustrating the ease with which these genes spread.

ST11 was the dominant ST in *K. pneumoniae* in this study, which is in accordance with another study of our hospital.[33] It also showed that ST11 was the main ST type of CRKP, although the CRKP were isolated from clinical samples. It may indicate there was some correlation between colonization and clinical infection. In this study, we found a big cluster of ST11 in *K. pneumoniae* from various wards had identical PFGE patterns, and the results were indicative that there was a clonal transmission of ST11-type CRKP in the hospital. Three novel ST1889 *K. pneumoniae* were indicative a nosocomial transmission in the pediatric ward. Considering their co-resistance to carbapenems and tigecycline, treatment could be very troublesome.

In this study, meropenem discs were used to screen rectal CRE. According to previous studies, meropenem discs screening method is a comparable method as that using entapenem discs or chromogenic medium for detecting rectal CRE.[19,34,35]

There are several limitations in our study. First, this is a single center study; however, our hospital is one of the largest hospitals in Southern China with 3500 beds and an annual admission of more than 130,000 patients from all over the region. Second, the stool samples were limited to those submitted for routine analysis and there may be some imbalance in stool samples submitted by the wards. However, our samples were from 65 of the 68 wards of the hospital, and represented a substantial proportion of patients passing through them. Third, the CRE screening method we used in this study may lack sensitivity for patients colonized with small numbers of CRE, or for patients colonized with CRE producing OXA-type carbapenemase; however, OXA seems to be rare in China. Despite these limitations, this study is the largest scale investigation of the prevalence and epidemiology of fecal CRE carriage in hospitalized patients in an entire hospital in China.

Conclusion

In conclusion, this study revealed a predominant fecal carriage of KPC-producing *K. pneumoniae* belonging to ST11 sequence type in a Chinese university hospital. There was also an emergence of *K. pneumoniae* ST1889, a new ST. Our data demonstrate ongoing cross-colonization of CRE in the hospital, and the urgent need for implementing infection control measures to control CRE dissemination. CRE carriage screening and de-colonization in specific populations may help to decrease the occurrence and control the transmission of CRE in hospitals.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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