CARBAPENEM-RESISTANT ENTEROBACTERIACEAE IN THE ASIA PACIFIC AND BEYOND

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CARBAPENEM-RESISTANT ENTEROBACTERIACEAE IN THE ASIA PACIFIC AND BEYOND

Topic Editors:
Yi-Wei Tang, Memorial Sloan Kettering Cancer Center, United States
Barry N. Kreiswirth, University of Medicine and Dentistry of New Jersey, United States
Liang Chen, Public Health Research Institute (PHRI), United States

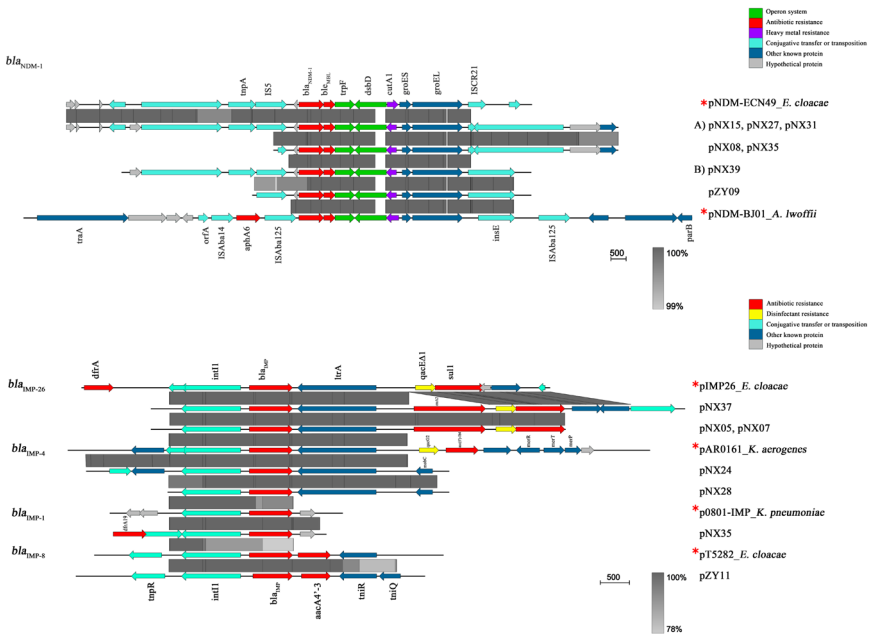

Image: Figure 4 from Cai et al. (2019)

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Molecular Characterization of Carbapenem-Resistant *Enterobacter cloacae* in 11 Chinese Cities

Chunmei Jin[1†], *Jiangang Zhang*[2†], *Qi Wang*[2], *Hongbin Chen*[2], *Xiaojuan Wang*[2], *Yawei Zhang*[2] *and Hui Wang*[2*]

[1] Department of Clinical Laboratory, Yanbian University Hospital, Yanji, China, [2] Department of Clinical Laboratory, Peking University People's Hospital, Beijing, China

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Edited by:
Yi-Wei Tang,
Memorial Sloan Kettering Cancer Center, United States

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Rong Zhang,
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Université Paris-Saclay, France

***Correspondence:**
Hui Wang
wanghui@pkuph.edu.cn;
whuibj@163.com

[†]These authors have contributed equally to this work and are co-first authors.

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Carbapenem-resistant *Enterobacteriaceae* (CRE) are usually resistant to most of antibiotics. Infections caused by such bacteria have a high mortality and pose a serious threat to clinical management and public health. *Enterobacter cloacae* ranks third among *Enterobacteriaceae* that cause nosocomial infections. In this study, the molecular characteristics of carbapenem-resistant *E. cloacae* in China were investigated. From November 2012 to August 2016, 55 non-repetitive strains of carbapenem-resistant *E. cloacae* were collected from 12 hospitals in 11 Chinese cities. The bacteria were identified with matrix-assisted laser desorption/ionization time of flight mass spectrometry. Antimicrobial susceptibility tests were determined by agar dilution method. Carbapenemase and other β-lactamase genes were detected with PCR and sequencing. Multilocus sequence typing and plasmid conjugation tests were performed. Among the 55 *E. cloacae* strains, 50 strains were detected to produce 8 types of carbapenemase including NDM-1, NDM-5, IMP-4, IMP-26, IMP-1, KPC-2, and VIM-1. NDM-1 accounted for 68.0% (34/50) among the carbapenemase-producing *E. cloacae*. A total of 24 sequence types were identified and ST418 was the most common, accounting for 20% (11/55). For further investigation, a pulsed-field gel electrophoresis (PFGE) assay was conducted to identify the PFGE patterns of the strains. These 23 isolates yielded 13 PFGE patterns, which were designated as type A–M. Eight isolates obtained from Shenzhen had the same PFGE pattern (type A) and the remaining 15 isolates belonged to the other 12 PFGE patterns (type B–M). The observation that 8 of the 15 bla_{NDM-1}-positive *E. cloacae* isolates obtained from Shenzhen with the same PFGE pattern (type A) suggested a transmission outbreak of a common strain. S1-nuclease PFGE and Southern blotting were also conducted to estimate the size of plasmids harbored by bla_{NDM-1}-positive strains. The results showed that the plasmids harboring the bla_{NDM-1} gene ranged in size from approximately 52–58 kilobases. Our study indicates that carbapenem-resistant *E. cloacae* strains that produce NDM carbapenemase have strong resistance. Early detection and monitoring of the prevalence of these strains are urgent.

Keywords: carbapenem-resistance, *Enterobacter cloacae*, carbapenemase, NDM-1, ST418

INTRODUCTION

In recent years, the emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) has become a serious issue both on community-acquired infections and healthcare-associated infections (van Duin and Doi, 2017). As well as other *Enterobacteriaceae*, *Enterobacter cloacae* (*E. cloacae*) is a conditional pathogen found in the intestine. Healthcare-associated infections caused by *E. cloacae* ranked third among all the *Enterobacteriaceae* (Dai et al., 2013). *Enterobacter cloacae* can produce chromosome mediated AmpC β-lactamase and has resistance to ampicillin, amoxicillin/clavulanic, cephamycin and first and second generation cephalosporin. A wide spectrum of antibacterial drugs such as carbapenems may be used in treatment more often. Thus, multidrug resistance has emerged rapidly under antibiotic selection pressure. Carbapenem-resistant *E. cloacae* infections have been reported in many countries such as Spain, Australia, the United States, India, and China (Kiedrowski et al., 2014; Fernández et al., 2015; Liu et al., 2015; Sidjabat et al., 2015). The emergence of carbapenem-resistant *E. cloacae* is an enormous challenge to clinical treatment. It is well known that the main mechanism for reduced susceptibility to carbapenems in *E. cloacae* is the deregulation of ACT (the natural cephalosporinase of *E. cloacae*), which is associated with a decrease in membrane permeability. In addition to this, producing carbapenemases is another important mechanism of *Enterobacteriaceae* in carbapenem resistance (Walsh et al., 2005; Nordmann et al., 2009; Tzouvelekis et al., 2012). Also, the mechanism of combinations of either ESBL or AmpC and mutation of porins may hold a certain proportion (Yang et al., 2010).

Up until now, there was a lack of multicenter research on carbapenem-resistant *E. cloacae* in China. So, we conducted this molecular epidemiological study on carbapenem-resistant *E. cloacae* to further understand the prevalence of the bacteria in China.

MATERIALS AND METHODS

Sample Collection

From November 2012 to August 2016, we collected 55 unrepeated strains of carbapenem-resistant [any carbapenem (imipenem, meropenem, or ertapenem) as determined by standard methods] *E. cloacae* from 12 hospitals in 11 Chinese cities (Beijing, Chengde, Zunhua, Ji'nan, Xuzhou, Xi'an, Wuhan, Xiamen, Guangzhou, Dongguan, and Shenzhen; **Figure 1**). The participating hospitals include Peking University People's Hospital, Peking Union Medical College Hospital, Affiliated Hospital of Chengde Medical University, People's Hospital of Zunhua, Qilu Hospital of Shandong University, Affiliated Hospital of Xuzhou Medical University, Xijing Hospital, Tongji Hospital, The First Affiliated Hospital of Xiamen University, The First Affiliated Hospital Sun Yat-sen University, Donghua Hospital Sun Yat-sen University, and Shenzhen Second People's Hospital.

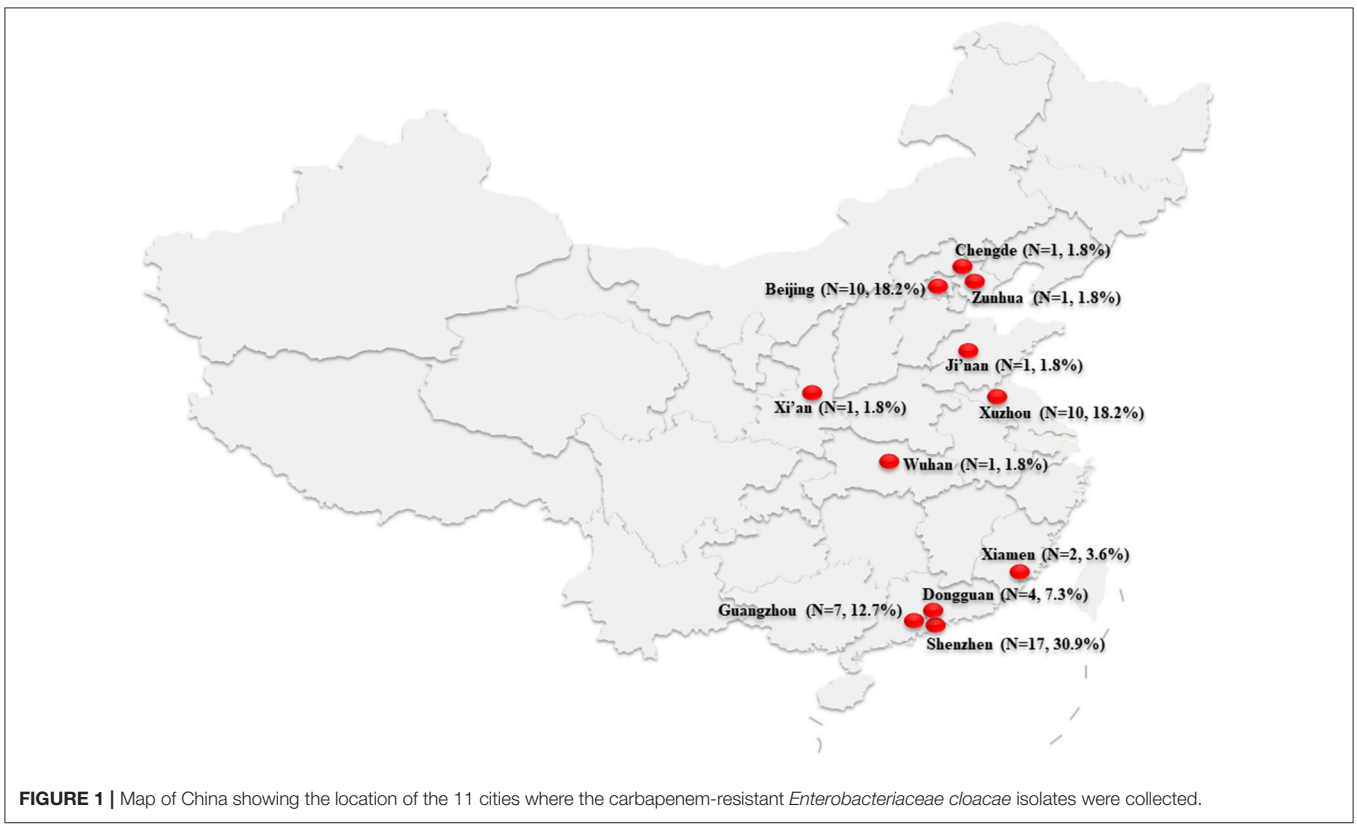

FIGURE 1 | Map of China showing the location of the 11 cities where the carbapenem-resistant *Enterobacteriaceae cloacae* isolates were collected.

Identification of the Bacterial Strains and Antimicrobial Susceptibility Tests

All isolates were identified with matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany). Minimum inhibitory concentrations (MICs) were determined by the agar dilution method according to CLSI guidelines (M100-S27). The tested drugs included ceftriaxone (Roche China, Shanghai, China), cefotaxime, ceftazidime, cefepime, aztreonam, amikacin, levofloxacin, minocycline, fosfomycin (National Institute for Food and Drug Control of China, Beijing, China), piperacillin/tazobactam, tigecycline (Pfizer, NY, USA), imipenem (Merck Sharp & Dohme, Hangzhou, China), meropenem (Sumitomo Pharmaceuticals, Suzhou, China), ciprofloxacin (Bayer, Leverkusen, Germany), and polymyxin B (Amresco, Solon, USA). Strains used in quality control were *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. The results were interpreted according to 2017 CLSI standards (M100-S27). The tigecycline test was performed according to the Food and Drug Administration standards.

Detection of Antimicrobial Resistance Genes

Phenotypic screening for the resistance genes of carbapenem-resistant *E. cloacae* strains was based on the 2017 CLSI guidelines. Modified Hodge test (MHT), imipenem-EDTA double-disk synergy test (DDST) (Lee et al., 2001), and modified carbapenem inactivation method (mCIM) were used to test carbapenemase production. Polymerase chain reaction (PCR) was used to detect carbapenemase genes (bla_{NDM}, bla_{KPC}, bla_{IMP}, bla_{IMI}, bla_{NMC}, bla_{GES}, bla_{SME}, bla_{SIM}, bla_{VIM}, and bla_{OXA-48}) and other β-lactamase genes (bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{DHA}, and bla_{CMY}) (Lewis et al., 2007; Yang et al., 2010). The products were submitted for sequencing.

Multilocus Sequence Typing (MLST)

MLST was performed according to a previously described method (https://pubmlst.org/ecloacae/). New alleles and sequence types were submitted to the MLST website and approved. Sequence Type Analysis and Recombinational Tests 2 (START2) (http://pubmlst.org/software/analysis/start2/) software was used to generate the phylogenetic tree (Jolley et al., 2001).

Plasmid Conjugation Test

The plasmid conjugation test was used to test carbapenem-resistant gene transfer. Ten strains were selected for the test. *Escherichia coli* EC600 (rifampicin resistant) was used as the recipient. Conjugants were screened using China blue lactose agar plates containing rifampicin (300 μg/ml) and imipenem (1 μg/ml). The donor and the recipient were mixed at a ratio of 1:1 for 24 h. Transconjugants were selected on China blue lactose agar plates (OXOID, Basingstoke Hampshire, UK), supplemented with rifampicin (100 μg/ml) and imipenem (1 μg/ml). PCR was used to screen for bla_{NDM-1}, bla_{VIM-1}, bla_{KPC-2}, and bla_{IMP-1} as previously described (Wang et al., 2014).

Pulsed-Field Gel Electrophoresis (PFGE)

Enterobacter cloacae isolates were characterized by PFGE according to the previously published protocol by Ribot et al., with modifications (Ribot et al., 2002). We selected 23 bla_{NDM-1}-positive isolates (including the 15 isolates from Shenzhen, 6

TABLE 1 | *In vitro* activities of antimicrobial agents against carbapenemase-producing *Enterobacteriaceae*.

Antimicrobials	All isolates ($n = 55$)			Isolates with bla_{NDM} ($n = 36$)			Isolates with other carbapenemase genes except bla_{NDM} ($n = 14$)			Comparison between the two groups	
	%S	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%S	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%S	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	χ^2	*P*-value
Piperacillin/tazobactam	20	256	>256	0	>256	>256	64.3	8	128	25.451	<0.001
Ceftazidime	1.8	>256	>256	0	>256	>256	7.1	256	>256	–	0.265
Cefotaxime	1.9	>256	>256	0	>256	>256	7.1	64	>256	–	0.286
Ceftriaxone	1.9	>256	>256	0	>256	>256	7.1	32	>256	–	–
Cefepime	1.8	64	128	0	128	128	7.1	8	64	–	–
Aztreonam	17.4	256	>256	6.7	256	>256	50	4	256	6.003	0.014
Imipenem	12.7	8	32	0	8	32	42.9	2	4	–	0.001
Meropenem	16.4	8	32	0	8	64	57.1	1	4	–	0.019
Amikacin	89.1	4	>256	86.1	4	>256	100	2	8	0.781	0.377
Ciprofloxacin	25.5	32	128	13.9	32	128	50	1	128	–	0.011
Levofloxacin	30.9	16	128	22.2	32	128	50	2	32	1.704	0.192
Fosfomycin	80	16	128	86.1	8	128	75	16	256	–	0.19
Minocycline	52.7	16	128	30.6	32	128	57.1	4	32	4.276	0.039
Polymyxin B	100	0.125	0.25	100	0.125	0.25	100	0.125	0.25	–	–
Tigecycline	78.2	1	8	72.2	1	8	91.7	1	1	0.838	0.36

isolates involved in the conjugation experiments, and another 2 representative isolates).

Electrophoresis conditions were altered to have an initial switch time of 2.16 s and a final switch time of 54.17 s, and gels were run for 18 h. The resulting PFGE patterns were analyzed in BioNumerics software (Applied Maths, Austin, TX, USA) with dendrograms based on the Dice coefficient with a band position tolerance of 1%. Patterns with no discernible differences were considered indistinguishable and given the same PFGE pattern designation.

S1-Nuclease PFGE and Southern Blotting

S1-nuclease PFGE and Southern blotting were performed to estimate the size of plasmids harbored by bla_{NDM-1}-positive

TABLE 2 | Microbiological and molecular characteristics of 34 bla_{NDM-1}-positive *Enterobacter cloacae* strains.

Isolate	Date of isolation	City	Gender/ Age (Year)	Ward	Specimen	mCIM (mm)	MHT	EDTA-DDST	DHA	CTX-M	ST	PFGE pattern	Plasmid size, harboring bla_{NDM} (kb)
ecl408	2015/6/8	Dongguan	M/24	ICU	ur	6	+	+	–	–	418	–	–
ecl409	2015/3/28	Dongguan	M/47	ICU	ur	6	+	+	–	–	418	–	–
ecl411	2015/6/25	Dongguan	F/49	ICU	ur	6	+	+	DHA-1	–	418	–	–
cas471	2015/12/28	Zunhua	M/36	ICU	ur	6	+	+	–	CTX-M-3	920	F	~54
ecl497	2015/6/16	Ji'nan	F/59	Outpatient	sp	6	+	+	DHA-1	CTX-M-3	51	G	~52
ecl645	2014/5/23	Guangzhou	F/66	Neurology	ur	6	+	+	–	CTX-M-3	93	–	–
ecl759	2015/1/28	Shenzhen	F/60	Hepatobiliary surgery	dr	6	±	+	DHA-1	–	88	B	~52
ecl760	2015/2/6	Shenzhen	F/60	Hepatobiliary surgery	wd	6	+	+	DHA-1	–	88	B	~52
ecl766	2015/5/22	Shenzhen	M/77	Respiratory	ca	6	+	+	DHA-1	–	93	C	~52
ecl767	2015/5/22	Shenzhen	M/77	Respiratory	bl	6	+	+	–	–	93	C	~52
ecl768	2015/6/8	Shenzhen	M/47	Neurosurgery	sp	6	+	+	–	–	418	A	~52
ecl771	2015/6/18	Shenzhen	F/45	Neurosurgery	sp	6	+	+	–	–	418	A	~52
ecl774	2015/8/28	Shenzhen	F/61	Neurosurgery	ur	6	+	+	–	–	418	A	~52
ecl776	2015/9/14	Shenzhen	M/38	Neurosurgery	ur	6	+	+	–	–	418	A	~52
ecl777	2015/10/20	Shenzhen	M/34	Neurosurgery	sp	6	+	+	–	–	93	D	~52
ecl778	2015/11/22	Shenzhen	M/83	Neurosurgery	ur	6	+	+	–	–	93	D	~52
ecl779	2015/12/8	Shenzhen	F/42	EICU	sp	6	+	+	–	–	418	A	~52
ecl780	2015/12/21	Shenzhen	M/70	Neurosurgery	sp	6	+	+	–	–	93	E	~54
ecl782	2015/12/18	Shenzhen	F/42	EICU	ur	6	+	+	–	–	418	A	~52
ecl784	2015/12/25	Shenzhen	M/84	Nephrology	bl	6	+	+	–	–	418	A	~52
ecl786	2016/1/8	Shenzhen	F/42	EICU	ba	6	+	+	–	–	418	A	~52
ecl828	2015/1/11	Xuzhou	M/55	EICU	sp	6	+	+	DHA-1		51	–	–
ecl830	2015/1/23	Xuzhou	M/40	Neurosurgery	sp	6	+	+	–	CTX-M-3	51	–	–
ecl844	2015/12/22	Xuzhou	M/77	EICU	sp	6	+	+	–	CTX-M-3	51	H	~52
ecl886	2015/5/8	Xiamen	M/85	ICU	ab	6	+	+	–	–	171	I	~52
ecl932	2016/5/27	Xiamen	M/61	Urology Surgery	ur	6	+	+	–	–	78	–	–
ecl979	2016/6/26	Wuhan	M/46	ICU	ur	6	+	+	–	–	78	J	~58
ecl982	2016/5/31	Xi'an	F/57	Hepatobiliary surgery	dr	6	+	+	–	–	78	–	–
ecl1017	2016/7/3	Beijing	F/35	Respiratory	bl	6	+	+	–	–	121	K	~56
ecl1028	2016/8/28	Beijing	F/59	Hematology	sp	6	+	+	–	–	127	–	–
ecl1045	2016/4/26	Xuzhou	M/59	Urology Surgery	bl	6	+	+	–	CTX-M-3	78	–	–
ecl1102	2016/6/22	Xuzhou	M/68	ICU	bl	6	+	+	–	–	231	–	–
ecl1115	2016/6/13	Xuzhou	M/74	ICU	sp	6	+	+	–	CTX-M-3	97	L	~52
ecl1127	2016/7/27	Xuzhou	M/50	EICU	sp	6	+	+	DHA-1	CTX-M-14	97	M	~55

ecl, Enterobacter cloacae; cas, Enterobacter asburiae; ICU, intensive care unit; EICU, emergency intensive care unit; ba, broncho-alveolar lavage; bl, blood; ca, catheter; dr, drainage; sp, sputum; ur, urine; wd, wound; EDTA-DDST, EDTA double-disk synergy test.

strains as described previously. We selected 23 bla_{NDM-1}-positive isolates as mentioned above.

The bla_{NDM-1} gene was detected by digoxigenin-labeled specific probes (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche Diagnostics, Mannheim, Germany). *Salmonella enterica* H9812 was used as a size marker.

Statistical Analyses

WHONET (version 5.6) software (http://www.whonet.org/software.html) and SPSS (version 22.0) software (SPSS Inc., Chicago, IL, USA) were used for statistical analyses.

Ethical Approval

This study was approved by the research ethics board at Peking University People's Hospital. Informed consent was not needed as this study was retrospective and participants were anonymized. Medical records and patient's information were retrospectively reviewed and collected.

FIGURE 2 | Multilocus sequence typing (MLST) phylogenetic tree of the 55 carbapenem-resistant *E. cloacae* strains. ab, abdominal fluid; ba, broncho-alveolar lavage.

RESULTS

Characteristics of Collected Samples

The most common specimens were respiratory tract (20 cases, 36.4%), followed by urine (13 cases, 23.6%), blood (11 cases, 20%), ascitic fluid (7 cases, 12.7%), bile (2 cases, 3.6%), catheter (1 case, 1.8%), and wound (1 case, 1.8%).

Antimicrobial Susceptibility Tests

Of all the antimicrobials tested, the most susceptible antimicrobial was polymyxin B (100%, 55/55), followed by amikacin (89.1%, 49/55), fosfomycin (80%, 44/55), tigecycline (78.2%, 43/55), minocycline (52.7%, 29/55), levofloxacin (30.9%, 17/55), and ciprofloxacin (25.5%, 14/55). All of the 36 bla_{NDM}-positive strains were resistant to piperacillin-tazobactam, ceftazidime, cefotaxime, ceftriaxone, cefepime, imipenem, and meropenem. It is worth noting that the most susceptible antimicrobials to bla_{NDM}-positive strains was polymyxin B (100%, 36/36), followed by amikacin (86.1%, 31/36), and fosfomycin (86.1%, 31/36). There was some differences between bla_{NDM}-positive strains and strains with other carbapenemases (**Table 1**).

Genotype Analysis

Among the 55 strains, 50 were confirmed to produce 8 types of carbapenemases including NDM-1, NDM-5, IMP-4, IMP-26, IMP-1, KPC-2, and VIM-1. The corresponding numbers of the strains that produced the foregoing types of carbapenemases were 34, 2, 6, 3, 2, 2, and 1. Other carbapenemase genes were not detected. No strains contained two or more carbapenemase genes. NDM-1-producing carbapenem-resistant *E. cloacae* was primarily distributed in Shenzhen (**Table 2**). Carbapenemase genes were not detected in the other 5 strains.

NDM-1-producing *E. cloacae* isolates were mainly collected from Shenzhen (44.1%, 15/34), followed by Xuzhou (20.6%, 7/34), Beijing, Dongguan, Guangzhou, Ji'nan, Xi'an, Xiamen, Wuhan, and Zunhua. These samples were primarily collected from the Intensive Care Unit and the Emergency Intensive Care Unit (41.2%, 14/34), followed by the department of neurosurgery (23.5%, 8/34). NDM-1-producing *E. cloacae* isolates were most commonly identified in sputum samples (35.3%, 12/34), followed by urine samples (32.4%, 11/34). All strains were positive for MHT, imipenem-EDTA-DDST, and mCIM. Results of the three tests were consistent. In addition, 6 strains also produced the AmpC enzyme DHA-1, 8 strains produced CTX-M-3, and 2 strains produced both CTX-M and DHA-1.

MLST

The results of the MLST are shown in **Figure 2**. A total of 24 sequence types were detected in the 55 *E. cloacae* strains. ST418 was the most common (20%, 11/55), followed by ST93 (14.5%, 8/55).

TABLE 3 | Antibiotic susceptibilities of *E. cloacae* isolates and their transconjugants (μg/ml).

Isolate	City	Carbapenemase	ST	MEM	IMP	FEP	CAZ	TZP	ATM	AMK	CIP	LVX	PB	TGC
***E. cloacae* isolates**														
cas471	Zunhua	NDM-1	920	8	16	64	>256	>256	256	8	16	16	0.25	1
ecl497	Ji'nan	NDM-1	51	8	4	32	>256	256	>256	>256	2	2	0.25	1
ecl591	Guangzhou	VIM-1	175	0.5	4	8	256	128	0.032	1	<=0.016	<=0.016	0.125	0.5
ecl763	Shenzhen	KPC-2	53	0.5	4	4	8	256	128	1	4	8	0.25	0.5
ecl844	Xuzhou	NDM-1	51	2	8	16	>256	128	128	4	0.25	0.5	0.125	0.5
ecl886	Xiamen	NDM-1	171	2	4	32	>256	256	128	1	2	2	0.5	1
ecl979	Wuhan	NDM-1	78	>32	>32	>256	>256	>256	–	1	>64	64	0.25	4
ecl1017	Beijing	NDM-1	121	8	8	64	>256	>256	–	2	64	16	0.125	0.5
ecl1025	Beijing	IMP-1	78	2	2	32	>256	8	–	0.5	32	32	0.125	0.25
***E. coli* transconjugant strains**														
471TC		NDM-1		2	8	32	>256	256	128	1	0.125	0.5	0.25	0.125
497TC		NDM-1		4	8	32	>256	128	128	1	0.125	0.25	0.125	0.125
591TC		VIM-1		2	4	128	>256	>256	0.25	1	0.125	0.25	0.125	0.125
763TC		KPC-2		4	4	8	32	>256	>256	1	2	2	0.125	0.125
844TC		NDM-1		2	8	16	>256	128	64	1	0.125	0.25	0.125	0.25
886TC		NDM-1		2	8	16	>256	128	128	1	0.125	0.25	0.125	0.25
979TC		NDM-1		4	8	16	>256	128	4	0.125	2	4	0.125	2
1017TC		NDM-1		8	16	128	>256	256	>256	2	0.125	1	0.125	0.125
1025TC		IMP-1		1	2	32	>256	16	0.125	1	0.125	0.25	0.25	0.125
EC600		–		0.032	0.25	0.064	0.25	2	0.125	1	0.125	0.25	0.25	0.25

MEM, meropenem; IMP, imipenem; FEP, cefepime; CAZ, ceftazidime; TZP, piperacillin/tazobactam; ATM, aztreonam; AMK, amikacin; CIP, ciprofloxacin; LVX, levofloxacin; PB, polymyxin B; TGC, tigecycline; TC, transconjugant strain; EC600, recipient strain.

Plasmid Conjugation Test

Plasmids from 9 carbapenemase-producing *E. cloacae* strains were successfully transferred to *E. coli* EC600. Drug sensitivity tests showed the MICs of meropenem increased by 5- to 8-fold in the 9 conjugators; for imipenem, cefepime, ceftazidime, and piperacillin/tazobactam, the MICs increased by 3∼6-, 7∼11-, 7∼10-, and 3∼7-fold, respectively (**Table 3**).

PFGE, S1-Nuclease PFGE and Southern Blotting

When typed by PFGE to determine if they were related, the 23 isolates yielded 13 PFGE patterns, which were designated as type A–M. Eight isolates (ecl768, ecl771, ecl774, ecl776, ecl779, ecl782, ecl784, and ecl786) obtained from Shenzhen had the same PFGE pattern (type A) and the remaining 15 isolates belonged to the other 12 PFGE patterns (type B–M) (**Table 2**). The observation that 8 of the 15 $bla_{\mathrm{NDM-1}}$-positive *E. cloacae* isolates with the same PFGE pattern (type A) and the same sequence type (ST418) suggested a transmission outbreak of a common strain.

The results of S1-nuclease PFGE and Southern blotting showed that the plasmids harboring the $bla_{\mathrm{NDM-1}}$ gene ranged in size from approximately 52–58 kilobases, respectively (**Table 2**). The plasmids harboring the $bla_{\mathrm{NDM-1}}$ gene of the 8 isolates obtained from Shenzhen were the same size (approximately 52 kilobases).

DISCUSSION

Carbapenemase-producing *E. cloacae* has been reported in many countries, such as strains producing OXA-48 and VIM-1 have been reported in Spain. In Brazil, Australia and America, strains producing NDM-1, IMP-4, and KPC-3, respectively, have been reported (Kiedrowski et al., 2014; Rozales et al., 2014; Villa et al., 2014; Fernández et al., 2015; Sidjabat et al., 2015), while in Chongqing, Henan and Ningxia of China, strains producing NDM-1 have been identified (Dai et al., 2013; Liu et al., 2015; Shi et al., 2017). Strains that produced other carbapenemases have also been reported in the Sichuan province of China (Huang et al., 2015). In the present study, we found that the *E. cloacae* prevalent in China mainly produced NDM-1 (68.0%, 34/50) and IMP-4 (12.0%, 6/50). NDM-1 was found in the highest proportion and may represent a significant drug-resistant mechanism of carbapenem-producing *Enterobacteriaceae* in China.

The plasmid conjugation test was completed with 6 $bla_{\mathrm{NDM-1}}$-positive strains. Conjugants were all detected to have the $bla_{\mathrm{NDM-1}}$. Susceptibility results showed that compared with the receptor bacteria EC600, the conjugants have a higher MIC value on cephalosporins and carbapenems. There were no MIC promotions on polymyxin B and tigecycline between conjugants and EC600. Many studies have demonstrated that the plasmid owned bla_{NDM} also have other resistant genes, such as $bla_{\mathrm{TEM-1}}$, bla_{CMY}, qnrA6, and qnrB1 for quinolone resistance, armA, rmtA, and rmtC for aminoglycoside resistance (Poirel et al., 2011a,b; Kocsis et al., 2016). But all strains in this study have no $bla_{\mathrm{TEM-1}}$ and bla_{CMY}. Five of the conjugants have no MIC difference on quinolone with the EC600. Maybe the relative plasmid did not harbor the quinolone resistant gene.

MLST showed subtype diversity. A total of 24 sequence types were detected in 55 *E. cloacae* strains. ST418 was detected the most frequently (11/55, 20%), and the second was ST93 (14.5%, 8/55). Three new sequence types were found, namely ST916, ST919, and ST920. Our study reveals the diversity of carbapenem-resistant *E. cloacae* and the difference in genetic affinity, which is consistent with the study of Gomez-Simmonds et al. (2016). Our study showed that ST418 is the main epidemic strain in Shenzhen in China; while in America, Central de Asturias of Spain, and the Henan province of China, the main epidemic strains were ST171, ST74, and ST120, respectively (Fernández et al., 2015; Liu et al., 2015; Gomez-Simmonds et al., 2016). We found that ST418 was genetically closer to ST127 and ST755 with START2 analysis. Studies have found that all ST418 strains produced NDM-1 carbapenemase, indicating that there might be a small outbreak of NDM-1-ST418 carbapenem-resistant *E. cloacae* in Shenzhen and Dongguan City of Guangdong province of China. In this study, ST78-NDM-1-type carbapenem-resistant *E. cloacae* was also found in Xuzhou, Xi'an, Wuhan, and Xiamen, which should be taken seriously concern.

In conclusion, our study indicates that ST418, which produces NDM-1 carbapenemase, is the main epidemic strain of carbapenem-resistant *E. cloacae* in Shenzhen and Dongguan City of China. Early detection and monitoring are necessary to prevent the further spread of the bacteria.

AUTHOR CONTRIBUTIONS

HW conceived and designed the study. CJ and JZ wrote this paper. CJ, QW, and JZ performed the experiments. QW and JZ analyzed the data. HC, XW, and YZ assisted CJ and JZ to finish the experiments. All authors approved the final version.

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Characterizing Mobilized Virulence Factors and Multidrug Resistance Genes in Carbapenemase-Producing *Klebsiella pneumoniae* in a Sri Lankan Hospital

*Chendi Zhu[1†], Veranja Liyanapathirana[2†], Carmen Li[1], Vasanthi Pinto[3], Mamie Hui[1], Norman Lo[1], Kam T. Wong[1], Nilanthi Dissanayake[2] and Margaret Ip[1]**

[1] Department of Microbiology, The Chinese University of Hong Kong, Shatin, Hong Kong, [2] Department of Microbiology, Faculty of Medicine, University of Peradeniya, Kandy, Sri Lanka, [3] Department of Anesthesiology and Critical Care, Faculty of Medicine, University of Peradeniya, Kandy, Sri Lanka

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Edited by:
Yi-Wei Tang,
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Liang Li,
Los Angeles Biomedical Research Institute, United States

***Correspondence:**
Margaret Ip
margaretip@cuhk.edu.hk

[†] These authors have contributed equally to this work

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Limited data is available on the epidemiology and characteristics of carbapenem-resistant *Enterobacteriaceae* (CRE) and their associated plasmids or virulence determinants from Sri Lanka. Through whole genome sequencing of CREs from the intensive care units of a Sri Lankan teaching hospital, we identified a carbapenemase gene, $bla_{OXA-181}$ in 10 carbapenemase-producing *Klebsiella pneumoniae* isolates (two strains of ST437 and eight strains of ST147) from 379 respiratory specimens. $bla_{OXA-181}$ was carried in three variants of ColE-type plasmids. *K. pneumoniae* strains with *ompK36* variants showed high minimum inhibitory concentrations to carbapenem. Furthermore, genes encoding for extended spectrum β-lactamases (ESBL), plasmid-mediated quinolone resistance (PMQR) determinants (*qnr*, *aac(6′)-Ib-cr*, and *oqxAB*) were present in all 10 strains. Amino acid substitution in chromosomal quinolone resistance-determining regions (QRDRs) *gyrA* (Ser83Ile) and *parC* (Ser80Ile) were also observed. All strains had yersiniabactin genes on mobile element ICE*kp*. Strict infection control practices and judicious use of antibiotics are warranted to prevent further spread of multidrug-resistant *K. pneumoniae*.

Keywords: OXA-181, quinolone, yersiniabactin, *Klebsiella pneumoniae*, Sri Lanka

INTRODUCTION

Carbapenem-resistant *Enterobacteriaceae* (CRE) is a global threat and infections caused by CRE are associated with high morbidity and mortality (Centers for Disease Control and Prevention, 2013). Among all the resistance mechanisms, plasmid-mediated horizontal transfer of carbapenemase genes is the main route for acquiring resistance in CRE (Nordmann et al., 2012). These mobile elements are capable of transferring resistance between different lineages and thus, pose a potential for dissemination. Three types of carbapenemases (class A: bla_{KPC}; class B: bla_{NDM}, bla_{IMP}, and bla_{VIM}; class D: $bla_{OXA-48-like}$) can hydrolyze carbapenems at varying levels along with other mechanisms, such as porin mutation and overexpression of efflux pump proteins (Poirel et al., 2012; Zhang et al., 2014; Lunha et al., 2016). *Klebsiella pneumoniae* clonal complex 258 (CC258)

have been known to associate with epidemic plasmids carrying numerous antimicrobial resistance genes and virulence factors like yersiniabactin. These interactions were hypothesized to provide a survival advantage for these clones (Holt et al., 2015). Sri Lanka sits in the southern tip of the Indian subcontinent. Although the epidemiology and diversity of carbapenemases, have been reported in India, the epidemiology and characteristics of the CRE and resistance determinants in Sri Lanka is lacking. Furthermore, the single study currently available on CRE from Sri Lanka does not contain information on associated plasmids or virulence determinants (Hall et al., 2014). Hence, we sequenced the CRE strains isolated from patient specimens in the intensive care units of one Sri Lankan hospital using whole genome sequencing (WGS) to describe their antimicrobial resistance and genetic profiles so as to provide a more in-depth view of the CRE in Sri Lanka.

MATERIALS AND METHODS

Bacterial Isolates

Single patient isolates were obtained from the respiratory specimens received from inpatients admitted to the intensive care units of the Teaching Hospital, Peradeniya, Sri Lanka between February to September 2015. During this period, a total of 379 respiratory specimens were processed and 64 coliforms were obtained. Among these coliforms, 2.6% (10 isolates) were found to be resistant to carbapenems using Stokes sensitivity testing method (Sri Lanka College of Microbiologists, 2011) and were saved for further study. The study was approved by the Institutional Ethical Committee of the Faculty of Medicine, University of Peradeniya.

Bacterial Identification and Antimicrobial Resistance

Bacterial strains were plated on blood agar and incubated at 37°C overnight and the identification of all strains were confirmed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry at the Department of Microbiology, the Chinese University of Hong Kong. Minimum inhibitory concentrations (MICs) of antimicrobials were determined by the microbroth dilution method according to Clinical and Laboratory Standards Institute (CLSI) guideline (CLSI, 2018). The following antibiotics were tested: amikacin (AK); ceftazidime (CAZ); ciprofloxacin (CIP); gentamicin (GN); colistin (CT); cefotaxime (CTX); ertapenem (ETP); fosfomycin (FOS); imipenem (IPM); meropenem (MEM); and tigecycline (TG).

Whole Genome Sequencing and Data Analysis

Bacterial DNA was extracted with Wizard genomic DNA purification kit (Promega, Madison, WI, United States). WGS was performed using the Illumina HiSeq 2500 platform, and unique index-tagged libraries were created for each sample to generate 90 bp paired-end reads (Global Biologics, LLC). The libraries gave 100× average coverage for each strain. Quality control of the raw reads was performed by FastQC (Andrews, 2010). Genomes were assembled using SPAdes assembler (version 3.5.0) (Bankevich et al., 2012). Contigs of ≥500 bp from each genome were included in the analyses. Prokka (version 1.9) software was used for genome annotation, including ORF finding and gene function annotation (Seemann, 2014). Raw reads and assembled contigs were used for multilocus sequence typing (MLST) analysis. SRST2 (Version 0.1.5) was used to map raw reads to the following databases: pubMLST database for sequence types; ARG-ANNOT V3 database for resistance genes; PlasmidFinder database (Updated 20170220) for plasmid replicons; and *K. pneumoniae* BIGSdb virulence gene database for virulence genes at http://bigsdb.web.pasteur.fr (Accession date: 20180313) (Carattoli et al., 2014; Gupta et al., 2014; Inouye et al., 2014). The contig containing carbapenemase for each genome was matched against the public database with NCBI BLAST to find the top hit plasmids of at least 95% identity and 95% coverage and were matched back to the plasmids in our genome dataset to extract possible plasmid contigs (Altschul et al., 1990). Extracted contigs were further aligned to the reference plasmid using Mauve Alignment software to check for similarity and coverage (Darling et al., 2004). Gaps were filled by PCR with primers designed from our genome data. Pan-genome dendrogram was created by Roary (Page et al., 2015). Contig files of all 10 strains in this study were deposited to GenBank BioProject under the accession number PRJNA439172.

RESULTS

Antimicrobial Susceptibilities and Molecular Characteristics of the CRE Isolates

Of the 10 CRE isolates included in the study, three were from the subsidiary ICU and seven were from the main ICU. All strains were confirmed *K. pneumoniae* and the susceptibility testing revealed all strains to be resistant to CIP, CTX, and sensitive to FOS and AK, while MIC for CT was ≤0.5 mg/L and MIC for TG was ≤2 mg/L (**Table 1**). All except one strain (SL50) were resistant to CAZ. All of the strains except SL33, SL49, and SL54 were resistant to ETP or MEM with MICs ≥16 and ≥8 mg/L, respectively, while resistance to IPM varied among isolates. In previous studies, mutations of *K. pneumoniae* outer membrane proteins ompK35 and ompK36 were found to confer increased MIC to carbapenems (Garcia-Fernandez et al., 2010; Zhang et al., 2014; Lunha et al., 2016). There were neither mutations nor insertions at ompK35 and its promoter in all of our strains when we aligned them to the wild-type gene in public database (GenBank Accession No. AJ011501). OmpK36 was identical in six highly resistant strains (ompK36-sl1). SL33 and SL49 shared the same gene (ompK36-sl2), but SL54 contained a unique variation (ompK36-sl3). The difference of ompK36-sl1 and ompK36-sl2 was the insertion of amino

TABLE 1 | Characteristics of the $bla_{OXA-181}$ producing *K. pneumoniae* isolates.

Strain No	Unit**	Stay length*** (days)	Specimen	ST	CUHK_SL	Other plasmids replicons	*ompK36* PEFXG insertion	MICs(mg/L)*										
								ETP	IPM	MEM	CTX	CAZ	AK	GN	CIP	TG	CT	FOS
SL33	S	–	ET secretion	437	A	IncFIB, IncFII	No	2	0.25	0.25	>128	64	2	64	64	1	0.12	4
SL49	M	7	ET secretion	437	A	IncFIB	No	4	0.5	0.25	128	128	4	0.5	128	1	0.25	4
SL54	S	–	ET secretion	147	C	IncFIB, IncR	No	4	1	0.5	>128	64	4	64	128	2	0.12	8
SL34	M	4	Sputum	147	B	IncFIB, IncA/C2, IncR	Yes	32	4	32	>128	>128	4	64	128	0.5	0.5	8
SL35	M	6	Sputum	147	B	IncFIB, IncR	Yes	32	4	32	>128	>128	4	128	64	0.5	0.25	16
SL36	M	17	Sputum	147	B	IncFIB, IncFII	Yes	32	8	32	>128	32	2	1	8	1	0.12	16
SL46	M	9	ET secretion	147	B	IncFIB, IncFII	Yes	16	1	8	128	16	4	0.5	8	1	0.12	8
SL50	M	13	ET secretion	147	B	IncFIB, IncR	Yes	32	8	16	4	0.5	1	128	32	1	0.25	32
SL52	S	–	ET secretion	147	B	IncFIB, IncR	Yes	32	2	32	>128	>128	4	128	16	1	0.25	64
SL68	M	4	Sputum	147	B	IncFIB, IncR	Yes	32	8	32	>128	>128	16	1	8	1	0.25	64

AK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; GN, gentamicin; CT, colistin; CTX, cefotaxime; ETP, ertapenem; FOS, fosfomycin; IPM, imipenem; MEM, meropenem; TG, tigecycline.

***M – Main study unit; S – Subsidiary unit.*

****Length of stay from date of specimen collection.*

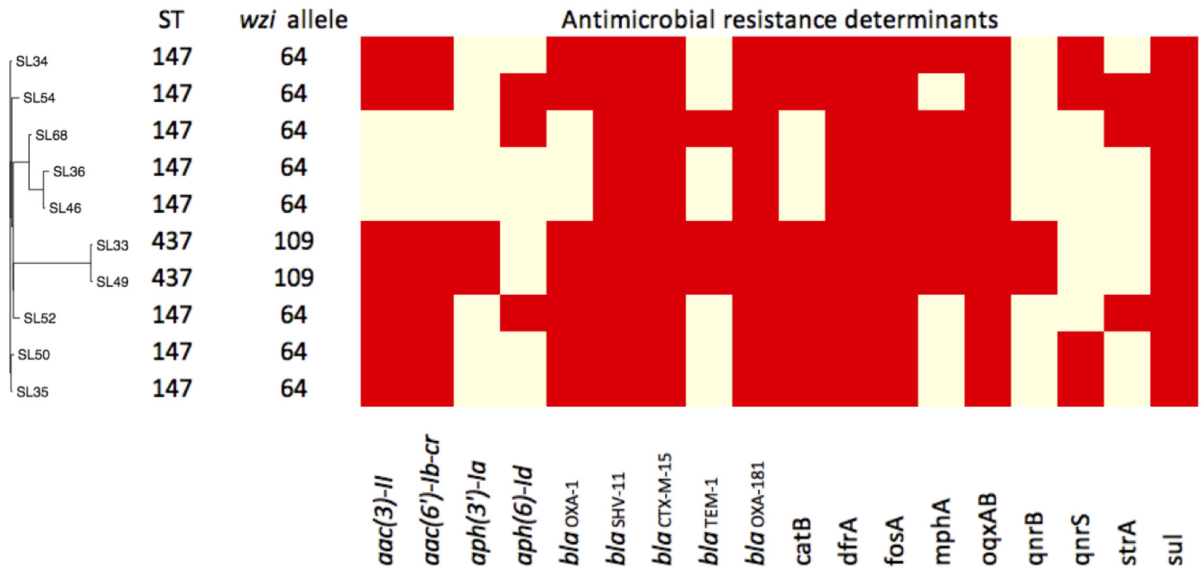

FIGURE 1 | Pan-genome dendrogram of 10 Sri Lanka strains with annotation. *Red boxes indicate presence and yellow for absence.

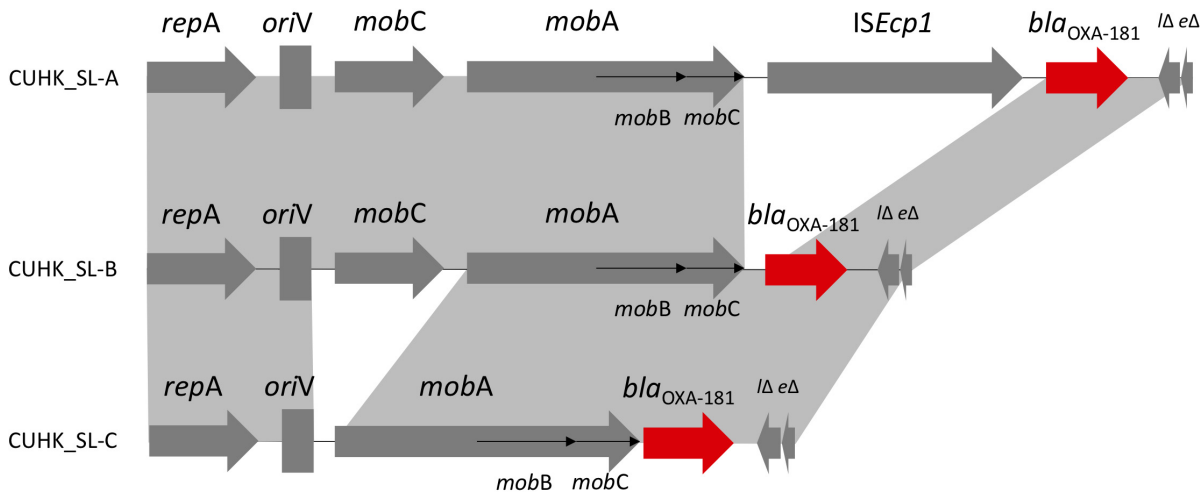

FIGURE 2 | ColE-type plasmids in Sri Lanka. *CUHK_SL-B had IS*Ecp1* deletion; CUHK_SL-C had IS*Ecp1* and *mobC* deletion.

acids glycine and aspartate after PEFXG domain within the L3 loop and one mutation in loop L4 and alpha-helix, respectively. The PEFXG domain (porin size determinant) insertion was also observed in several studies that addressed strains with high resistance to carbapenems (Garcia-Fernandez et al., 2010; Hall et al., 2014; Zhang et al., 2014). OmpK36-sl3 was different from the others in the region between loop L3 and loop L6, but there was no insertion interruption in the PEFXG domain.

The *K. pneumoniae* isolates belonged to ST437 (2 strains) and ST147 (8 strains) (**Table 1**). Capsular genes showed two *wzi* alleles 64 and 109 in strains ST147 and ST437, respectively (**Figure 1**). Only one carbapenemase gene, *bla*$_{OXA-181}$ was found in all isolates. All isolates harbored β-lactamase genes, including *bla*$_{TEM-1}$, and *bla*$_{OXA-1}$, *bla*$_{SHV-11}$, and to extended-β-lactamase, *bla*$_{CTX-M-15}$. Plasmid-mediated quinolone resistance (PMQR) determinants (*qnr*, *aac(6′)-Ib-cr*, and *oqxAB*) were also detected. We also found two amino acid substitutions at *gyrA* (S83I) and *parC* (S80I) in all strains, which have been frequently reported in quinolone-resistant *K. pneumoniae* worldwide (Aldred et al., 2014).

The virulence profiles among the 10 strains were the same and all carried yersiniabactin genes (*ybt*, *irp1*, *irp2*, and *fuyA*) and *kfu*, *mrk*, but were absent of *rmpA* or *rmpA2* genes. Yersiniabactin genes were found on mobile element ICE*kp*. 4437 genes and 2182 accessory genes were used to establish the dendrogram of their pan-genomes (**Figure 1**). The distinct accessory gene profiles of ST437 and ST147 indicated that ST437 was a distant cluster.

The pan-genome dendrogram also revealed a cluster of ST147 (SL36, SL46, and SL68) that contained fewer resistance genes (**Figure 1**) and were sensitive to GN and with lower MIC (8 mg/L) to CIP compared with other ST147 strains (**Table 1**).

Plasmids Harboring $bla_{OXA-181}$

Three different ColE-type plasmids were identified (**Figure 2**). One plasmid (CUHK_SL-A) was identical to KP3 (GenBank accession no. JN205800) and was found in the two ST437 strains. CUHK_SL-A was a short plasmid (7,606 bp) harboring only one resistance gene and was previously well described (Potron et al., 2011a). Another plasmid (CUHK_SL-B) was found in 7 ST147 strains of this study with a deletion of an insertion sequence (IS*Ecp1*) when compared to CUHK_SL-A. Several studies from the United States, Germany, and France have reported this plasmid in both *K. pneumoniae* and *Escherichia coli* (GenBank Accession No. CP006802, CP016038, and JX423831). The third plasmid (CUHK_SL-C) was found in strain SL54 with a **mobile gene deletion (*mobC*) when compared to CUHK_SL-B. This plasmid was not reported before.

DISCUSSION

Carbapenemase $bla_{OXA-181}$ was first described in India as a Class D $bla_{OXA-48-like}$ enzyme from clinical samples in year 2006 and 2007 (Castanheira et al., 2011). It was thought to originate from an environmental strain as a chromosomal gene (Potron et al., 2011b). Although it has been detected worldwide, most of the patients have a travel history to the Indian subcontinent, especially India (Poirel et al., 2012). In 2014, $bla_{OXA-181}$ and bla_{NDM} were reported in *K. pneumoniae* in Sri Lanka, and included mainly ST14 and ST147 (Hall et al., 2014). ST437 belongs to CC258 and is a single locus variant of the globally prevalent ST258, carrying bla_{KPC} in Brazil and bla_{NDM}, $bla_{OXA-245}$ (plasmid: IncL/M) in Spain (Seki et al., 2011; Oteo et al., 2013). $bla_{OXA-181}$ was previously described in plasmid ColE, IncT and in the chromosome, which were all isolated from patients transferred from India, as well as described in plasmid IncX3 from China (Potron et al., 2011a; Dimou et al., 2012; Villa et al., 2013; Kayama et al., 2015; Liu et al., 2015; Partridge et al., 2015). ColE plasmid encoding various β-lactamase genes and $bla_{OXA-181}$ is related to transposon Tn*2013*. Insertion sequence IS*Ecp1* was considered to be associated with $bla_{OXA-181}$ acquisition, and its deletion may stabilize the resistance gene in the plasmid (Potron et al., 2013). *mobC* gene deletion in the strains may affect the frequency of conjugal plasmid mobilization (Zhang and Meyer, 1997).

In this study, all strains harbored quinolone-resistant determinants with quinolone resistance-determining region (QRDR) mutation on their chromosomes. A recent epidemiology study has shown the correlation of quinolone consumption and CRE in United States military health system, and another case-control study of CRE outbreaks in the Netherlands have determined quinolone use as the only risk factor for the acquisition of $bla_{OXA-48-like}$ producing *Enterobacteriaceae* compared to other antibiotics use (Lesho et al., 2015; Dautzenberg et al., 2016). Possible mechanisms may be due to co-transfer of two plasmids bearing dual resistance mechanisms or recombination into one plasmid as described before (Rodríguez Martínez et al., 2014; Liu et al., 2015). Furthermore, the impact of different plasmids and fluoroquinolone resistant determinants on fitness cost has been found to vary with strains of different STs. This may have contributed to the spread of this particular clone (Tóth et al., 2014; Johnson et al., 2015). It was suggested that energetically favorable mutations with the double serine mutations in the QRDR region in the current study have been described to favor the fitness and dissemination of such clones (Fuzi et al., 2017). Although all strains were non-hypervirulent *K. pneumoniae* (negative for *rmpA*/*rmpA2* genes, non K1/K2 capsule serotypes), all strains encoded several yersiniabactin genes. These genes being on the integrative conjugative elements (ICE*Kp*) can contribute to the spread of *K. pneumoniae* in the population and may serve as a predictor of invasive infection (Lam et al., 2018).

In conclusion, we explored the genetic profile of multidrug resistant CRE in Sri Lanka. $bla_{OXA-181}$ (through ColE-type plasmid) and yersiniabactin genes have disseminated to different STs of *K. pneumoniae*. We recommend active surveillance of high risk inpatients and long term studies to determine possible intra-unit transfer and facilitate infection control, especially as the length of stay in all instances where data was available is >72 h. Judicious antibiotics use especially that of quinolones is also recommended.

AUTHOR CONTRIBUTIONS

VP and ND conceptualized, collected, preliminarily analyzed, and approved the manuscript. MI, MH, and VL conceptualized, conducted, analyzed, drafted, and approved the manuscript. CZ, NL, KW, and CL contributed to sample storage and preparation, molecular analysis, and approval of the manuscript. CZ prepared the first draft of the manuscript.

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Genotypic and Phenotypic Characterization of IncX3 Plasmid Carrying *bla*$_{NDM-7}$ in *Escherichia coli* Sequence Type 167 Isolated From a Patient With Urinary Tract Infection

*Yingying Hao, Chunhong Shao, Yuanyuan Bai and Yan Jin**

Department of Clinical Laboratory, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, China

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Edited by:
Yi-Wei Tang,
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Zhejiang University School of Medicine, China

***Correspondence:**
Yan Jin
sdslyyjy@163.com

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Infections due to New Delhi metallo-beta lactamase (NDM)-7-producing *Escherichia coli* are infrequent and sporadic. In this study, we report one case of recurrent urinary tract infection caused by *bla*$_{NDM-7}$-producing *E. coli* belonging to phylogenetic group A, sequence type (ST) 167. In this study, we aimed to describe the genotype and phenotype of *bla*$_{NDM-7}$-producing *E. coli* in China. The isolate exhibited resistance to β-lactam antimicrobials, trimethoprim-sulfamethoxazole, quinolones, and aminoglycosides. *bla*$_{NDM-7}$ is located on a conjugative plasmid designated pJN05NDM-7 belonging to type IncX3. pJN05NDM-7 was fully sequenced and compared with all publicly available *bla*$_{NDM-7}$-harboring plasmids. pJN05NDM-7 is almost identical to pKpN01-NDM7 and pKW53T, although the plasmids are geographically unrelated. The comparison of IncX3 plasmids harboring *bla*$_{NDM}$ in China showed high similarity, with genetic differences within insertion fragments. Notably, the differences in plasmids of animal and human origin were insignificant, because only one plasmid showed deletion inside the ISAba125 region compared with pJN05NDM7. Our study demonstrates that *E. coli* carrying IncX3 plasmids play an important role as a reservoir and in the spread of *bla*$_{NDM}$. Further studies should be performed to control the dissemination of *bla*$_{NDM}$ among food animals.

Keywords: NDM-7, carbapenemase, *Escherichia coli*, multi-drug resistance, China

INTRODUCTION

New Delhi metallo-beta-lactamase (NDM)-producing bacteria are spread worldwide and pose a serious threat to public health, and is highly disseminated in China (Zhang et al., 2017; Liu et al., 2018). The surveillance for carbapenem-resistant *Enterobacteriaceae* (CRE) showed that *bla*$_{NDM}$ production was the second major mechanism of carbapenem resistance *in Escherichia coli*, and *bla*$_{NDM-1}$ was the most frequent variant (Khan et al., 2017). Since the first report on NDM-1

in 2009, 20 variants of NDM have been assigned in the Lahey Clinic database (Liu et al., 2018). NDM-7, which differs from NDM-1 by two point mutations corresponding to amino acid substitutions, was described in 2013 with increased carbapenemase activity compared with NDM-1 (Cuzon et al., 2013). bla_{NDM-7} is infrequently detected, and sporadic cases of infections due to bla_{NDM-7}-producing enterobacteria have been reported in France, India, the United States of America, and Japan (Cuzon et al., 2013; Chen et al., 2015; Wang et al., 2016; Devanga Ragupathi et al., 2017; Pal et al., 2017; Sugawara et al., 2017; Espinal et al., 2018). In China, bla_{NDM-7}-producing *E. coli* ST131 was first reported in 2016; however, the genetic content of bla_{NDM-7}-harboring plasmids was not clearly described (Wang et al., 2016). Considering its global distribution, increasing attention should be paid to epidemiological survey of bla_{NDM-7}.

In this study, we detected a bla_{NDM-7}-producing *E. coli* isolate from a patient without a history of traveling admitted in a Chinese Hospital. To elucidate the molecular epidemiology and evolutionary dynamics involved in the dissemination of bla_{NDM}, the genomic content and in-depth molecular characterization of the strains was determined in this study.

MATERIALS AND METHODS

Bacterial Strains

The carbapenem-resistant *E. coli* strain JN05 was recovered from urine sample of a 61-year-old woman with recurrent urinary tract infection at a teaching hospital in Shandong Province of China in 2015. The patient was diagnosed with vesicovaginal fistula secondary to cervical cancer after chemotherapy and electrocautery surgery in 2009. According to the abdominal ultrasonography, the patient was diagnosed with hydronephrosis and hydroureter of upper segment on admission. After the treatment with multiple antibiotics failed, nephrostomy was performed to improve hydronephrosis. There was no history of traveling abroad. Informed consent was signed by the patient involved in this study. The methods in this study were approved by the Ethics Committee of Shandong Provincial Hospital and were carried out in accordance with the approved guidelines. The strain obtained from the patient was identified as *E. coli* by using Vitek-2 compact system and confirmed by Vitek-MS system (BioMérieux, France). Phenotypic detection of carbapenemases was performed using carbapenem inactivation method (CIM) and EDTA-modified CIM (eCIM) test.

Antibiotic Susceptibility Assay

Susceptibility assay of antibiotics was performed on Mueller-Hinton (MH) agar plates using E test strips (**Table 1**). Susceptibility assay results were interpreted by Clinical Laboratory Standards Institute (CLSI) breakpoints (CLSI, 2017), with the exception of tigecycline, polymyxin B, and fosfomycin, which were interpreted by EUCAST breakpoints (EUCAST, 2017).

TABLE 1 | Antibiotic susceptibilities of *Escherichia coli* JN05 and its transconjugant.

Isolates	Minimal inhibitory concentrations (μg/mL)														
	TZP	ATM	CZO	CRO	CAZ	FEP	FOX	IMP	MEM	ETP	AK	CN	CIP	LEV	SXT	FOS	TGC
JN05	> = 256	> = 256	> = 256	> = 256	> = 256	> = 256	> = 256	> = 32	> = 32	> = 32	> = 256	> = 256	> = 32	> = 32	> = 32	2	0.38
J05	> = 256	0.64	> = 256	> = 256	> = 256	16	> = 256	16	8	12	1	0.5	< = 0.02	< = 0.02	0.32	2	0.38
J53Azi^R	< = 0.016	< = 0.016	< = 0.016	< = 0.016	< = 0.016	< = 0.016	< = 0.016	< = 0.02	< = 0.02	< = 0.02	< = 0.016	< = 0.016	< = 0.02	< = 0.02	< = 0.02	2	0.38

TZP, piperacillin/tazobactam; ATM, aztreonam; CZO, cefazolin; CRO, cefatriaxone; FEP, cefepime; FOX,cefoxitin; IPM, imipenem; MEM, meropenem; ETP, ertapenem; AK, amikacin; CN,gentamicin; CIP, ciprfloxacin; LEV,levofloxacin; STX, trimethoprim/sulfamethoxazole; FOS, fosfomycin; and TGC, tigecycline.

Molecular Typing

Multilocus sequence typing (MLST) and phylogenetic typing was performed for molecular typing of the isolate as previous described (Wirth et al., 2006; Wang et al., 2016). The virulence factors of extraintestinal pathogenic *E. coli* (ExPEC)-associated genes were screened by PCR-based assays (Wang et al., 2016).

Screening of Antibiotic Resistance Genes

Antimicrobial resistance genes were screened by PCR and DNA sequencing as described previously (Zhu et al., 2016). These antimicrobial resistance genes included carbapenemase-encoding genes, extended-spectrum β-lactamase genes, AmpC β-lactamase genes, 16S rRNA methylase genes, fosfomycin resistance genes, quinolone resistance genes, and polymyxin B resistance genes (mcr-1) (Du et al., 2016; Zhu et al., 2016).

Analysis of bla_{NDM}-Carrying Plasmids

Conjugation test was performed by mixed broth method using *E. coli* J53Azi^R as the recipient strain. Transconjugants were selected on MH agar plates containing 6 μg/mL ceftazidime and 100 μg/mL sodium azide. The antimicrobial susceptibility test of the transconjugant was carried out as antibiotic susceptibility assay of clinical strain.

The size and amounts of plasmids carried by the clinical isolate and transconjugant were evaluated by S1-pulsed-field gel electrophoresis (PFGE) as previously described (Liu et al., 2018).

Plasmid Sequencing

The plasmid pJN05NDM carrying bla_{NDM-7} (present in strain JN05) was extracted and sequenced using an Illumina Hiseq platform and assembled by SOAPdenovo at the MajorBio Co (Shanghai, China). The gaps were closed through PCR and Sanger Sequencing at Sangon Biotech (Shanghai, China). The plasmid sequences were annotated by BLAST against the non-redundant protein database. PlasmidFinder was used for detection and typing of the plasmid.

RESULTS

Resistance Profile of JN05 Strain

The carbapenem-resistant *E. coli* isolate JN05 was identified as metallo-beta-lactamase (MBL)-producing strains by eCIM. The JN05 strain was resistant to aztreonam, carbapenems, cephalosporins, quinolones, aminoglycosides, piperacillin-tazobactam, and trimethoprim-sulfamethoxazole, but was susceptible to fosfomycin, polymyxin B, and tigecycline (**Table 1**).

Molecular Grouping, Resistance Genotyping, and Virulence Genotyping

The *E. coli* strain JN05 was assigned to ST167 and belonged to phylogenetic group A. It carried *papG* II, which may play an important role in the pathogenic process. Multiple antimicrobial resistance genes, including bla_{NDM-7}, $bla_{CTX-M-3}$, $bla_{CTX-M-14}$, bla_{TEM-1}, *qnrS, armA*, and *acc(6′)-Ib* genes, are responsible for the resistance profile of strain JN05.

Analysis of the Plasmid Harboring NDM

New Delhi metallo-beta-lactamase -harboring plasmid of strain JN05 was successfully transferred into *E. coli* J53Azi^R by conjugation experiment. The presence of NDM-7 in the transconjugant was confirmed using PCR, and MLST was used to distinguish the transconjugants from the clinical strain. The transconjugant J05 was susceptible to aztreonam, quinolones, and aminoglycosides, but resistant to carbapenems and cephalosporin. S1-PFGE showed that the clinical strain JN05 harbored six plasmids, and the transconjugant J05 contained a single plasmid, which was approximately 46 Kb (**Supplemental Figure S1**).

pJN05NDM-7 is a 46,161-bp plasmid belonging to the IncX3 incompatibility group. The complete sequence of plasmid pJN05NDM-7 was submitted to GenBank under accession number MH523639. In pJN05NDM-7, bla_{NDM-7} was preceded by IS*3000*-IS*Aba125*-IS*5* in the upstream region and followed by *ble-trpF-dsbC*-IS*26-ΔumuD* in the downstream region. This bla_{NDM} genetic structure was common in *Enterobacteriaceae* for the horizontal transfer of bla_{NDM} (Pal et al., 2017).

The full published sequences of seven plasmids harboring NDM-7 were downloaded and compared, including pKW53T-NDM (Accession No. KX214669), pEC50-NDM-7 (Accession No. KX470735), pKPN01-NDM-7 (Accession No. NZ_CP012990), pOM26-1 (Accession No. KP776609), pM110_X3 (Accession No. AP018141), pABC218-NDM (Accession No. KX214670), and pABC133-NDM (Accession No. KX214671) (Espedido et al., 2015; Pal et al., 2017). Sequence alignments revealed that pJN05NDM-7 was 100% identical to the previously described plasmid pKW53T-NDM of *E. coli* isolated in Kuwait (KW53T). pJN05NDM-7 plasmid showed high overall nucleotide identity (99%) with pEC50-NDM-7 from China and pKPN01-NDM-7 from Canada. In addition, pJN05NDM-7 was similar to the plasmid pOM26-1 isolated from Oman, and pABC218-NDM and pABC133-NDM from UAE. However, they lacked mobile genetic elements or even the topoisomerase III gene (**Figure 1**).

To explore the geographic distribution of IncX3 plasmids harboring bla_{NDM} in China, 29 plasmids were screened and analyzed, including two plasmids from North China (pEc1929, pNDM5-E6CN), 14 plasmids from East China (NUHL24835, p112298-NDM, pAD-19R, pNDM-5_IncX3, pNDM5-SSH006, pNDM-20, pNDM-QD28, pNDM-QD29, RJA274, pYE315203, pYQ13500-NDM, pZHDC33, pZHDC40, and pJN05NDM), six plasmids from South China (p112298-NDM, pCREC-A6-NDM, pNDM-HF727, pP785-NDM5, pP788A-NDM5, and pP855-NDM5), four plasmids from Central China (pEC50-NDM-7, pNDM-HN380, pP744T-NDM5, and pP768-NDM-5), and three plasmids from West China (pECNDM101, p3-NDM, and pSCE516-2) (**Supplementary Figure S2**).

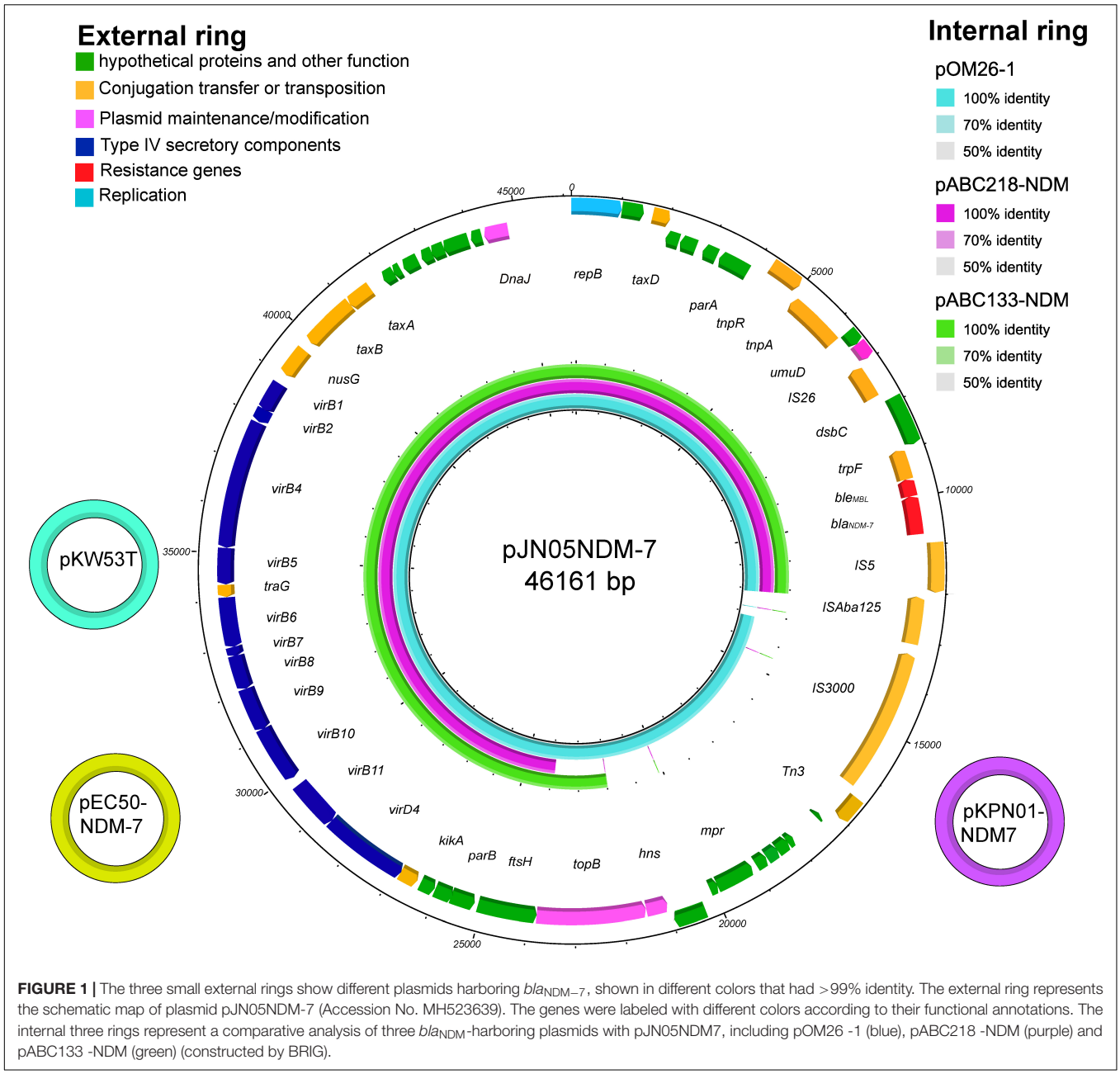

FIGURE 1 | The three small external rings show different plasmids harboring bla_{NDM-7}, shown in different colors that had >99% identity. The external ring represents the schematic map of plasmid pJN05NDM-7 (Accession No. MH523639). The genes were labeled with different colors according to their functional annotations. The internal three rings represent a comparative analysis of three bla_{NDM}-harboring plasmids with pJN05NDM7, including pOM26 -1 (blue), pABC218 -NDM (purple) and pABC133 -NDM (green) (constructed by BRIG).

Multiple NDM variants were harbored in the plasmids, including NDM-1, NDM-5, NDM-7, NDM-13, NDM-17, and NDM-20. We observed that the IncX3 plasmids carrying bla_{NDM-5} originating from different provinces of China showed high similarity, except three plasmids with various lengths of insertion sequences (pP744, pRJA274, and pZHDC40). Six IncX3 plasmids originating from pigs and one plasmid from chicken were identical to pNDM-HN380, thus confirming that this mobile NDM vector is widespread in China (He et al., 2017; Ho et al., 2018).

As obvious differences were observed among sequences of pJN05NDM-7, pP744, pRJA274, and pZHDC40, linear structural comparison of whole genome sequences of pJN05NDM with the above plasmids was performed (**Figure 2**). The backbone of these plasmids showed high degrees of conservation and similarity, with sequence polymorphism at the region of additional insertion around the NDM gene. The plasmids did not carry any resistance genes other than NDM, except pRJA274. pRJA274 is a 53,134-bp circular IncX3 type plasmid haboring two resistance genes including bla_{NDM-1} and bla_{SHV-12}. pRJA274 is almost identical to pJN05NDM-7, but the IS*Aba125* element (935-bp) between IS*3000* and IS*5* at the nucleotide position 40,753 was missing. In addition, the backbone of pRJA274 shared identity with plasmid pIncX-SHV. Compared with pJN05NDM-7, the inserted sequence of pP744 was different, with a deletion of 543 bp at IS*Aba125* located downstream of IS*5*. In pZHDC40,

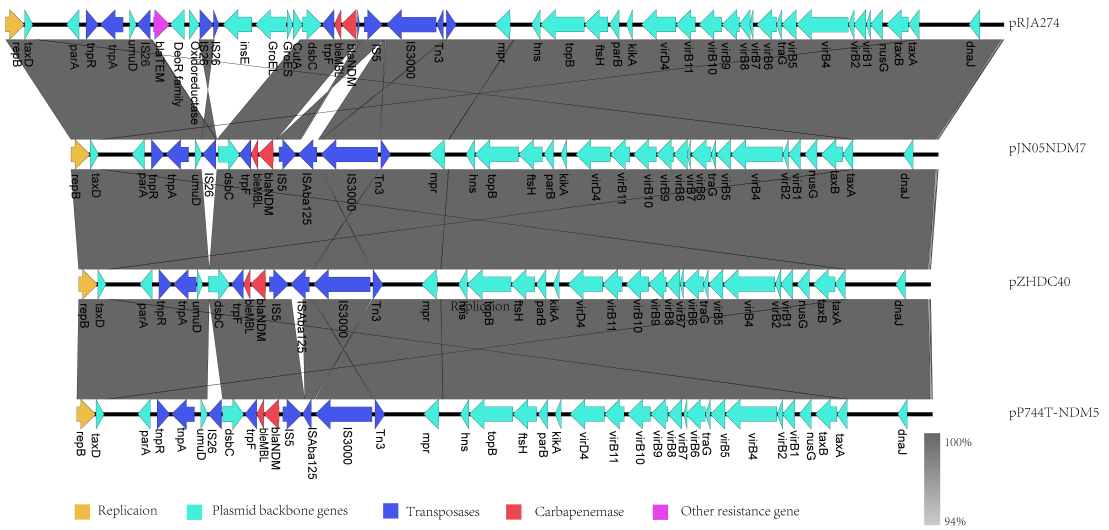

FIGURE 2 | Comparison of the IncX3 plasmids harboring bla_{NDM} from China with pJN05NDM7.

deletion of 816 bp at IS*26* downstream of NDM-7 was observed.

DISCUSSION

In this study, we aimed to evaluate the genotype and phenotype of bla_{NDM-7} -producing *E. coli* in China and found that *E. coli* carrying IncX3 plasmids play an important role as a reservoir and in the spread of bla_{NDM}.

Although ST131 is the most prevalent strain type of *E. coli* worldwide, ST167 is considered to be related to clinical infections in China (Yang et al., 2014). In this study, JN05 assigned to ST167 was isolated from a 61-year-old woman with recurrent urinary tract infection. The isolate JN05 belonged to phylogroup A and was positive for *papG II*, which increased the ability of P-fimbriae adhesin (Wang et al., 2016). In addition, this isolate contained multiple resistance genes, including bla_{TEM-1},$bla_{CTX-M-3}$, $bla_{CTX-M-14}$, bla_{TEM-1}, *qnrS, armA, and acc(6′)-Ib* genes; therefore, showed multidrug resistance and increased resistance to β-lactam drugs.

According to the surveillance of CRE strains in China, bla_{NDM} was mainly responsible for carbapenemase resistance in *E. coli*, while bla_{NDM-7} was relatively uncommon. Since the first report on clinical infection due to bla_{NDM-7} in France, this is the first report on fully sequenced plasmid carrying bla_{NDM-7} isolated from China.

Escherichia coli isolates carrying bla_{NDM-7} belonging to different STs were sporadic reported worldwide (Cuzon et al., 2013; Wang et al., 2016; Devanga Ragupathi et al., 2017; Pal et al., 2017; Espinal et al., 2018). According to previous reports, bla_{NDM-7} gene can be carried by several Enterobacter species and multiple types of plasmids including IncX3, IncF, and IncA/C groups, with sizes ranging from 37 to > 100 kb. IncX3, a self-conjugative plasmid, was most frequently observed to be the carrier of bla_{NDM-7}.

Interestingly, the plasmid pJN05 was identical to the plasmid pKW53T-NDM-7 isolated in Kuwait. Geographical contiguity or travel history could not be considered as a cause of resistance gene transmission, suggesting that the plasmids maybe native and not imported. We proved that plasmids harboring bla_{NDM} were hidden in the environment and in the human gut worldwide long before we identified them. It is possible that IncX3 plasmids carrying different variants originated from the same plasmid, but point mutations during transmission and evolution generated the differences. Exposure to carbapenem agents speed up the evolution of plasmids carrying bla_{NDM} variants and enhance enzyme activity toward carbapenems.

Notably, bla_{NDM}-producing isolates of animal origin increased, indicating that food animals have become the reservoir of bla_{NDM} (He et al., 2017; Kong et al., 2017). To understand the geographical distribution and gene polymorphism among the plasmids originating from different region, IncX3 plasmids carrying bla_{NDM} isolated from different areas were compared. Six plasmids harboring bla_{NDM} originating from pigs or chickens showed high similarity (> 99%) to those from patients. Because carbapenems were not approved for use in food animals in China, we assumed that the NDM-producing isolates were introduced to the farm via contaminated feed and water. The food animals contaminated by bacteria harboring bla_{NDM} accelerated the spread of resistance genes among healthy population. Nonetheless, more data are needed to explain the dissemination of bla_{NDM} among animals and humans.

CONCLUSION

In conclusion, this study identified self-transmissible IncX3 plasmids carrying bla_{NDM}, which were disseminated in geographically segregated areas in China and other countries in the world. This study emphasizes the important role of IncX3

plasmids in transmission of bla_{NDM} in China. Effective measures should be taken to monitor and control the rapid dissemination of bla_{NDM}.

AUTHOR CONTRIBUTIONS

YH and YJ contributed to experiment conception, design, and wrote the paper. CS and YB performed data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02468/full#supplementary-material

FIGURE S1 | S1-pulsed-field gel electrophoresis (S1-PFGE) patterns of clinical isolate JN05. **(A)** PFGE of clinical isolate JN05; **(B)** PFGE of transconjugant J05; M, marker, Salmonella enterica serotype Braenderup H9812.

FIGURE S2 | Alignment of pJN05NDM7 with 28 IncX3-typed plasmids harboring bla_{NDM} in China identfied in GenBank (To April 2018).

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The Simplified Carbapenem Inactivation Method (sCIM) for Simple and Accurate Detection of Carbapenemase-Producing Gram-Negative Bacilli

*Xiaopeng Jing[1], Huan Zhou[2], Xiaochun Min[1], Xing Zhang[1], Qing Yang[3], Shuaixian Du[4], Yirong Li[5], Fangyou Yu[6], Min Jia[7], Yu Zhan[8], Yi Zeng[8], Bo Yang[8], Yunjun Pan[9], Binghuai Lu[10], Rong Liu[2] and Ji Zeng[1]**

[1] Department of Clinical Laboratory, Wuhan Fourth Hospital, Puai Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Department of Pathophysiology, School of Basic Medicine, Key Laboratory of Ministry of Education of China for Neurological Disorders, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [3] Department of Clinical Laboratory, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China, [4] Department of Clinical Laboratory, Union Hospital, Huazhong University of Science and Technology, Wuhan, China, [5] Department of Clinical Laboratory, Zhongnan Hospital, Wuhan University, Wuhan, China, [6] Department of Clinical Laboratory, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China, [7] Department of Clinical Laboratory, Wuhan First Hospital, Wuhan, China, [8] Department of Clinical Laboratory, The Central Hospital of Wuhan, Wuhan, China, [9] Department of Clinical Laboratory, Renmin Hospital, Hubei University of Medicine, Shiyan, China, [10] Department of Laboratory Medicine, Civil Aviation General Hospital, Peking University Civil Aviation School of Clinical Medicine, Beijing, China

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Edited by:
Yi-Wei Tang,
Memorial Sloan Kettering Cancer Center, United States

Reviewed by:
Xian-Zhi Li,
Health Canada, Canada
Maurizio Sanguinetti,
Università Cattolica del Sacro Cuore, Italy

***Correspondence:**
Ji Zeng
whzjmicro@163.com

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This study reports the simplified carbapenem inactivation method (sCIM) to detect carbapenemase-producing gram-negative bacilli in a simple and accurate manner. This method is based on the modified carbapenem inactivation method (mCIM) with the improvement of experimental procedures. Instead of incubating the antibiotic disk in the organism culture media, the organism to be tested was smeared directly onto the antibiotic disk in the sCIM. For evaluating the sensitivity and specificity of the method, a total of 196 *Enterobacteriaceae*, 73 *Acinetobacter baumannii*, and 158 *Pseudomonas aeruginosa* isolates were collected. Polymerase chain reaction (PCR) was used to detect the carbapenemase genes. Phenotypic evaluations were performed using both the sCIM and the mCIM. PCR results showed that, of the 196 *Enterobacteriaceae* strains, 147 expressed the carbapenemase genes bla_{KPC-2} (58.5%), bla_{IMP-4} (21.8%), bla_{IMP-2} (2.0%), bla_{VIM-1} (6.1%), bla_{NDM-1} (10.2%), and bla_{OXA-48} (1.4%). sCIM results had high concordance with PCR results (99.5%) and mCIM results (100%) with the exception of one *Klebsiella pneumoniae* strain, which had an minimal inhibitory concentration (MIC) for imipenem of 0.25 mg/L. PCR demonstrated that 53 of the 73 *A. baumannii* isolates expressed the carbapenemase genes bla_{OXA-23} (98.1%) and bla_{VIM-2} (1.8%). sCIM and PCR results corresponded but all *A. baumannii* isolates were carbapenemase negative by the mCIM. PCR demonstrated that 25 of the 158 *P. aeruginosa* isolates expressed carbapenemase genes bla_{VIM-1} (52%), bla_{VIM-2} (8%), bla_{VIM-4} (36%), and bla_{IMP-4} (4%). sCIM results had high concordance with PCR results (100%) and the mCIM results (99.4%) with the exception of one *P. aeruginosa* isolate that expressed

the bla_{VIM-4} gene. The sCIM offers specificity and sensitivity comparable to PCR but has the advantage of being more user-friendly. This method is suitable for routine use in most clinical microbiology laboratories for the detection of carbapenemase-producing gram-negative bacilli.

Keywords: carbapenemase, modified carbapenem inactivation method, gram-negative bacilli, *Enterobacteriaceae*, simplified carbapenem inactivation method

INTRODUCTION

Carbapenemases can be divided based on their molecular characteristics into class A, B, or D using the Ambler classification system. Class A and D carbapenemases require serine at their active sites, whereas class B carbapenemases, also called metallo-β-lactamases (MBLs), require zinc for β-lactam hydrolysis (Jean et al., 2015; Bonomo, 2017; Khan et al., 2017). The most common class A carbapenemases are KPC enzymes, while notable transmissible class B carbapenemase include IMP, VIM, and NDM enzymes. Common class D carbapenemases include OXA-23-like, OXA-24-like, OXA-48-like, and OXA-58-like enzymes (Jean et al., 2015).

The prevalence of carbapenemases in gram-negative bacilli, especially carbapenemase-producing *Enterobacteriaceae* (CPE), *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, has increased markedly in the past 10 years (Jean et al., 2015; Logan and Weinstein, 2017). Genes encoding carbapenemases are often located on plasmids, facilitating the spread of carbapenem resistance between different bacteria (Jean et al., 2015). Carbapenem-resistant strains have caused difficulties in the clinical treatment and prevention of nosocomial infections.

The convenient and accurate detection of carbapenemases are of great clinical importance. CLSI (2010) introduced the modified Hodge test for carbapenemase detection, but this method can only be used for the accurate detection of KPC-type carbapenemases in *Enterobacteriaceae*. CLSI (2012) recommended the Carba NP test method for the detection of carbapenemases in gram-negative bacilli; however, the preparation of the reagents required for this test is complicated and the solutions cannot be stored for extended periods, limiting its clinical application. van der Zwaluw et al. (2015) designed a new detection method, carbapenem inactivation method (CIM), which is easy to operate and highly sensitive in the detection of carbapenemases. In 2017, based on the CIM method, CLSI recommended the modified carbapenem inactivation method (mCIM). This method is effective at detecting a variety of carbapenemases (CLSI, 2017; Pierce et al., 2017). However, it is a relatively complex method and can only be used to detect carbapenemases in *Enterobacteriaceae* and *P. aeruginosa* (CLSI, 2018). In the present study, based on the mCIM, we designed a simplified carbapenem inactivation method (sCIM) for simple and accurate detection of carbapenemases in gram-negative bacilli and compared it with polymerase chain reaction (PCR) and mCIM methods.

MATERIALS AND METHODS

Bacteria

To validate the sCIM, we collected 194 *Enterobacteriaceae*, 73 *A. baumannii*, and 158 *P. aeruginosa* clinical isolates from eight hospitals in China during 2017, and two OXA-48-producing *Klebsiella pneumoniae* and *Escherichia coli* from clinically conserved strains (Yu et al., 2017). Microorganisms were identified using the Microflex LT system (Bruker Daltonik GmbH, Bremen, Germany) and minimal inhibitory concentration (MIC) of imipenem, meropenem, and ertapenem were determined using the broth microdilution method. The 194 *Enterobacteriaceae* clinical isolates included 104 strains of *K. pneumoniae*, 72 strains of *E. coli*, and 18 strains of *Enterobacter cloacae*, in which 146 strains were resistant to imipenem and meropenem and five strains were resistant to ertapenem but susceptible to imipenem and meropenem. The two OXA-48-producing *K. pneumoniae* and *E. coli* were intermediate to imipenem, meropenem, and ertapenem. Fifty-three strains of *A. baumannii* and 149 strains of *P. aeruginosa* were resistant to imipenem and meropenem.

Modified Carbapenem Inactivation Method

In the mCIM, 1 μL loopfuls of *Enterobacteriaceae* or 10 μL loopfuls of *P. aeruginosa* or *A. baumannii* from blood agar plates was emulsified in 2 mL trypticase soy broth (TSB). A meropenem disk was then immersed in the suspension and incubated for a minimum of 4 h at 35°C. A 0.5 McFarland suspension of *E. coli* ATCC 25922 was prepared in saline using the direct colony suspension method. A Mueller–Hinton agar (MHA) plate was inoculated with *E. coli* ATCC 25922 using the routine disk diffusion procedure. The meropenem disk was removed from the TSB and placed on an MHA plate previously inoculated with the *E. coli* ATCC 25922 indicator strain. Plates were incubated at 35°C in ambient air for 18−24 h. An inhibition zone diameter of 6–15 mm or colonies within a 16–18 mm zone was considered to be a positive result, and a zone of inhibition ≥19 mm was considered to be a negative result (CLSI, 2017; Pierce et al., 2017; CLSI, 2018).

Simplified Carbapenem Inactivation Method

The sCIM is based on the mCIM with improvement of experimental procedures. Instead of incubating the antibiotic disk in the organism culture media for 4 h as in the mCIM, the organism to be tested was smeared directly onto an antibiotic

disk in the sCIM. To perform the sCIM, for *Enterobacteriaceae*, a 0.5 McFarland standard suspension (using the direct colony suspension method) of *E. coli* ATCC 25922 was inoculated onto the MHA plate, following the routine disk diffusion procedure; for *A. baumannii* and *P. aeruginosa,* a 0.5 McFarland standard suspension (using direct colony suspension method) of *E. coli* ATCC 25922 was diluted 1:10 in saline and inoculated onto the MHA plate, following the routine disk diffusion procedure. Plates were allowed to dry for 3–10 min. Then, 1–3 overnight colonies of the test organisms grown on blood agar were smeared onto an imipenem disk (10 μg; Oxoid, Hampshire, United Kingdom) (**Figure 1**) to allow one side of the disk was evenly coated with the test bacteria; immediately afterward, the side of the disk having bacteria was placed on the MHA plate previously inoculated with *E. coli* ATCC 25922. An imipenem disk placed on an MHA plate was used as the control. All plates were incubated at 35°C for 16–18 h in ambient air. Bacterial strains that produced carbapenemase can hydrolyze imipenem; hence. the susceptible indicator strain grew unchecked. In contrast, the zone of inhibition around the disk shows a diameter of 6–20 mm (**Figure 2**), or the satellite growth of colonies of *E. coli* ATCC 25922 around the disk with a zone diameter ≤22 mm (**Figure 3**), indicating that the isolate was capable of producing carbapenemase; a zone of inhibition ≥26 mm was considered to be a negative result; a zone of inhibition of 23–25 mm was considered to be a carbapenemase indeterminate result.

PCR Detection of Carbapenemase Genes

To perform PCR, primers were designed to detect the bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, $bla_{\mathrm{OXA-48-like}}$, and $bla_{\mathrm{OXA-23-like}}$ genes (**Table 1**). PCR was performed according to previously described procedures (Yigit et al., 2001; Woodford et al., 2006; Doyle et al., 2012). Briefly, 25 μL of PCR Master Mix (CWBio, Beijing, China) was mixed with 4 μL of forward and reverse primers and water to a final volume of 45 μL. Then, 5 μL of sample lysate from each test isolate was added to the mix. For *Enterobacteriaceae* and *P. aeruginosa*, the PCR program consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 10 s, annealing at 56°C for 30 s, elongation at 72°C for 90 s, and a final extension at 72°C for 2 min. For *A. baumannii,* the PCR program consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 25 s, annealing at 52°C for 40 s,

FIGURE 1 | The sCIM testing procedure. Bacteria to be tested were grown overnight on a blood agar plate. One to three overnight colonies were smeared onto an imipenem disk and the disk was then placed on the testing plate.

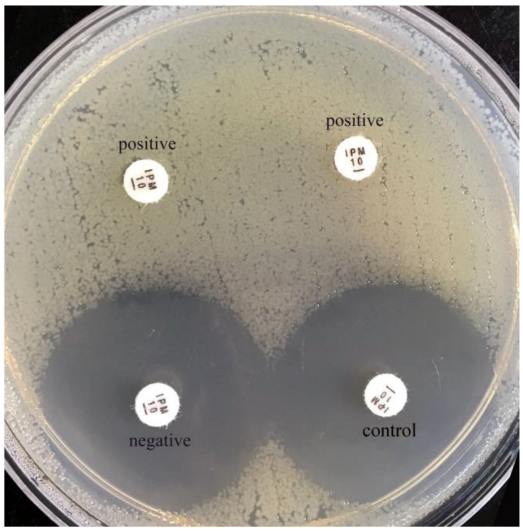

FIGURE 2 | Results of sCIM testing of gram-negative bacilli. The zones of inhibition of the negative isolate and the control were similar, whereas the zones of inhibition of the positive isolates (the left is a *K. pneumonia* producing KPC-2; the right is an *E. coli* producing NDM-1) were 6 mm.

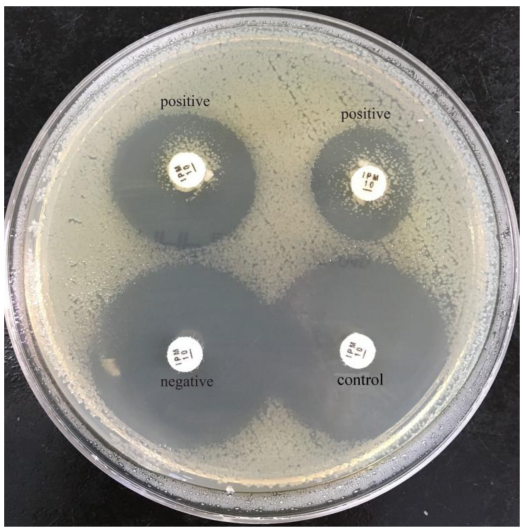

FIGURE 3 | Results of sCIM testing of gram-negative bacilli. Positive isolates (the left is a *P. aeruginosa* producing VIM-4; the right is an *A. baumannii* producing OXA-23) demonstrate satellite growth within the zone of inhibition around the disk.

TABLE 1 | Primers for the detection of carbapenemase-producing *Enterobacteriaceae.*

Carbapenemase gene	Primer sequences[a]	Amplicon size (bp)	Reference
bla_{KPC}	5′-TGTCACTGTATCGCCGTC-3′	1010	Khan et al., 2017
	5′-CTCAGTGCTCTACAGAAAACC-3′		
bla_{IMP}	5′-GAAGGCGTTTATGTTCATAC-3′	587	Jean et al., 2015
	5′-GTACGTTTCAAGAGTGATGC-3′		
bla_{VIM}	5′-GTTTGGTCGCATATCGCAAC-3′	389	Jean et al., 2015
	5′-AATGCGCAGCACCAGGATAG-3′		
bla_{NDM}	5′-GCAGCTTGTCGGCCATGCGGGC-3′	782	Jean et al., 2015
	5′-GGTCGCGAAGCTGAGCACCGCAT-3′		
$bla_{OXA-48-like}$	5′-GCGTGGTTAAGGATGAACAC-3′	438	Jean et al., 2015
	5′-CATCAAGTTCAACCCAACCG-3′		
$bla_{OXA-23-like}$	5′-GATCGGATTGGAGAACCAGA-3′	501	Li et al., 2012
	5′-ATTTCTGACCGCATTTCCAT-3′		

[a] *The first and second primers for each gene are forward and reverse primers, respectively.*

elongation at 72°C for 50s, and a final extension at 72°C for 6 min. PCR products were selected for sequencing and sequences were aligned using the BLAST software tool[1].

sCIM Detection on Positive Blood Cultures

To further confirm the sensitivity of the sCIM in clinical detection, in August 2018, 47 gram-negative bacilli of positive blood cultures collected from three hospitals were directly tested with the sCIM and antimicrobial susceptibility tests at the same time. The positive rate of carbapenemases-producing strains was analyzed to assess if the method may shorten the turnaround time (TAT).

RESULTS

Sensitivity and Specificity of sCIM

Of the 196 *Enterobacteriaceae* tested, 148 were shown to produce carbapenemase by the sCIM test, whereas 147 were shown to carry a carbapenemase-encoding gene by PCR. Both the OXA-48-producing *K. pneumoniae* and *E. coli* were carbapenemase positive by the sCIM and their MICs for imipenem were 2 mg/L. One *K. pneumoniae* isolate with an MIC for imipenem of 0.25 mg/L harbored CTX-M-15 and was found to be carbapenemase positive by the sCIM but carbapenemase negative by PCR. One *K. pneumoniae* isolate with an MIC for imipenem of 16 mg/L was found to be carbapenemase negative by both PCR and the sCIM, suggesting that this isolate may be a carbapenemase negative CRE (**Table 2**).

Of the 73 *A. baumannii* strains, 53 were resistant to imipenem. These 53 strains were all found to be carbapenemase positive by the sCIM. Fifty-two strains were found to carry the bla_{OXA-23} gene and one strain carry the bla_{VIM-2} gene by PCR. Twenty imipenem-susceptible *A. baumannii* strains were negative by both the sCIM and PCR. The concordance rate of the sCIM and PCR for *A. baumannii* was 100% (**Table 3**).

[1] https://blast.ncbi.nlm.nih.gov/Blast.cgi

Of the 158 *P. aeruginosa* strains tested, 149 were resistant to imipenem and 25 were found to be carbapenemase positive by the sCIM. The concordance rate of the sCIM and PCR for *P. aeruginosa* was 100% (**Table 3**).

In our experiment, the zones of inhibition of strains found to be carbapenemase negative by the sCIM ranged in size from 28 to 32 mm. In contrast, the zones of inhibition of strains found to be carbapenemase positive by the sCIM were 6 mm for *Enterobacteriaceae* isolates, 15 ± 4 mm for *A. baumannii* isolates, and 7 ± 3 mm for *P. aeruginosa* isolates (**Figures 2**, **3** and **Table 4**). Many small colonies were observed in the zones ≥12 mm around the imipenem disks, demonstrating the "satellite phenomenon" (**Figure 3**).

Carbapenemase Enzymes Produced by Tested Strains

In this study, 225 isolates were found to be positive for carbapenemase-encoding genes by PCR. The carbapenemases identified in *Enterobacteriaceae* isolates were KPC-2 (58.5%), IMP-4 (21.8%), IMP-2 (2.0%), VIM-1 (6.1%), NDM-1 (10.2%), and OXA-48 (1.4%) (**Table 2**). The carbapenemases identified in *A. baumannii* isolates were OXA-23 (98.1%) and VIM-2 (1.9%). The carbapenemases identified in *P. aeruginosa* were VIM-1 (52%), VIM-2 (8%), VIM-4 (36%), and IMP-4 (4%) (**Table 3**).

The Sensitivity and Specificity of the sCIM Is Comparable to the mCIM

The results of tests of 196 *Enterobacteriaceae* showed that the concordance rate of the sCIM and the mCIM was 100%, including one false positive. Of the 158 *P. aeruginosa* isolates, 25 were found to be carbapenemase positive by the sCIM and 24 were found to be carbapenemase positive by the mCIM. The results were inconsistent for one VIM-4-producing isolate. Of the 73 *A. baumannii* isolates, 53 were found to be carbapenemase positive by the sCIM but all were found to be carbapenemase negative by the mCIM (**Tables 2**, **3**). These results showed that the sCIM and the mCIM had similar rates of detection for CPE and *P. aeruginosa* and that the sCIM may be superior to the mCIM for the detection of carbapenemase-producing *A. baumannii*.

TABLE 2 | Laboratory detection of 196 *Enterobacteriaceae* isolates.

Enterobacteriaceae (n)	PCR (n)	MIC of imipenem (mg/L)			MIC of meropenem (mg/L)			MIC of ertapenem (mg/L)			sCIM		mCIM	
		≥4	2	≤1	≥4	2	≤1	≥2	1	≤0.5	+	−	+	−
K. pneumoniae (105)	KPC-2 (54)	54	0	0	54	0	0	54	0	0	54	0	54	0
	IMP-4 (19)	19	0	0	19	0	0	19	0	0	19	0	19	0
	IMP-2 (1)	1	0	0	1	0	0	1	0	0	1	0	1	0
	VIM-1 (6)	6	0	0	6	0	0	6	0	0	6	0	6	0
	NDM-1 (3)	3	0	0	3	0	0	3	0	0	3	0	3	0
	OXA-48(1)	0	1	0	0	1	0	0	1	0	1	0	1	0
	None (21)	1	0	20	1	0	20	4	0	17	1	20	1	20
E. coli (73)	KPC-2 (31)	31	0	0	31	0	0	31	0	0	31	0	31	0
	IMP-4 (9)	9	0	0	9	0	0	9	0	0	9	0	9	0
	IMP-2 (2)	2	0	0	2	0	0	2	0	0	2	0	2	0
	NDM-1 (5)	5	0	0	5	0	0	5	0	0	5	0	5	0
	OXA-48(1)	0	1	0	0	1	0	0	1	0	1	0	1	0
	None (25)	0	0	25	0	0	25	2	0	23	0	25	0	25
E. cloacae (18)	KPC-2 (1)	1	0	0	1	0	0	1	0	0	1	0	1	0
	IMP-4 (4)	4	0	0	4	0	0	4	0	0	4	0	4	0
	VIM-1 (3)	3	0	0	3	0	0	3	0	0	3	0	3	0
	NDM-1 (7)	7	0	0	7	0	0	7	0	0	7	0	7	0
	None (3)	0	0	3	0	0	3	0	0	3	0	3	0	3

+, positive; −, negative; sCIM, simplified carbapenem inactivation method; mCIM, modified carbapenem inactivation method.

TABLE 3 | Laboratory detection of carbapenemase-producing *A. baumannii* and *P. aeruginosa*.

Species (n)	PCR (n)	MIC of imipenem (mg/L)		MIC of meropenem(mg/L)		sCIM		mCIM	
		≥8	≤2	≥8	≤2	+	−	+	−
P.aeruginosa (158)	IMP-4 (1)	1	0	1	0	1	0	1	0
	VIM-1 (13)	13	0	13	0	13	0	13	0
	VIM-2 (2)	2	0	2	0	2	0	2	0
	VIM-4 (9)	9	0	9	0	9	0	8	1
	None (133)	124	9	124	9	0	133	0	133
A.baumannii (73)	OXA-23 (52)	52	0	52	0	52	0	0	52
	VIM-2 (1)	1	0	1	0	1	0	0	1
	None (20)	0	20	0	20	0	20	0	20

+, positive; −, negative; sCIM, simplified carbapenem inactivation method; mCIM, modified carbapenem inactivation method.

sCIM Detection on Positive Blood Cultures

A total of 47 gram-negative bacteria isolated from blood cultures in three hospitals were tested by the sCIM. Eight *K. pneumoniae* producing KPC-2 and one *A. baumannii* producing OXA-23 isolates were positive, with a positive rate of 19.1% (9/47). No false-negative strains were found. These data indicate that the sCIM can directly detect blood culture positive strains and report the enzyme-producing strains 1 day earlier than the routine antimicrobial susceptibility test.

DISCUSSION

The detection principles of the sCIM and the mCIM are similar, based on the fact that carbapenemases can hydrolyze carbapenem (Queenan and Bush, 2007; Jean et al., 2015; van der Zwaluw et al., 2015; Bonomo, 2017; Pierce et al., 2017). However, the strategies of the two methods to hydrolyze carbapenem are different. In the mCIM, the antibiotic disk is put into the TSB containing the test organisms for approximately 4 h, whereas in the sCIM, the test organisms are directly smeared onto the antibiotic disk. The mCIM requires TSB and MHA plates, while the sCIM only requires MHA plates. Thus, the operation in the sCIM experiment has fewer steps and is more convenient than the mCIM. When carbapenemase-producing bacteria is smeared on imipenem disks, the enzyme spreads and hydrolyzes the antibiotics on the paper, leading to a reduction in the size of the zone of inhibition.

Another difference of the sCIM compared with the mCIM is that the imipenem disk is selected. In general, the different types

TABLE 4 | Comparison of sCIM and mCIM results for selected strains.

Strain (n)	Type of carbapenemase	sCIM zone diameter (mm)	mCIM zone diameter (mm)
Enterobacteriaceae (147)	KPC-2, IMP-4, IMP-2, VIM-1, NDM-1, OXA-48	6	6
P. aeruginosa (23)	VIM-1, VIM-2, VIM-4	6	6–12
P. aeruginosa (1)	VIM-4	21*	22
P. aeruginosa (1)	IMP-4	6	6
A. baumannii (52)	OXA-23	6–20*	21–24
A. baumannii (1)	VIM-2	18*	24

*sCIM, simplified carbapenem inactivation method; mCIM, modified carbapenem inactivation method. *Satellite growth observed around the disk within the zone of inhibition.*

of carbapenemases have different hydrolysis rates for different carbapenem (Jean et al., 2015). For most carbapenemases, the *K*m values for imipenem is much larger than that for meropenem (Queenan and Bush, 2007). Therefore, we compared the efficiency of different carbapenem disks in detecting carbapenemases in the current study. We found that the diameter of the zone of inhibition was 6 mm for all the testing CPEs when using the imipenem disk. When using the meropenem disk, the diameter of the zone of inhibition varied considerably in different CPEs, indicating that imipenem was more rapidly hydrolyzed by carbapenemases compared to meropenem. For an easier interpretation of the experimental results, we chose an imipenem disk for the sCIM test. Because a few of ESBLs or AmpC produced by *Enterobacteriaceae* might hydrolyze imipenem with low activity (Carvalhaes et al., 2010; Bialek-Davenet et al., 2017; van Boxtel et al., 2017), it may result in few false positives in the sCIM test. In our study, we discovered a false positive result in a *K. pneumoniae* strain producing CTX-15. but the MIC of imipenem was less than 2 mg/L for this isolate. However, no false-positive isolate was discovered from those with a MIC for imipenem $\geq$2 mg/L. Therefore, the sCIM may be suitable for the determination of carbapenem-nonsusceptible strains, while the carbapenem-susceptible strains may have few false positives.

The concentration of bacteria in the disk diffusion method can affect the diameter of the zone of inhibition. At the same concentration of the drug, the lower the concentration of the tested bacterial used, the larger the diameter of the zone of inhibition that will be obtained. Correspondingly, when the concentration of bacteria is lower, the change of the zone of inhibition is also greater upon the variation of antibiotics concentration. In general, the hydrolysis rate of carbapenem by carbapenemase-producing non-fermenting bacteria is weaker than that of CPE. Therefore, reducing the concentration of *E. coli* ATCC 25922 can increase the sensitivity of the sCIM in detecting non-fermenting bacteria. For initial experiments with non-fermenting bacteria, the concentration of *E. coli* ATCC 25922 was adjusted from the 0.5 McFarland standard suspension by 5-fold and 10-fold dilutions. We found that the results obtained using these two dilutions were similar for *A. baumannii*. However, only 24 strains of *P. aeruginosa* were carbapenemase positive at the fivefold dilution, whereas 25 strains were carbapenemase positive at the 10-fold dilution. Therefore, the concordance rate with PCR was higher at the 10-fold dilution than the 5-fold dilution. Based on these results, we selected a 10-fold dilution of the 0.5 McFarland standard suspension of *E. coli* ATCC25922 for experiments with *P. aeruginosa* and *Acinetobacter* spp.

PCR results showed that KPC, VIM, NDM, IMP, and OXA-type enzymes were produced by *Enterobacteriaceae*, *A. baumannii*, and *P. aeruginosa,* and the sCIM can be used for sensitive (100%) and specific (99.6%) detection of carbapenemase-producing gram-negative bacilli. Only one of the 226 sCIM positive isolates, a CTX-15-producing *K. pneumoniae* strain, caused a false positive in sCIM tests. Pierce et al. (2017) reported that TEM-1 and TEM-52 enzymes might cause false positives in mCIM tests, too. CLSI (2018) recommended that the mCIM be used to detect carbapenemases in *Enterobacteriaceae* and *P. aeruginosa*. We found that only one isolate carrying the gene encoding VIM-4 was carbapenemase negative by the mCIM in our tests. Therefore, we concluded that the sensitivity of the sCIM for detecting CPE and *P. aeruginosa* was similar to that of the mCIM.

The carbapenemases identified in *Enterobacteriaceae* isolates were mainly KPC-2 and IMP-4. Because mainland China is not an OXA-48-endemic area, no OXA-48 positive strains were found in our collected isolates. These results were similar to the distribution of enzymes reported in mainland China by Zhang et al. (2017). To confirm the capability of the sCIM to detect OXA-48 carbapenemase, we collected two clinically conserved OXA-48-producing strains. Both strains were positive by the sCIM and the mCIM. However, we still cannot completely evaluate the capability of the sCIM to detect OXA-48 because of the small number of tests, and more investigations are needed to further confirm the reliability of the sCIM in detecting OXA-48-expressing isolates.

Our experimental data showed that the majority of CRE was CPE (99.3%), and only one *K. pneumoniae* isolate with an MIC for imipenem of 16 mg/L was not producing carbapenemase. This isolate was resistant to beta-lactams and susceptible to aminoglycosides and fluoroquinolones. The ratio of carbapenemase-producing isolates was 16.8% in carbapenem-resistant *P. aeruginosa*, and was slightly higher than the data reported by Yin et al. (2018). Carbapenemase-producing isolates are only a small part of carbapenem-resistant *P. aeruginosa*, and the loss or alteration of OprD is thought to be the most prevalent mechanism for carbapenem resistance in *P. aeruginosa*

(Li et al., 2012; Yin et al., 2018). Among the 53 carbapenem-resistant *A. baumannii*, 52 produced OXA-23 type enzyme, and one produced VIM-2 type enzyme. The resistance mechanism of carbapenem-resistant *A. baumannii* is mainly related to carbapenemase (Jean et al., 2015).

Compared to other carbapenemase detection methods, the sCIM has several obvious advantages. First, the sCIM does not require special equipment or reagents, hence is less costly. Second, the sCIM is easy to perform using the conventional paper diffusion method and is not complicated. Third, the results can be easily assessed. Finally, the sCIM has a wide range of detection and can be used to detect CPE, *Acinetobacter* spp., and *P. aeruginosa*. The sCIM is suitable for routine use in most clinical microbiology laboratories to detect carbapenemase-producing bacteria and can contribute to the reduction of carbapenemase-producing gram-negative bacilli in hospitals.

AUTHOR CONTRIBUTIONS

XJ, XM, XZ, QY, SD, YL, MJ, YiZ, YuZ, BY, YP, FY, and BL isolated the bacteria and performed the laboratory measurements. JZ, XJ, and HZ made substantial contributions to conception and design. JZ and RL wrote and revised the manuscript. JZ drafted the manuscript. All authors read and approved the final manuscript.

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High Prevalence of *bla*$_{NDM}$ Variants Among Carbapenem-Resistant *Escherichia coli* in Northern Jiangsu Province, China

Ruru Bi[1†], *Ziyan Kong*[1], *Huimin Qian*[2], *Fei Jiang*[3], *Haiquan Kang*[3], *Bing Gu*[1,3*] and *Ping Ma*[1,3*]

[1] Medical Technology School, Xuzhou Medical University, Xuzhou, China, [2] Jiangsu Provincial Center for Disease Control and Prevention, Nanjing, China, [3] Department of Laboratory Medicine, Affiliated Hospital of Xuzhou Medical University, Xuzhou, China

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Liang Chen,
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Jian-Hua Liu,
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***Correspondence:**
Bing Gu
gb20031129@163.com
Ping Ma
672443193@qq.com

†Present Address:
Ruru Bi,
Department of Laboratory Medicine, Suzhou Science and Technology Town Hospital, Suzhou, China

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The continuous emergence of carbapenem-resistant *Escherichia coli* (CRECO) presents a great challenge to public health. New Delhi metallo-lactamase (NDM) variants are widely disseminated in China, so the research on the prevalence and transmission of diverse *bla*$_{NDM}$ variants is urgently needed. In the present study, 54 CRECO isolates were collected from 1,185 *Escherichia coli* isolates in five hospitals in Northern Jiangsu Province, China from September 2015 to August 2016. Antimicrobial susceptibility tests, PCR detection of resistance determinants, multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) were performed to characterize these strains. Plasmid conjugation experiments were carried out to determine the transferability of resistant genes from selected isolates. PCR-based replicon typing (PBRT), S1 nuclease-PFGE, and Southern blotting were conducted for plasmid profiling. Carbapenemase genes were detectable in all CRECO isolates, among which thirty-one CRECO isolates were found to carry *bla*$_{NDM-5}$ (54.7%), while, *bla*$_{NDM-1}$, *bla*$_{NDM-7}$, *bla*$_{NDM-4}$, *bla*$_{NDM-9}$, and *bla*$_{KPC-2}$ were identified in 14, five, two, one, and one isolates, respectively. MLST results revealed 15 different STs and four new STs were first reported to be linked with NDM-producing isolates. PFGE typing showed that no more than two isolates with the same ST appeared to the same band pattern except three ST410 isolates. Twenty-six selected NDM-producing isolates were successfully transferred to *E. coli* J53 by conjugation experiments. Notably, 50.0% (13/26) of *bla*$_{NDM}$ variants were found to be carried by ∼55 kb IncX3 plasmid. Our study reported a high prevalence of *bla*$_{NDM}$ variants, especially *bla*$_{NDM-5}$, in Northern Jiangsu province, China. Diverse *bla*$_{NDM}$ variants were mainly carried by ∼55 kb IncX3 plasmids, suggesting that the fast evolution and high transferability of this kind of plasmid promote the high prevalence of *bla*$_{NDM}$ variants. Therefore, large-scale surveillance and effective infection control measures are also urgently needed to prevent diverse *bla*$_{NDM}$ variants from becoming epidemic in the future.

Keywords: carbapenem, *Escherichia coli*, *bla*$_{NDM}$ variants, diversity, plasmid

INTRODUCTION

Carbapenem, a β-lactam that is highly potent against Gram-negative bacteria, has been recognized as a last resort for treating of infections caused by multidrug-resistant bacteria. However, the increasing number of carbapenem-resistant *Enterobacteriaceae* (CRE) is unexpected despite infection control efforts, and it poses a great challenge to clinic (Zilberberg and Shorr, 2013). Carbapenem resistance is predominantly attributed to the presence of carbapenemases, among which Class A (bla_{KPC}), Class B (bla_{NDM}, bla_{VIM}, bla_{IMP}), and Class D (bla_{OXA-48}) types are most common for *Enterobacteriaceae* (Walsh, 2010; Albiger et al., 2015). An emerging carbapenemase, New Delhi metallo-lactamase (NDM), was first reported in a Swedish patient with a hospitalization history in India, it exhibited resistant to all β-lactams except for monobactams, and has great potential to cause global health crisis (Yong et al., 2009). Initially, bla_{NDM} gene was endemic to India subcontinent and NDM-producing isolates tested worldwide have geographical links with these high prevalence areas. However, an increasing number of regions worldwide have reported that patients with bla_{NDM-}positive isolates have never been abroad, indicating that bla_{NDM} genes are also associated with some special clones (Leverstein-Van et al., 2010).

In China, since the first report of bla_{NDM} gene in four carbapenem-resistant *Acinetobacter baumannii* isolates (Chen et al., 2011), increasing *Enterobacteriaceae* have been identified as carriers of the bla_{NDM} gene. *Escherichia coli*, an important member of *Enterobacteriaceae*, are often spread globally through some epidemiological lineages. Although the prevalence of NDM-producing CRE strains is low, outbreaks caused by bla_{NDM}-positive isolates have been identified in several regions of China, indicating high transferability of the bla_{NDM} gene and the severity of infections caused by bla_{NDM-1}-positive organisms (Wang et al., 2014; Jin et al., 2015; Yu et al., 2016). Furthermore, Kaase et al. (2011) first reported a novel bla_{NDM} variant, bla_{NDM-2}, which differs by one amino acid substitution (Pro28Ala) from bla_{NDM-1}, and the subsequent discovery of other bla_{NDM} variants highlights the rapid evolution of this multi-drug resistance gene. In 2012, the NDM enzyme reservoir, India, first reported diverse bla_{NDM} variants among *Enterobacteriaceae* and bla_{NDM} variants exhibited higher minimum inhibitory concentration (MIC) levels of carbapenem compared with bla_{NDM-1} (Rahman et al., 2014). Although the bla_{NDM} gene is continuously recoverable in China, data on the prevalence and characteristics of bla_{NDM} variants among *Enterobacteriaceae* are still needed for preventing its transmission. Notably, a study conducted by Hu et al. (2017) have discovered that various species of bacteria harbored several kinds of bla_{NDM} variants in China, which were mainly carried by diverse plasmids with different sizes. In the present study, we reported a high prevalence of bla_{NDM} variants among *E. coli* from five hospitals in Northern Jiangsu Province, China. Moreover, these diverse bla_{NDM} variants were mainly located on the same plasmid.

MATERIALS AND METHODS

Study Design

From September 2015 to July 2016, five hospitals (two in Xuzhou, two in Suqian, and one in Lianyungang) in Northern Jiangsu Province of China collected 1,185 *E. coli* isolates to examine the prevalence and molecular epidemiology of carbapenem-resistance isolates. Initial species identification and antimicrobial susceptibility testing was performed by the Vitek 2 system (bioMe'rieux, France) and MALDI-TOF MS (Bruker Microflex LT, Bruker Daltonik GmbH, Bremen, Germany) according to the manufacturer's instructions.

Antimicrobial Susceptibility Testing

Initial susceptibility testing was examined by Vitek 2 system. Further MICtesting was conducted by agar dilution method for cefoxitin, ceftriaxone, ceftazidime, cefepime, aztreonam, amikacin, ciprofloxacin, tigecycline, and piperacillin/tazobactam. The MICs of imipenem, meropenem, and ertapenem were determined by E-tests. For colistin, MIC values were tested by broth microdilution method. The agar dilution method and E-test were performed according to the standard Clinical and Laboratory Standards Institute guideline (M100-S26) (CLSI, 2017). The breakpoints of Food and Drug Administration (FDA) and European Committee on Antimicrobial Susceptibility Testing were used for tigecycline and polymyxin, respectively.

Molecular Detection of Resistance Genes

DNA templates were prepared by alkaline lysis method using the kit (MoBio, USA). Carbapenemase genes (bla_{KPC} bla_{NDM}, bla_{SME}, bla_{GES},bla_{VIM}, bla_{IMP}, and bla_{OXA-48}) (Senda et al., 1996; Queenan et al., 2000; Poirel et al., 2004; Endimiani et al., 2008; Yang et al., 2012; Pereira et al., 2015; Al-Agamy et al., 2017), extended spectrumβ-lactamase genes (bla_{SHV}, bla_{TEM}, $bla_{CTX-M-1group}$, $bla_{CTX-M-2group}$, $bla_{CTX-M-8group}$, and $bla_{CTX-M-9group}$) (Schmitt et al., 2007; Yu et al., 2007), and plasmid-mediated AmpC genes(bla_{ACC}, bla_{FOX}, bla_{MOX}, bla_{DHA}, $bla_{CIT/SPM}$, and bla_{EBC}) (Pérez-Pérez and Hanson, 2002) were examined by PCR. *E. coli* ATCC25922 was used for the quality control. Positive amplifications were subject to Sanger sequencing (GENEWIZ Company, Suzhou, China).

PFGE and MLST

Molecular typing of 54 NDM-producing *E. coli* isolates was performed by pulsed-field gel electrophoresis (PFGE). The plugs containing genomic DNA were prepared according to the procedure described by Pereira et al. (2015). The DNA fragments digested with restriction endonuclease *XbaI* (TaKaRa Biotechnology, Dalian, China) were separated by PFGE on 1% SeaKem Gold agarose (Lonza, Rockland, ME, USA) using the CHEF Mapper XA PFGE system (Bio-Rad, USA) for 18 h at 14°C. The electrophoretic switch times were 6.8–35.4 s. *Salmonella* H9812 was used as reference marker. Dice coefficients was used to calculate the similarity of PFGE patterns. Dendrograms were constructed by the unweighted pair group method with arithmetic averages (UPGMA) using BioNumerics software

version 5.10. Isolates were categorized to be of the same cluster when their dice similarity index was ≥85%. Multi-locus sequence typing of *E. coli* was conducted by PCR as previously described (Wirth et al., 2006). The allelic profiles and sequence types were identified by amplifying and sequencing the seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA, recA*) according to the reference website (https://enterobase.warwick.ac.uk/species/index/ecoli). A minimum spanning tree of 54 bla_{NDM}-positive isolates was also constructed by BioNumerics software version 5.10.

Conjugation Assay

The conjugation experiment was implemented by mix broth mating among 26 selected isolates. The donor (clinical strains harboring the bla_{NDM} gene) and recipient (sodium azide-resistant *E. coli* J53) were mixed and cultured in broth at 37°C overnight. The transconjugants were selected on MH agar with sodium-azide (180 μg/mL) and imipenem (1 μg/mL).. Initial species identification was conducted by Vitek MS system. Transformants were regarded as transconjugants when it exhibited resistance to carbapenem and harbored the bla_{NDM} gene.

Plasmid Analysis

Twenty-six selected isolates, including bla_{NDM-5} and bla_{NDM-1} gene with different STs from the aforementioned five different hospitals and all bla_{NDM-4} (one isolates was lost in transit), bla_{NDM-7}, and bla_{NDM-9}-positive isolates, were subjected to further plasmid analysis. Incompatibility groups of plasmids extracted from transconjugants were determined by PCR-based replicon typing as described previously (Carattoli et al., 2005; Johnson et al., 2012). S1-PFGE and Southern blotting were conducted to isolate and locate resistance plasmids. Briefly, the gel plugs embedded with bla_{NDM}-positive isolates were digested with S1 nuclease (TaKaRa Biotechnology, Dalian, China) and linear plasmids were separated by CHEF-Mapper XA PFGE system (Bio-Rad) as described above. The universal primers (F: GAAGCTGAGCACCGCATTAG; R: GGGCCGTATGAGTGATTGC) were used for probe synthesis. The plasmid DNA were transferred to positive-charged nylon membranes (Millipore, USA), and DIG-labeled bla_{NDM-}specific probe served to hybridize plasmids according to the instructions of the DIG High Prime DNA Labeling and Detection Starter Kit (Roche, USA).

RESULTS

Clinical Data and Prevalence of Carbapenemase Genes Among CRECO

A total of 54 (4.56%, 54/1185) non-duplicate *E. coli* isolates that exhibited resistance to imipenem or meropenem were obtained from five hospitals in Northern Jiangsu Province, China. The Affiliated Hospital of Xuzhou Medical University (Hosptial A, $n = 18$), the Children's Hospital of Xuzhou (Hosptial B, $n = 13$), the People's Hospital of Suqian (Hosptial C, $n = 11$), the First People's Hospital of Suqian (Hosptial D, $n = 6$), and the Second People's Hospital of Lianyungang (Hosptial E, $n = 6$) were included. Among 54 CRECO isolates, 53 (98.1%, 53/54) were found to be bla_{NDM}-positive and 1 was bla_{KPC-2}-positive. Interestingly, five different bla_{NDM} variants were identified in this collection (**Figure 1**). Among them, the bla_{NDM-5} was the prevailing variant, accounting for 58.5% (31/53) of bla_{NDM}-positive isolates, followed by bla_{NDM-1} (26.4%, 14/53). Moreover, bla_{NDM-7}, bla_{NDM-4}, and bla_{NDM-9} genes were also identified in 5, 2, and 1 isolates, respectively. NDM variations in amino acid substitutions at various positions are shown in **Table 1**. The distribution of the bla_{NDM} variants in hospitals is depicted in **Figure 1**.

Antimicrobial Susceptibility Patterns and Prevalence of Additional Resistance Genes of NDM-Producing CRECO

Fifty-three NDM-producing CRECO isolates were resistant to all cephalosporins (cefoxitin, ceftriaxone, ceftazidime, and cefepime) and enzyme inhibitors (piperacillin/tazobactam) but remained susceptible to colistin and tigecycline. The resistance rates to aztreonam, amikacin, and ciprofloxacin were 84.9, 22.6, and 92.4%, respectively. As shown in **Table 2**, among 31 bla_{NDM-5}-positive isolates, 93.5% were resistant to ciprofloxacin, 74.2% to aztreonam, and 32.2% to amikacin. Resistance to aztreonam and ciprofloxacin were 85.7 and 92.9% among 14 bla_{NDM-1}-producing isolates and were susceptible to amikacin. As for bla_{NDM-7}-positive isolates, susceptibility was only found for amikacin. The bla_{NDM-4} and bla_{NDM-9} isolates were resistant to all antibiotics tested in this study except for colistin and tigecycline. One bla_{KPC-2}-positive isolate was resistant to amikacin, colistin, and tigecycline. Molecular features revealed that most CRECO isolates carried the ESBLs gene, AmpC gene, or both. Overall, bla_{CTX-M}, bla_{SHV}, and bla_{TEM} were identified in 42, 11, and 32 isolates, respectively. $bla_{CTX-M-15}$ was the most common ESBLs gene in our study, accounting for 26.4% followed by $bla_{CTX-M-55}$ ($n = 9$), $bla_{CTX-M-65}$ ($n = 8$), $bla_{CTX-M-14}$ ($n = 8$). $bla_{CTX-M-64}$ ($n = 1$), $bla_{CTX-M-90}$ ($n = 1$), and $bla_{CTX-M-123}$ ($n = 1$). All bla_{TEM} positive isolates were identified as bla_{TEM-1}. Moreover, 6 bla_{CMY-2}, 5 bla_{CMY-42}, and 1 bla_{DHA-1} were also identified.

Molecular Typing of CRECO

A total of 15 STs were identified in 54 CRECO isolates (**Figure 2**). Among 53 bla_{NDM}-producing isolates, ST167 was the most prevalent, accounting for 35.8% (19/53), followed by ST410 (16.9%, 9/53), ST617 (13.2%, 7/53), ST405 (5.6%, 3/53), ST155 (3.8%, 2/53), ST156 (3.8%, 2/53), ST361 (3.8%, 2/53), and ST2659 (3.8%, 2/53). ST90, ST224, ST46, ST648, ST2376, and ST2083 were identified in one isolate. ST167 and ST617 are different by one allele and both correspond to clonal complex CC10. Moreover, ST410 and ST90 belong to clonal complex CC23. One KPC-2-producing isolate belong to ST131. PFGE typing revealed that no more than two isolates with the same ST type appeared to the same band pattern except for three ST410 isolates from hospital B (**Figure 3**). Surprisingly, the two isolates (E11 and E28) from hospital A and B shared the same patterns, indicating the occurrence of cross-transmission between hospitals.

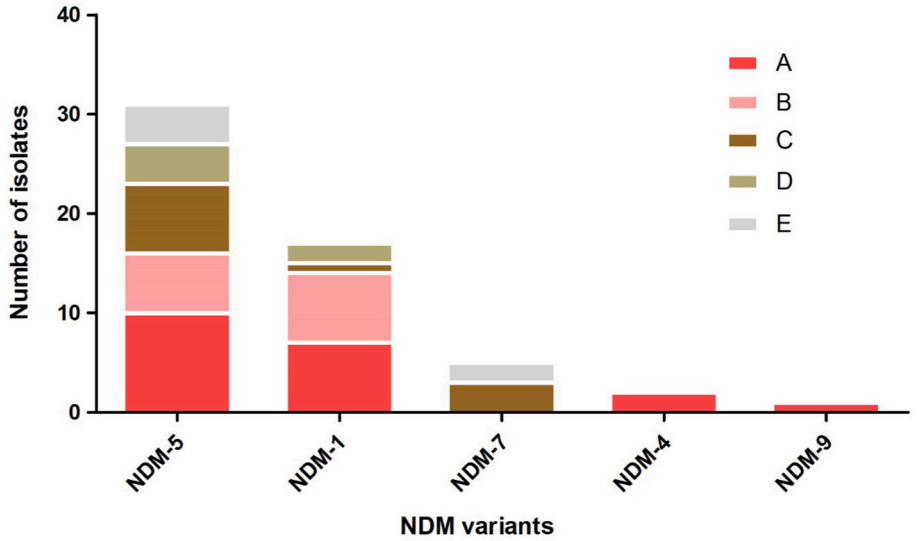


FIGURE 1 | The distribution of bla_{NDM} variants among different hospitals. Hospital A, the Affiliated Hospital of Xuzhou Medical University; Hospital B, the Children's Hospital of Xuzhou; Hospital C, the People's Hospital of Suqian; Hospital D, the First People's Hospital of Suqian; Hospital E, the Second People's Hospital of Lianyungang.

TABLE 1 | Amino acid substitutions of initially reported NDM variants.

NDM-type	Val 88	Asp 130	Glu 152	Met 154	Isolates	Country	Tourism history	GenBank accession no.
NDM-1	–	–	–	–	*K. pneumoniae, E. coli*	Sweden	India	FN396876
NDM-4	–	–	–	Leu	*E. coli*	Cameroon	Pakistan	JQ348841
NDM-5	Leu	–	–	Leu	*E. coli*	UK	India	JN104597
NDM-7	–	Asn	–	Leu	*E. coli*	UK	Spain	JX262694
NDM-9	–	–	Lys	–	*K. pneumoniae*	China	No	KC999080

Characteristic of Plasmids Harboring the bla_{NDM} Gene

All of plasmids haboring bla_{NDM} gene from 26 selected CRECO isolates were successfully transferred to *E. coli* J53, and transconjugants exhibited resistance to carbapenem, cephalosporins and enzyme inhibitors (**Table 3**). As shown in **Figure 4**, S1-PFGE and Southern blotting revealed that all bla_{NDM-5} genes were located on the same size (∼55 kb) plasmids, which was associated with IncX3 ($n = 5$), IncFI ($n = 3$), IncFII ($n = 1$), and untypeable replicon ($n = 3$). The bla_{NDM-1} genes were carried by 55∼210 kb plasmids, including IncX3 ($n = 5$), IncFI ($n = 1$), and IncFII ($n = 1$) replicon types. Among five bla_{NDM-7} positive isolates, four harbored ∼55 kb IncX3 plasmids with DIG-labeled bla_{NDM-7}, and the remaining one was carried by ∼110 kb IncFI plasmid. The bla_{NDM-4} and bla_{NDM-9} genes were located on ∼55 kb IncX3 and ∼110 kb IncI1 plasmids, respectively. Surprisingly, E5 isolates harbored three bla_{NDM-5}–positive plasmids of ∼55, ∼105, and ∼320 kb in size, suggesting high insertion efficiency of the bla_{NDM} gene. Further plasmid sequencing from E5 isolates revealed that the respective GenBank accession numbers for ∼55 and ∼105 kb plasmids were NC_022740.1 and AP018144.1, however, the nucleotide sequences of ∼320 kb plasmid could not be completely obtained.

DISCUSSION

Metallo-lactamase, NDM, is an emerging carbapenem-resistant β-lactamase that is of major public concern due to its high medical and economic burden (Otter et al., 2017), especially for developing countries such as India, Pakistan, and the Balkan countries. As the most populous country in the world, there are major difficulties in preventing the dissemination of multidrug resistant genes in China. Therefore, comprehensive, extensive studies on diverse bla_{NDM} variant-positive *E. coli* are needed to provide clear information to optimize antibiotic policy in endemic areas.

Generally, the prevalence of the bla_{NDM} gene has continuously increased worldwide. As of now, the NDM enzyme has been identified in almost all of the world, including many countries in Asia, Africa, Europe, the Americas, and Australia (Berrazeg et al., 2014). A study from India also analyzed the occurrence of the bla_{NDM} gene among carbapenem resistant isolates, and it accounted for 45.4% of them (Rahman et al., 2018). Recently,

TABLE 2 | Antimicrobial susceptible patterns of NDM-producing *Escherichia coli*.

Antibotic	Total ($n = 53$)		NDM-5 ($n = 31$)		NDM-1 ($n = 14$)		NDM-7 ($n = 5$)		NDM-4 ($n = 2$)		NDM-9 ($n = 1$)	
	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
IMP	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
MEP	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
ETP	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
FOX	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
CRO	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
CAZ	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
FEP	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
ATM	15.1	81.1	22.6	74.2	7.1	85.7	0.0	100.0	0.0	100.0	0.0	100.0
AMK	75.4	22.6	67.7	32.2	92.9	0.0	60.0	40.0	0.0	100.0	0.0	100.0
CIP	5.7	92.4	6.5	93.5	7.1	92.9	0.0	80.0	0.0	100.0	0.0	100.0
TZP	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
TGC	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0
COL	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0

IMP, imipenem; ETP, ertapenem; MEP, meropenem; PB, colistin; TGC, tigecycline; ATM, aztreonam; FOX, cefoxitin; FEP, cefepime; CA, ceftazidim; CRO, cefatriaxone; YZP, piperacillin/tazobactam; AMK, amikacin; CIP, ciprofloxacin.

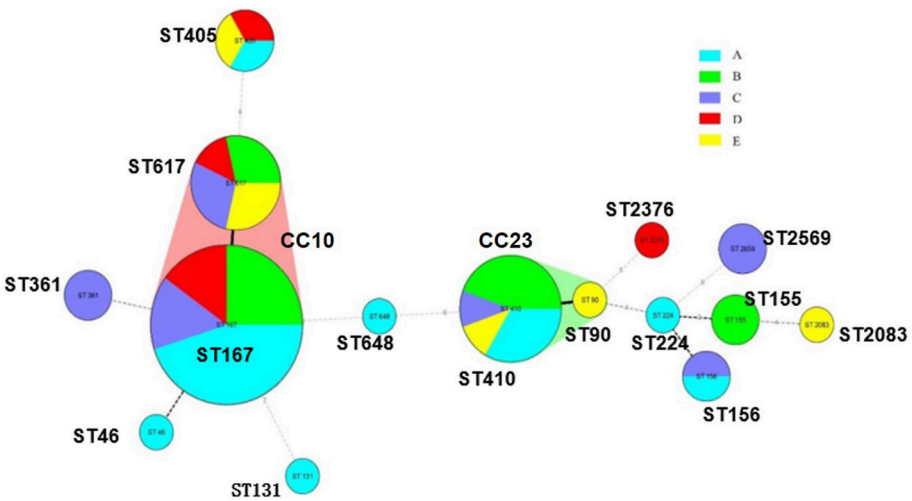
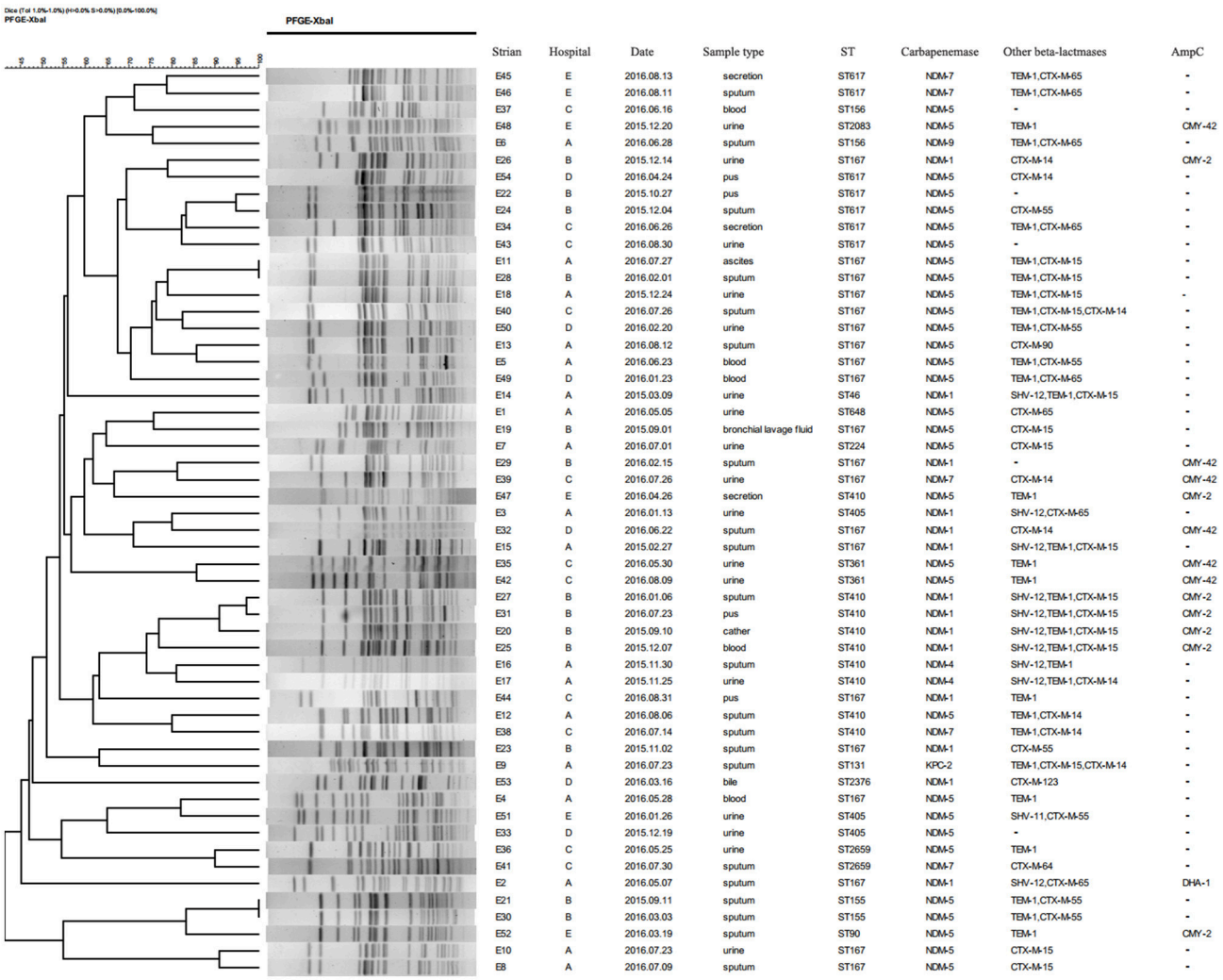


FIGURE 2 | Minimum spanning tree of 54 CRECO isolates from different hospitals.

a survey from the French National Reference Center revealed that among 140 carbapenem-resistant isolates 21% were NDM producer (Gauthier et al., 2018). In 2017, a nationwide study of clinical CRE strains in China demonstrated that 49% were NDM producer among carbapenem-resistant *E. coli* (Zhang et al., 2017). Furthermore, a multicenter study of the China CRE network revealed that among 39 carbapenem-resistant *E. coli* isolates, 74.4% were NDM producer, suggesting that there is a serious challenge in combating infections caused by this "superbug" in China (Zhang et al., 2018). In the present study, we identified 53 bla_{NDM}-carrying isolates among 54 CRECO, which is much higher than in any other region of China (Wang S. et al., 2016; Hu et al., 2017; Liang et al., 2017). To the best of our knowledge, this is also the first report on the bla_{NDM} gene in Northern Jiangsu Province. Moreover, the emergence of such a high prevalence of bla_{NDM} variants indicates that the bla_{NDM} gene is increasing in this area.

Since its first identification in 2009, the bla_{NDM} gene has evolved at a fast pace during the past 10 years. Twenty-one bla_{NDM} variants have been identified in different countries, all of which are archived at http://www.lahey.org/Studies/other.asp. Khan et al. (2017) reported that the Asian continent, especially China and India, was a reservoir of NDM producers, in which about a 58.2% abundance of the bla_{NDM-1} variants was found. Among these bla_{NDM} variants, the bla_{NDM-1} gene has been reported as the most prevalent type worldwide (Nordmann and Poirel, 2014). In our study, five bla_{NDM} variants were identified as responsible for MBL production, with the bla_{NDM-5}



Strian	Hospital	Date	Sample type	ST	Carbapenemase	Other beta-lactmases	AmpC
E45	E	2016.08.13	secretion	ST617	NDM-7	TEM-1,CTX-M-65	-
E46	E	2016.08.11	sputum	ST617	NDM-7	TEM-1,CTX-M-65	-
E37	C	2016.06.16	blood	ST156	NDM-5	-	-
E48	E	2015.12.20	urine	ST2083	NDM-5	TEM-1	CMY-42
E6	A	2016.06.28	sputum	ST156	NDM-9	TEM-1,CTX-M-65	-
E26	B	2015.12.14	urine	ST167	NDM-1	CTX-M-14	CMY-2
E54	D	2016.04.24	pus	ST617	NDM-5	CTX-M-14	-
E22	B	2015.10.27	pus	ST617	NDM-5	-	-
E24	B	2015.12.04	sputum	ST617	NDM-5	CTX-M-55	-
E34	C	2016.06.26	secretion	ST617	NDM-5	TEM-1,CTX-M-65	-
E43	C	2016.08.30	urine	ST617	NDM-5	-	-
E11	A	2016.07.27	ascites	ST167	NDM-5	TEM-1,CTX-M-15	-
E28	B	2016.02.01	sputum	ST167	NDM-5	TEM-1,CTX-M-15	-
E18	A	2015.12.24	urine	ST167	NDM-5	TEM-1,CTX-M-15	-
E40	C	2016.07.26	sputum	ST167	NDM-5	TEM-1,CTX-M-15,CTX-M-14	-
E50	D	2016.02.20	urine	ST167	NDM-5	TEM-1,CTX-M-55	-
E13	A	2016.08.12	sputum	ST167	NDM-5	CTX-M-90	-
E5	A	2016.06.23	blood	ST167	NDM-5	TEM-1,CTX-M-55	-
E49	D	2016.01.23	blood	ST167	NDM-5	TEM-1,CTX-M-65	-
E14	A	2015.03.09	urine	ST46	NDM-1	SHV-12,TEM-1,CTX-M-15	-
E1	A	2016.05.05	urine	ST648	NDM-5	CTX-M-65	-
E19	B	2015.09.01	bronchial lavage fluid	ST167	NDM-5	CTX-M-15	-
E7	A	2016.07.01	urine	ST224	NDM-5	CTX-M-15	-
E29	B	2016.02.15	sputum	ST167	NDM-1	-	CMY-42
E39	C	2016.07.26	urine	ST167	NDM-7	CTX-M-14	CMY-42
E47	E	2016.04.26	secretion	ST410	NDM-5	TEM-1	CMY-2
E3	A	2016.01.13	urine	ST405	NDM-1	SHV-12,CTX-M-65	-
E32	D	2016.06.22	sputum	ST167	NDM-1	CTX-M-14	CMY-42
E15	A	2015.02.27	sputum	ST167	NDM-1	SHV-12,TEM-1,CTX-M-15	-
E35	C	2016.05.30	urine	ST361	NDM-5	TEM-1	CMY-42
E42	C	2016.08.09	urine	ST361	NDM-5	TEM-1	CMY-42
E27	B	2016.01.06	sputum	ST410	NDM-1	SHV-12,TEM-1,CTX-M-15	CMY-2
E31	B	2016.07.23	pus	ST410	NDM-1	SHV-12,TEM-1,CTX-M-15	CMY-2
E20	B	2015.09.10	cather	ST410	NDM-1	SHV-12,TEM-1,CTX-M-15	CMY-2
E25	B	2015.12.07	blood	ST410	NDM-1	SHV-12,TEM-1,CTX-M-15	CMY-2
E16	A	2015.11.30	sputum	ST410	NDM-4	SHV-12,TEM-1	-
E17	A	2015.11.25	urine	ST410	NDM-4	SHV-12,TEM-1,CTX-M-14	-
E44	C	2016.08.31	pus	ST167	NDM-1	TEM-1	-
E12	A	2016.08.06	sputum	ST410	NDM-5	TEM-1,CTX-M-14	-
E38	C	2016.07.14	sputum	ST410	NDM-7	TEM-1,CTX-M-14	-
E23	B	2015.11.02	sputum	ST167	NDM-1	CTX-M-55	-
E9	A	2016.07.23	sputum	ST131	KPC-2	TEM-1,CTX-M-15,CTX-M-14	-
E53	D	2016.03.16	bile	ST2376	NDM-1	CTX-M-123	-
E4	A	2016.05.28	blood	ST167	NDM-5	TEM-1	-
E51	E	2016.01.26	urine	ST405	NDM-5	SHV-11,CTX-M-55	-
E33	D	2015.12.19	urine	ST405	NDM-5	-	-
E36	C	2016.05.25	urine	ST2659	NDM-5	TEM-1	-
E41	C	2016.07.30	sputum	ST2659	NDM-7	CTX-M-64	-
E2	A	2016.05.07	sputum	ST167	NDM-1	SHV-12,CTX-M-65	DHA-1
E21	B	2015.09.11	sputum	ST155	NDM-5	TEM-1,CTX-M-55	-
E30	B	2016.03.03	sputum	ST155	NDM-5	TEM-1,CTX-M-55	-
E52	E	2016.03.19	sputum	ST90	NDM-5	TEM-1	CMY-2
E10	A	2016.07.23	urine	ST167	NDM-5	CTX-M-15	-
E8	A	2016.07.09	sputum	ST167	NDM-5	CTX-M-15	-

FIGURE 3 | Dendrogram of PFGE profiles of 54 CRECO isolates**.** The UPGMA algorithm was used to construct dendrogram based on the dice similarity coefficient. Isolates were categorized to be of the same cluster when their dice similarity index was ≥ 85%.

gene being the most prevalent. Compared with NDM-1, NDM-5 producers exhibited higher hydrolytic activity and toxicity toward carbapenem and cephalosporin (Mei et al., 2017). The *bla*$_{NDM-5}$ gene is an emerging *bla*$_{NDM}$ variants and has tended to surpass *bla*$_{NDM-1}$ recently, and it differs from the NDM-1 enzyme at two amino acid substitutions, exhibited increased carbapenem resistance (Zhang et al., 2016). The *bla*$_{NDM-4}$ and *bla*$_{NDM-7}$ genes have been discovered among *E. coli* in China with relatively low prevalence, involving in 4 and 6 patients, respectively. Moreover, clinical *bla*$_{NDM-9}$-positive *E. coli* has only been found in Taiwan (Lai et al., 2017), with this being the first report on the mainland China. Moreover, not merely NDM-5, amino acid substitutions in NDM-4 (M154L), NDM-7 (D130A), and NDM-9 (E152A) could also result in high levels of carbapenem resistance (Düzgün, 2018). Stewart et al. (2017) reported that the substitution in M154L, which is found in most *bla*$_{NDM}$ variants, could enhance resistance to ampicillin at low zinc(II) concentrations relevant to infection sites.

bla$_{NDM}$ variant-positive isolates exhibited multi-drug resistance. Variable resistances to aztreonam, amikacin, and ciprofloxacin were identified in NDM-1 and NDM-5-producing *E. coli*. The NDM-7, NDM-4, and NDM-9-producing isolates exhibited pan-resistant phenotypes, showing resistance to almost all commonly used clinical antibiotics except for colistin and tigecycline. In view of this, the expert recommended polymyxins was regarded as the last resort for NDM-producing isolates (Yamamoto and Pop-Vicas, 2014).

ST167 was confirmed as significant carrier in the present study, and is reported to be associated with the production of the NDM enzyme, especially NDM-5 (Chen et al., 2016). Notably, four *bla*$_{NDM}$-positive ST617 strains were identified in the world, but we identified 6 isolates in the present study. Moreover, a novel new variant of *bla*$_{NDM}$, *bla*$_{NDM-21}$, belonged to ST617, which

TABLE 3 | Antimicrobial susceptible patterns and characteristics of 26 selected NDM-producing *Escherichia coli* (μg/mL).

Isolates	Antimicrobial susceptible													Hospital	Carbapenemase	MLST	Imp and size (kb)
	ETP	IMP	MEP	FOX	CRO	CAZ	FEP	ATM	AMK	CIP	TZP	TGC	PB				
E5	>32	32	>32	>256	>256	>256	>256	>256	>256	32	>256	0.5	0.5	A	NDM-5	ST167	X3/55[a], FII/105[b],UT/320
E5-J53	8	2	4	>256	>256	>256	16	64	0.5	≤0.06	256	0.25	0.25				
E7	8	8	4	>256	256	>256	32	16	>256	64	128	0.25	0.5	A	NDM-5	ST224	FII/55
E7-J53	4	2	4	>256	256	>256	8	16	1	≤0.06	128	0.125	0.25				
E12	16	8	4	>256	>256	>256	256	2	2	128	>256	0.5	0.25	A	NDM-5	ST410	UT/55
E12-J53	8	4	8	>256	>256	>256	32	2	0.5	≤0.06	256	0.5	0.25				
E22	>32	24	>32	>256	>256	>256	>256	4	2	64	>256	0.5	1	B	NDM-5	ST617	X3/55
E22-J53	8	2	6	>256	>256	>256	32	1	1	≤0.06	>256	0.25	0.5				
E21	>32	12	>32	>256	>256	>256	128	>256	4	1	>256	0.5	0.5	B	NDM-5	ST155	X3/55
E21-J53	16	6	8	>256	>256	>256	16	32	2	≤0.06	256	0.5	0.25				
E28	>32	>32	>32	>256	>256	>256	>256	>256	128	64	256	0.5	0.5	B	NDM-5	ST167	X3/55
E28-J53	16	4	8	>256	>256	>256	32	16	0.5	≤0.06	256	0.125	0.5				
E36	>32	>32	>32	>256	>256	>256	256	1	4	64	>256	0.25	0.25	C	NDM-5	ST2659	FI/55
E36-J53	8	2	4	>256	>256	>256	64	2	1	≤0.06	>256	0.25	< 0.125				
E37	>32	>32	>32	>256	>256	>256	256	1	2	128	>256	0.5	1	C	NDM-5	ST156	X3/55
E37-J53	8	2	4	>256	>256	>256	32	2	1	≤0.06	256	0.25	0.25				
E49	>32	>32	>32	>256	>256	>256	>256	>256	2	256	>256	0.125	0.5	D	NDM-5	ST167	X3/55
E49-J53	16	6	8	>256	>256	>256	32	128	0.5	≤0.06	>256	0.125	0.5				
E33	>32	>32	>32	>256	>256	>256	256	>256	>256	128	128	0.5	0.5	D	NDM-5	ST405	FI/55
E33-J53	4	2	2	>256	>256	>256	16	1	32	≤0.06	>256	0.25	0.125				
E47	>32	>32	>32	>256	>256	>256	256	4	8	8	>256	0.25	0.5	E	NDM-5	ST410	UT/55
E47-J53	8	4	6	>256	>256	>256	16	2	0.5	≤0.06	>256	0.25	0.5				
E48	>32	>32	>32	>256	>256	>256	256	4	4	128	>256	0.5	0.5	E	NDM-5	ST2083	FI/55
E48-J53	4	2	3	>256	>256	>256	16	1	0.5	≤0.06	>256	0.125	0.5				
E15	>32	8	8	>256	>256	>256	>256	>256	2	64	>256	0.25	0.25	A	NDM-1	ST167	FII/210
E15-J53	8	4	4	>256	>256	>256	32	2	0.25	≤0.06	>256	0.25	< 0.125				
E3	32	4	8	>256	>256	>256	128	256	4	64	>256	0.25	0.125	A	NDM-1	ST405	X3/55
E3-J53	12	4	4	>256	>256	>256	16	>256	2	≤0.06	>256	0.25	< 0.125				
E20	>32	>32	>32	>256	>256	>256	128	256	2	256	>256	0.25	0.5	B	NDM-1	ST410	X3/55
E20-J53	8	4	4	>256	>256	>256	128	256	0.25	≤0.06	>256	0.25	0.125				
E8	>32	8	8	>256	>256	>256	>256	256	2	128	>256	0.5	0.5	B	NDM-1	ST167	X3/210
E8-J53	16	4	8	>256	>256	>256	16	256	1	≤0.06	>256	0.5	0.25				
E44	>32	12	12	>256	>256	>256	64	1	2	256	128	0.125	0.25	C	NDM-1	ST167	X3/170
E44-J53	16	2	4	>256	>256	>256	32	>256	0.5	≤0.06	128	0.125	0.25				
E32	>32	>32	>32	>256	>256	>256	256	256	4	256	>256	0.5	1	D	NDM-1	ST167	FI/55

(Continued)

TABLE 3 | Continued

Isolates	Antimicrobial susceptible													Hospital	Carbapenemase	MLST	Imp and size (kb)
	ETP	IMP	MEP	FOX	CRO	CAZ	FEP	ATM	AMK	CIP	TZP	TGC	PB				
E32-J53	8	2	4	>256	>256	>256	16	2	0.5	≤0.06	256	0.125	0.125				
E53	16	8	6	>256	>256	>256	64	256	2	1	128	0.5	0.5	D	NDM-1	ST2376	X3/55
E53-J53	8	4	6	>256	>256	>256	16	1	0.25	≤0.06	128	0.5	0.25				
E6	16	8	8	>256	>256	>256	>256	>256	>256	64	>256	0.5	1	A	NDM-9	ST156	I1/105
E6-J53	6	2	2	>256	>256	>256	32	64	2	≤0.06	256	0.125	0.25				
E16	16	8	8	>256	>256	>256	128	>256	1	32	>256	0.25	0.25	A	NDM-4	ST410	X3/55
E16-J53	4	2	2	>256	>256	>256	16	1	0.5	≤0.06	>256	0.25	0.25				
E38	>32	>32	>32	>256	>256	>256	>256	>256	4	256	>256	0.125	0.5	C	NDM-7	ST410	FI/110
E38-J53	8	2	4	>256	>256	>256	16	4	2	≤0.06	256	0.125	0.125				
E39	>32	>32	>32	>256	>256	>256	256	>256	2	1	>256	0.5	0.5	C	NDM-7	ST167	X3/55
E39-J53	16	6	8	>256	>256	>256	32	128	1	≤0.06	256	0.25	0.25				
E41	>32	>32	>32	>256	>256	>256	256	>256	2	256	>256	0.25	1	C	NDM-7	ST2659	X3/55
E41-J53	8	4	6	>256	>256	>256	16	2	1	≤0.06	>256	0.25	0.25				
E45	>32	24	>32	>256	>256	>256	256	>256	>256	64	>256	0.5	0.5	E	NDM-7	ST617	X3/55
E45-J53	16	4	8	>256	>256	>256	16	256	2	≤0.06	>256	0.125	<0.125				
E46	>32	12	>32	>256	>256	>256	>256	>256	>256	128	>256	0.5	0.25	E	NDM-7	ST617	X3/55
E46-J53	8	4	6	>256	>256	>256	16	64	0.5	≤0.06	>256	0.5	0.125				
E.coli J53	≤0.125	0.25	≤0.125	4	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	2	0.125	<0.125				

IMP, imipenem; ETP, ertapenem; MEP, meropenem; PB, colistin; TGC, tigecycline; ATM, aztreonam; FOX, cefoxitin; FEP, cefepime; CAZ, ceftazidim; CRO, cefatriaxone; YZP, piperacillin/tazobactam; AMK, amikacin; CIP, ciprofloxacina: a
[a]*GenBank accession no. NC_022740.1;*
[b]*GenBank accession no. AP018144.1.*

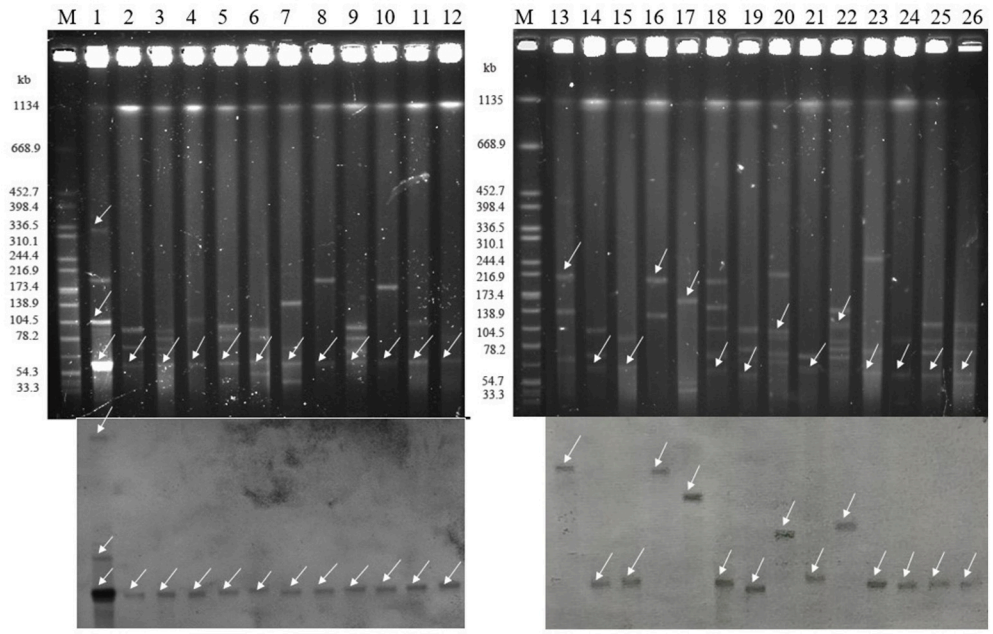


FIGURE 4 | Isolation and determination of plasmids harboring the bla_{NDM} gene in 26 clinical and transconjugants (**Top**: S1-nuclease PFGE patterns; **Bottom**: Southern blotting with a bla_{NDM} specific probe). The arrows represent thelocation of linear plasmid DNA hybridized with the bla_{NDM} specific probe. M: Braenderup H9812 Marker; lane 1: E5; lane 2: E7; lane 3: E12; lane4: E22; lane 5: E21; lane 6: E28; lane 7: E36; lane 8: E37; lane 9: E49; lane 10: E33; lane 11: E47; lane 12: E48; lane 13: E15; lane 14: E3; lane 15: E20; lane 16: E8; lane 17: E44; lane18: E32; lane 19: E53; lane 20: E6; lane 21: E16; lane 22: E38; lane 23: E39; lane 24: E41; lane 25: E45; lane 26: E46.

should deserve more attention (Liu et al., 2018). Furthermore, four new STs ST361, ST46, ST2376, and ST2083 were firstly reported to be linked with NDM-producing isolates.

The high efficiency of transfer renders the NDM producer ubiquitous throughout the world. Torres-González et al. (2015) reported an outbreak caused by bla_{NDM-1}-carrying plasmid, which is easily transferred between *E. coli* and *Klebsiella pneumoniae*. Similarly, in the present study, the plasmids of 26 selected CRECO isolates were also tested for transfer. All selected bla_{NDM-5} genes were identified to be located on 55 kb plasmid, among which IncX3 was the most common replicon type. Notably, bla_{NDM-5} gene was frequently reported to be carried by the IncX3 plasmid of 55 kb in size, which was widely reported in China (Yang et al., 2014), Inidia (Krishnaraju et al., 2015), Australia (Wailan et al., 2015), and Damark (Hammerum et al., 2015). Moreover, Li et al. (2018) reported that IncX3 type plasmids play an important role in the transmission of the bla_{NDM-5} gene in *Enterobacteriaceae* and this kind of plasmid occurred in different species. Further illustrated the challenge of preventing the dissemination of the bla_{NDM-5} gene. The widespread bla_{NDM-1}-carrying plasmid has been found to be associated with multiple replicon types, including IncX3, IncF, and IncA/C etc. In agreement with previous studies (Göttig et al., 2013; Wang L. H. et al., 2016), four bla_{NDM-7}-positive plasmids were identified as ∼55 kb IncX3 type, while the bla_{NDM-7} gene was also detected in the IncFI plasmid, which have been identified in India (Rahman et al., 2014). As for bla_{NDM-9}, to the best of our knowledge, this is the first report on ∼105 kb IncI1 type plasmid harboring bla_{NDM-9} gene. Overall, most plasmids harboring bla_{NDM} variants were identified as 55 kb IncX3 types, hinting that amino acid mutations might occur in the process of plasmid transfer, resulting in the emergence of bla_{NDM} variants. The high prevalence of the bla_{NDM} genes due in part to plasmid transfer, meanwhile, the fast evolution of this multidrug resistance gene also favors the persistence of such bacteria harboring it.

In summary, the present study reported high prevalence of bla_{NDM} variants, especially bla_{NDM-5}, among carbapenem-resistant *E. coli* in Northern Jiangsu Province. The presence of five different variants further increases the threat to public health because of the limited treatment options. Notably, diverse bla_{NDM} variants were mainly located on ∼55 kb IncX3 plasmids, indicating that the fast evolution and high transferability of this kind of plasmid has led to the high prevalence of bla_{NDM} variants. Timely detection of NDM enzyme and antimicrobial susceptibility testing are necessary so that infections caused by NDM producers receive appropriate and effective therapy. Similarly, large-scale surveillance and effective infection control measures are also urgently needed to prevent diverse bla_{NDM} variants from becoming epidemics in the future.

AUTHOR CONTRIBUTIONS

The laboratory measurements were performed by RB, ZK, and HQ. BG and PM participated in experimental design and manuscript revision. Data analysis were implemented by RB, ZK, HQ, FJ, and HK.

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SuperPolymyxin™ Medium for the Screening of Colistin-Resistant Gram-Negative Bacteria in Stool Samples

*Sara M. Przybysz[1], Carlos Correa-Martinez[2], Robin Köck[1,2,3], Karsten Becker[1] and Frieder Schaumburg[1]**

[1] Institute of Medical Microbiology, University Hospital Münster, Münster, Germany, [2] Institute of Hygiene, University Hospital Münster, Münster, Germany, [3] Institute of Hygiene, DRK Kliniken Berlin, Berlin, Germany

Colistin is one of the last resort antimicrobials for the treatment of infections caused by multidrug-resistant Gram-negative bacteria. After the emergence of transferable colistin resistance genes (*mcr*-1–5), a reliable culture-based screening method to detect colonization with colistin-resistant Gram-negative bacteria (CRGN) is needed. The objective of this study was to test the performance of SuperPolymyxin™ medium to screen for CRGN in stool samples and to compare different methods for the confirmation of colistin resistance (e.g., Etest, broth microdilution [BMD], and the Rapid Polymyxin™ NP test). Colonization with CRGN was analyzed in a prospective cohort study among travelers. Stool samples (Fecal Transwab™) taken before, during and after travel were cultured on SuperPolymyxin™ agar. Every phenotypically different colony was subcultured for species identification using MALDI-TOF mass spectrometry. Susceptibility to colistin was tested using Etest and confirmed by BMD and the Rapid Polymyxin™ NP test. In total, 128 participants provided 1,495 stool samples. After culture on SuperPolymyxin™ medium (37°C, 24–48 h), 1,851 phenotypically different colonies were isolated. Isolates belonging to intrinsically colistin-resistant genera (e.g., *Morganella, Providencia, Proteus*) or *Stenotrophomonas maltophilia* were excluded from further analysis ($n = 421$). Among the remaining 1,430 isolates, colistin resistance was confirmed in 279 by Etest® (19.5%) and 218 by BMD (15.3%). The Rapid Polymyxin™ NP test was compared with BMD (reference) to detect colistin resistance (specificity: 88.6%, sensitivity 71.1%). SuperPolymyxin™ medium is suitable to screen for fecal colonization with CRGN. The high proportion of colistin-susceptible isolates growing on SuperPolymyxin™ medium caused a high workload. The confirmation of CRGN with the Rapid Polymyxin™ NP Test could be a less labor-intensive alternative to BMD.

Keywords: Colistin, Resistance, Screening, Stool, *mcr*-1

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Shanghai Pulmonary Hospital, China

***Correspondence:**
Frieder Schaumburg
frieder.schaumburg@ukmuenster.de

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INTRODUCTION

The emergence and spread of antimicrobial resistance is currently one of the biggest challenges in health care worldwide. This is particularly true for Gram-negative bacteria. In the past decades, carbapenems were considered as safe and efficient antimicrobials for the treatment of infections with extended-spectrum β-lactamase (ESBL)-producing *Enterobacterales* or non-fermenting

bacteria (e.g., *Pseudomonas aeruginosa*, *Acinetobacter baumannii*). Today, the increase and global spread of carbapenem-resistant bacteria raise serious concerns and physicians can find themselves "beamed back" to the pre-antimicrobial era as only very few compounds are available to treat infections with these multidrug-resistant microorganisms. For example, 13 of 38 European countries reported in 2015 an inter-regional dissemination or even an endemic occurrence of carbapenemase-producing *Enterobacterales* (Magiorakos et al., 2013; Albiger et al., 2015). In addition, carbapenemase-producing *Enterobacterales* were also detected in livestock, companion animals, seafood and wildlife (Köck et al., 2018).

Although parenteral colistin (Polymyxin E) was not considered safe (e.g., nephro- and neurotoxicity) and effective in the past, it experiences a revival in human medicine nowadays due to the lack of alternative antimicrobial agents to treat infections due to carbapenem-resistant bacteria. Moreover, colistin is widely used in veterinary medicine to treat diarrhea in poultry and pig production systems. In 2012, the European Union estimated that the use of polymyxins in food-producing animals was 600-times higher than in humans (Skov and Monnet, 2016).

Colistin is a decapeptide with a poor resorption after oral administration due to its hydrophilic properties. For the treatment of multidrug-resistant Gram-negative bacteria, it is given intravenously as the prodrug colistin methanesulfonate (colistimethate) (Grégoire et al., 2017). Colistin interacts with lipopolysaccharides on the outer membrane of Gram-negative bacteria and causes membrane damage leading to bacterial death (Grégoire et al., 2017). Some genera are intrinsically resistant to colistin (e.g., *Burkholderia, Hafnia, Morganella, Proteus, Providencia, Serratia*) (Leclercq et al., 2013; Jayol et al., 2017). However, acquired colistin resistance has been reported, which is either encoded on the bacterial chromosome (e.g., mutations in *lpxA*, *lpxC*, *lpxD, pmrA, pmrB*, *mgrB*), or on transferable plasmids (e.g., *mcr-1–5*) (Nordmann et al., 2016a; Grégoire et al., 2017). Recently, *Enterobacterales* harboring *mcr* genes have emerged in livestock (Irrgang et al., 2016; Kieffer et al., 2017; Wang et al., 2017). Hence, *mcr* genes (mainly *mcr-1*) are currently the main mechanism of colistin resistance in livestock farming and *mcr* positive colistin-resistant Gram-negative bacteria (CRGN) can now be found in humans, breeding animals (e.g., pigs, poultry) or even "filth flies"(Guenther et al., 2017; Wang et al., 2017; Onwugamba et al., 2018). International travel also contributes to the spread of colistin-resistant bacteria, particularly through tourists from South-East Asia (Arcilla et al., 2016).

Effective screening media are needed to detect individuals colonized with CRGN. Recently, a colistin resistance screening agar was developed by Nordmann et al. (SuperPolymyxin™ medium). To the best of our knowledge this is currently the only available selective agar for the screening of colistin-resistant bacteria (Nordmann et al., 2016a). SuperPolymyxin™ medium is based on eosin methylene blue, which is selective for Gram-negative bacteria. It also contains colistin, daptomycin and amphotericin B. Presumptive lactose-fermenting species grow in darkblue-brown colonies, while lactose-non-fermenters grow colorless or light lavender. In addition, the medium allows for the differentiation of lactose-fermenters as *Escherichia coli* colonies have a characteristic metallic green sheen and colonies of *Enterobacter* spp. and *Klebsiella* spp. are brown, dark-centered and mucoid. This SuperPolymyxin™ medium was already challenged with a selection of well-characterized colistin-susceptible and -resistant isolates (Nordmann et al., 2016a; Abdul Momin et al., 2017; Jayol et al., 2018).

Several test are available for the detection of colistin resistance but they are either labor intensive (broth microdilution [BMD]) or have a high rate of very major errors (Etest®) (Matuschek et al., 2018). Recently, the Rapid Polymyxin™ NP test was developed by Nordmann et al., which can detect colistin resistance within 2 h (Nordmann et al., 2016b). Briefly, the test is based on the metabolism of glucose by viable cells leading to a decrease of the pH-value. The change in pH is indicated by phenol-red. In case of colistin resistance, bacteria can grow in the presence of colistin (3.75 mg/L) and the test suspension turns from red to yellow (Nordmann et al., 2016b). This Rapid Polymyxin™ NP test could be a suitable alternative to BMD or Etest®.

The objective of this study was now to assess the test performance and applicability of SuperPolymyxin™ medium in routine screening of fecal samples and to compare different methods for the confirmation of colistin resistance (e.g. Etest®, BMD, and the Rapid Polymyxin™ NP test).

MATERIALS AND METHODS

Ethics

Ethical clearance was obtained from the ethical committee of the medical faculty of "Westfälische Wilhelms-Universität," Münster, Germany (approval number: 2014-013-f-S). All participants signed a written informed consent prior to any study-related procedures.

Fecal Samples and Media Characteristics

Fecal samples were self-collected by international travelers who took part in a prospective cohort study on the import of antimicrobial-resistant bacteria from abroad (Münster, Germany, April 2016–April 2018). Participants were trained to collect samples strictly avoiding cross-contamination from the environment by either sticking the whole swab in the stool or by swabbing the stool from the anal region before cleaning. The participants provided stool samples in Cary-Blair medium (Fecal Transwab™, MWE medical wire, Corsham, England) once before, during and after the trip (Arcilla et al., 2017). The fecal swabs were stored at room temperature until being cultured. For that purpose, 10 µl of the inoculated Cary-Blair medium were streaked on SuperPolymyxin™ medium (three-phase streak technique) and cultured for 24–48 h at 37°C under aerobic conditions (Nordmann et al., 2016a).

Bacterial Cultures

One of each phenotypically different colony growing on SuperPolymyxin™ medium was subcultured on Columbia blood agar for species identification using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass

spectrometry (Bruker, Bremen, Germany) applying the database MBT Compass 4.1. All intrinsically resistant species were removed from further susceptibility testing (e.g., *Hafnia alvei, Morganella morganii, Proteus hauseri, Proteus vulgaris*, *Proteus mirabilis*, *Providencia rettgeri*, *Providencia stuarti*, *Providencia alcalifaciens*, *Serratia liquefaciens*, *Serratia marcescens, Serratia ureolytica*) (Magiorakos et al., 2012; Leclercq et al., 2013; Jayol et al., 2017; Saly et al., 2017) (**Figure 1**). *Stenotrophomonas maltophilia* was excluded due to missing EUCAST breakpoints for colistin.

Susceptibility Testing

All isolates being not intrinsically resistant to colistin were further tested using colistin Etest® (bioMérieux, Marcy l'Étoile, France) on Mueller-Hinton agar (BD Diagnostics, Heidelberg, Germany) according to the manufacturer's instruction. Minimum inhibitory concentrations (MIC) were interpreted according to EUCAST clinical breakpoints (Version 8.0; colistin-susceptible: MIC ≤2 mg/L, colistin-resistant: MIC >2 mg/L). MICs from Etest® were rounded to the next doubling step for comparison with MIC from broth microdilution (Schaumburg et al., 2017).

Colistin resistance was confirmed by the BMD reference method (Sensititre™ FRCOL, Thermo Fisher Scientific, Wesel, Germany) according to the manufacturer's instruction. Briefly, bacterial suspensions (0.5 McFarland) were prepared in Mueller-Hinton broth with TES buffer and transferred to the microtitre plates (range of colistin concentration: 0.12–128 mg/L). The results were read after 18–24 h at 35°C. Test results with "skipped wells" (i.e., no growth in one well but growth in a well with higher colistin concentration) were considered non-interpretable (Poirel et al., 2017).

The Rapid Polymyxin™ NP Test was assessed as a rapid and cheap alternative to BMD to confirm colistin resistance (Nordmann et al., 2016b). The test was performed in triplicate. In case of inconsistent results, the final interpretation of the test was based on the result as suggested by the majority of the three test runs.

E. coli NCTC 13846 (DSMZ 105182, colistin-resistant) and *P. aeruginosa* ATCC 27853 (colistin-susceptible) were used as controls.

mcr-1 Screening

All isolates having a MIC >2–64 mg/L as tested by BMD were screened for the presence of *mcr-1* by isothermal amplification (eazyplex® SuperBug *mcr-1*, AmplexDiagnostics GmbH, Gars-Bahnhof, Germany). This range was chosen as almost all *mcr* positive CRGN showed a colistin MIC ≤ 64 mg/L (Nordmann et al., 2016a; Eiamphungporn et al., 2018; Poirel et al., 2018).

Statistics

Statistical analyses were performed with "R" (package "epiDisplay" and "caret"). The 95% confidence interval (95% CI) of error rates (major and very major errors) and the categorical agreement between BMD and Etest® or Rapid Polymyxin™ NP Test was calculated with Wilson procedure without a correction for continuity.

RESULTS

In total, 128 participants were included who provided 1,495 fecal swabs (median number of swabs per participant: 12, range: 2–23). Of these, 1,258 (84.2%) showed growth on SuperPolymyxin™ medium. No growth was detected on 237 plates (15.8%, **Figure 1**).

The median number of phenotypically different colonies growing on SuperPolymyxin™ medium was one colony (range: 1–3). In total, 1,851 phenotypically different colonies from SuperPolymyxin™ medium were subcultured for species identification (MALDI-TOF MS). We identified 47 different species.

In total, 421 isolates (22.7%) belonging to the following species were considered intrinsically colistin resistant and were excluded from further testing: *P. rettgeri* ($n = 116$), *M. morganii* ($n = 98$), *S. marcescens* ($n = 66$), *P. alcalifaciens* ($n = 52$), *P. mirabilis/vulgaris* ($n = 33$), *H. alvei* ($n = 8$), *P. hauseri* ($n = 3$), *S. ureolytica* ($n = 3$), *P. stuarti* ($n = 2$) and *S. liquefaciens* ($n = 2$, **Figure 1**). *S. maltophilia* ($n = 38$) was excluded due to missing EUCAST breakpoints.

Overall, 77.3% of the isolates from SuperPolymyxin™ agar ($n = 1{,}430$) were Gram-negative species that are normally susceptible to colistin, such as *Klebsiella pneumoniae* ($n = 363$), *E. coli* ($n = 251$), *Enterobacter cloacae* ($n = 188$), *Klebsiella variicola* ($n = 168$), *Enterobacter asburiae* ($n = 147$), *Pseudomonas aeruginosa* ($n = 72$), *Enterobacter kobei* ($n = 46$), *Raoultella ornithinolytica* ($n = 39$), *Enterobacter aerogenes* ($n = 38$), *Citrobacter freundii* ($n = 34$), *Comamonas testosteroni* ($n = 16$) and others ($n = 68$). Only one Gram-positive species (*Enterococcus* sp.) grew on the medium.

The non-intrinsically resistant species ($n = 1{,}430$) were screened for colistin resistance by Etest®. Colistin resistance was detected in 19.5% (279/1,430) of the isolates. Although Etest® is a convenient method to measure MICs, it is not recommended for colistin susceptibility testing due to false susceptible results (Matuschek et al., 2018). We confirmed colistin resistance with BMD in all isolates being tested resistant by Etest® ($n = 279$). BMD confirmed colistin resistance in 78.1% (218/279). Thus, 15.3% (218/1,430) of all non-intrinsically colistin resistant species growing on SuperPolymyxin™ medium were colistin resistant.

To assess the proportion of *mcr-1* mediated colistin resistance, all isolates exhibiting a colistin MIC between >2–64 mg/L ($n = 124$) were screened for *mcr-1* by PCR. This comprised *Acinetobacter junii* ($n = 1$), *Delftia acidovorans* ($n = 1$), *E. coli* ($n = 39$), *E. cloacae* ($n = 39$), *E. asburiae* ($n = 17$), *R. ornithinolytica* ($n = 5$), *E. kobei* ($n = 13$), *C. testosteroni* ($n = 6$), *Ochrobactrum* sp. ($n = 1$), *Pseudomonas protegens* ($n = 1$), and *Yokenella regensburgei* ($n = 1$). A total of 26.6% ($n = 34$) were *mcr-1* positive (33 *E. coli*, 1 *E. asburiae*).

Since Etest® can underreport colistin resistance, we tested the Etest® performance in our setting using BMD as reference. For that purpose, we selected 130 consecutively collected species from our samples. Twelve isolates were excluded due to non-interpretable results in BMD (skipped wells, **Table 1**).

The essential agreement between Etest® and BMD was 59.3% (70/118, 95%CI: 50.3%−67.7%) and the category agreement was

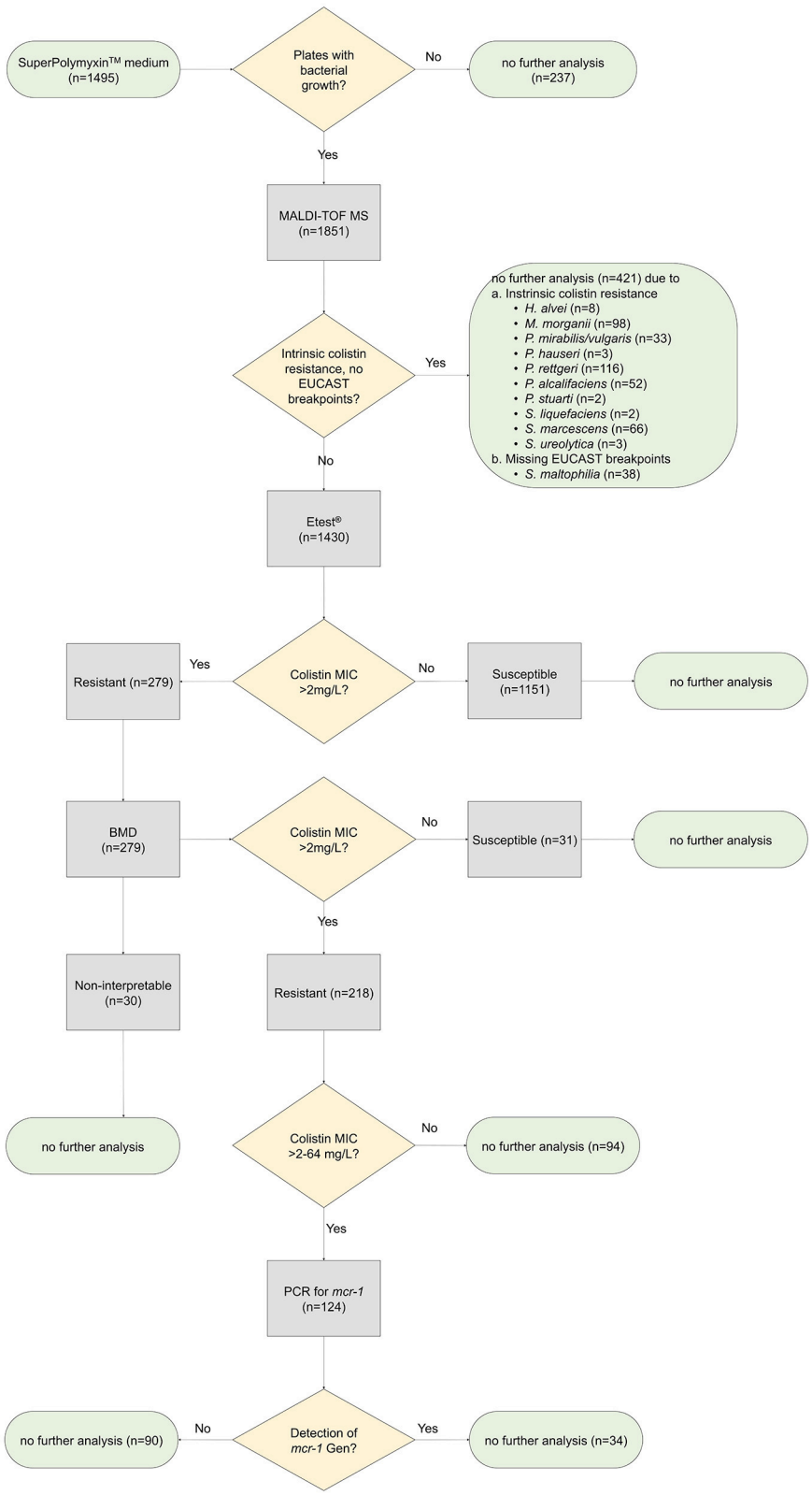

FIGURE 1 | Study procedure. Minimum inhibitory concentrations (MIC) of all colistin non-intrinsically resistant bacterial species growing on SuperPolymyxin™ medium were tested by gradient diffusion test (Etest®, bioMérieux) and broth microdilution (BMD) to confirm colistin resistance. Presence of the transferable resistance determinant *mcr-1* was tested by PCR.

TABLE 1 | Comparison of Etest® and broth microdilution for colistin susceptibility testing.

		Broth microdilution (*n*)		
		Resistant	**Susceptible**	**Total**
Etest® (*n*)	Resistant	37	3	40
	Susceptible	9	69	78
	Total	46	72	118[a]

[a] *12 isolates were excluded due to non-interpretable results in BMD (skipped wells).*

89.8% (106/118, 95%CI: 83.1–94.1%). Wrong Etest® results were either due to major errors (false resistant: 3/118, 2.5%, 95%CI: 0.9–7.2%) or very major errors (false susceptible: 9/118, 7.6%, 95%CI: 4.1–13.9%).

The specificity and sensitivity of Etest® (reference: BMD) was 95.8% and 80.4%, respectively. The negative and positive predictive values for the detection of colistin resistance using Etest® were 88.5 and 92.5%, respectively (accuracy: 89.8%).

BMD is recommended for colistin susceptibility testing, but it is labor intensive and time consuming. Therefore, we tested, if the Rapid Polymyxin™ NP Test can be applied to rapidly confirm or rule out colistin resistance. For that purpose, the same set of consecutively selected isolates ($n = 130$) was tested with Rapid Polymyxin™ NP Test (merged test results of triplicate runs) and BMD (reference).

Test results of the Rapid Polymyxin™ NP Test were not evaluable in three subsequent test runs for *P. alcalifaciens* ($n = 4$), *C. testosteroni* ($n = 2$), *Acinetobacter junii* ($n = 1$), *Pseudomonas fluorescens* ($n = 1$), *E. kobei* and *P. aeruginosa* ($n = 1$) due to no growth in the growth control. These isolates were excluded for the calculation of the test performance. Additionally, 12 isolates were excluded due to skipped wells in BMD.

The category agreement between Rapid Polymyxin™ NP Test and BMD was 82.1% (89/108, 95%CI: 74.1–88.4%). Disagreement was either due to major errors (false resistant: 8/108, 7.4%, 95%CI: 3.8–13.9%) or very major errors (false susceptible: 11/108, 10.2%, 95%CI: 5.8–17.3%).

The specificity and sensitivity of Rapid Polymyxin™ NP Test compared to BMD were 88.6 and 71.1%, respectively. The negative and positive predictive values to detect colistin resistance using Rapid Polymyxin NP Test were 84.9 and 77.1%, respectively (accuracy: 91%).

The test performance was impaired, if only the first test run of Rapid Polymyxin™ NP Test and not the merged results of the triplicate testing were considered (specificity: 85.7%, sensitivity: 63.4%, negative predictive value: 80.0%, positive predictive value: 72.2%).

DISCUSSION

We tested the applicability of SuperPolymyxin™ medium and found that it is suitable to detect colistin resistance (including *mcr-1* positive isolates) in human fecal samples but it was associated with high workload.

The high proportion of positive SuperPolymyxin™ medium (growth after 48 h, 84.2%) in our study is in contrast to a recent report on hospitalized patients where bacterial growth was found on 17/41 agars (41.5%) after inoculation from rectal swabs (Jayol et al., 2018). In our study, the participants stored the samples at room temperature during their travel and bacteria might have multiplied during this time. The different rates of growth on SuperPolymyxin™ medium (84.2 vs. 41.5%) might be due to an inoculum effect as colistin-susceptible isolates were shown to grow on SuperPolymyxin™ medium at $\geq 10^6$ CFU/mL (Nordmann et al., 2016a). If this played a role in our study, one should expect a higher proportion of colistin susceptible bacteria in early compared to late samples due to longer storage times. However, the proportion of susceptible isolates (according to Etest®) was similar throughout the study fluctuating between 16 and 27% (data not shown).

In total, 279 of 1,430 isolates were found to be colistin-resistant by Etest®, which was confirmed in 218 isolates (78.1%) by BMD. This is in line with a recent comparison of different Etest® manufacturers (reference: BMD) where the categorical agreement was 79–85% (Matuschek et al., 2018).

To detect the 218 isolates with acquired colistin resistance, we had to perform 1,851 MALDI-TOF MS tests (to rule out intrinsically resistant isolates) and 1,430 Etest® cultures, thus experiencing a relevant workload. Most likely, this might be due to an inoculum effect due to bacterial growth during storage at room temperature (Nordmann et al., 2016a). A low quality of in-house SuperPolymyxin™ medium is unlikely since we strictly followed the recommendations (e.g., 7 days shelf life, storage of colistin stock solution in glass tubes) (Nordmann et al., 2016a). Others recently showed that SuperPolymyxin™ medium could even be used for up to 4 weeks (Abdul Momin et al., 2017).

Several studies evaluating SuperPolymyxin™ medium did not use clinical samples but monocultures or defined mixed cultures of different species (n: 2–9) and therefore cannot be compared with our study as we used fecal samples (Nordmann et al., 2016a; Abdul Momin et al., 2017). Other reports analyzed rectal colonization rates with *mcr-1* positive isolates in pigs (98%) and hospitalized patients (0%) using SuperPolymyxin™ medium (Kieffer et al., 2017; Saly et al., 2017; Jayol et al., 2018). These studies did not focus in detail on the proportion of colistin-susceptible isolates growing on SuperPolymyxin™ medium. Notably the high number of colistin-susceptible isolates growing on SuperPolymyxin™ medium caused the major workload (**Figure 1**).

In a subset of 130 consecutively collected isolates, we studied the test performance of Etest® (reference: BMD) in our setting. Here, the categorical agreement (89.9%) was even slightly better compared to a recent study with 75 isolates (79–85%) depending on the manufacturer of the gradient diffusion test (**Table 1**) (Matuschek et al., 2018). Since the very major error rate (i.e., false susceptible isolates) of Etest® in our setting was 7.6%, we might have missed approximately 8% of non-intrinsically colistin-resistant isolates using our approach (i.e., screening for colistin resistance with Etest® and confirming with BMD, **Figure 1**). We decided to use Etest® as a screening method and BMD for confirmation as BMD was considered to cause

TABLE 2 | Comparison of the Rapid Polymyxin™ NP Test and broth microdilution to detect colistin resistance.

		Broth microdilution (*n*)		
		Resistant	**Susceptible**	**Total**
Rapid Polymyxin™ NP Test (*n*)	Resistant	27	8	35
	Susceptible	11	62	73
	Total	38	70	108[a]

[a] *10 isolates were excluded the due to non-evaluable results in Rapid Polymyxin™ NP Test (no growth in the growth control) and 12 isolates were excluded due to non-interpretable results in broth microdilution (skipped wells).*

an inapplicable workload due to the high number of isolates. However, an alternative to confirm/rule-out colistin resistance is the Rapid Polymyxin™ NP Test. (**Table 2**). However, Compared to Etest®, Rapid Polymyxin™ NP Test had a lower specificity (95.8 vs. 88.6%) and sensitivity (80.4 vs. 71.1%) to detect colistin resistance (**Tables 1**, **2**).

Although our study provides valuable data for the screening of colistin resistance in fecal samples, some limitations need to be addressed. First, we did not apply broth microdilution to confirm colistin resistance in all isolates growing on SuperPolymyxin™ medium but only used BMD for confirmation of colistin-resistant isolates according to Etest®. Since Etest® can have high false-susceptible rates, we might have missed additional colistin-resistant isolates. Second, we did not screen for newer *mcr*-members (i.e., *mcr-2–mcr-5*). These members are still very rare and the European Center for Disease Prevention and Control currently recommend only a screening for *mcr-1* (European Centre for Disease Prevention Control, 2016; Sun et al., 2018). Third, since we only tested fecal samples from international travelers, we are unable to draw any conclusions on the test performance of SuperPolymyxin medium™ when screening hospitalized patients, animals or environmental samples for colistin-resistant isolates.

In conclusion, SuperPolymyxin™ medium is suitable to screen for fecal colonization with colistin-resistant isolates but is associated with a high workload due to a high proportion of colistin-susceptible isolates growing on SuperPolymyxin™ medium. The confirmation of colistin resistance with the Rapid Polymyxin™ NP Test is a cheap and but less-accurate alternative to BMD.

AUTHOR CONTRIBUTIONS

KB, RK, SP, and FS designed the study. SP, CC-M, and FS performed sampling and microbiological analyses. SP and FS analyzed the data. SP and FS drafted the manuscript. All authors reviewed and agreed on the final version of the manuscript. All authors have commented and agreed on the final version of the manuscript.

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Complete Genome Sequence of *bla*$_{IMP-6}$-Positive *Metakosakonia* sp. MRY16-398 Isolate From the Ascites of a Diverticulitis Patient

*Tsuyoshi Sekizuka[1], Mari Matsui[2], Tomiyo Takahashi[3], Michiko Hayashi[2], Satowa Suzuki[2], Akihiko Tokaji[3] and Makoto Kuroda[1]**

[1] Pathogen Genomics Center, National Institute of Infectious Diseases, Tokyo, Japan, [2] Antimicrobial Resistance Research Center, National Institute of Infectious Diseases, Higashimurayama, Japan, [3] The Public Health Institute of Kochi Prefecture, Kōchi City, Japan

A novel species of carbapenemase-producing Enterobacteriaceae (CPE) was isolated from a patient diagnosed with sigmoid colon diverticulitis. At first, laboratory testing suggested it was *Klebsiella oxytoca* or *Pantoea* sp.; however, a complete genome sequence of the isolate, MRY16-398, revealed that it could be novel species, most similar to [*Kluyvera*] *intestini*, of which taxonomic nomenclature is still under discussion. Orthologous conserved gene analysis among 42 related bacterial strains indicated that MRY16-398 was classified as the newly proposed genus *Metakosakonia*. Further, MRY16-398 was found to harbor the *bla*$_{IMP-6}$ gene-positive class 1 integron (In722) in plasmid pMRY16-398_2 (IncN replicon, 47.4 kb in size). This finding implies that rare and opportunistic bacteria could be potential infectious agents. In conclusion, our results highlight the need for continuous monitoring for CPE even in nonpathogenic bacteria in the nosocomial environment.

Keywords: ***Kluyvera***, ***bla*$_{IMP-6}$**, **carbapenemase, IncN,** ***Metakosakonia***

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Yi-Wei Tang,
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Ghassan M. Matar,
American University of Beirut, Lebanon
Filipa Grosso,
Universidade do Porto, Portugal

****Correspondence:***
Makoto Kuroda
makokuro@niid.go.jp

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INTRODUCTION

Antimicrobial resistance (AMR) is a global issue linked to increased and often unrestricted antibiotic use in the clinical settings, which leads to the dissemination of carbapenem-resistant Enterobacteriaceae (CRE) in health care facilities (World Health Organization, 2017). Carbapenemases comprise three of the four Ambler classes as follows: Class A (*Klebsiella pneumoniae* carbapenemases, KPC, some variants of Guiana extended-spectrum β-lactamases, GES), Class B (metallo-β-lactamases, MBL including New Delhi metallo-β-lactamases, NDM, Verona integron-encoded metallo-β-lactamases, VIM, and imipenemase, IMP), and Class D (OXA-48-like carbapenemases) (Logan and Weinstein, 2017). These carbapenemase-producing Enterobacteriaceae (CPE) have the potential to facilitate the widespread transmission of antimicrobial resistance genes (ARGs) via mobile genetic elements through processes including natural competence, transformation, and plasmid transconjugation that can occur in any environment (Kelly et al., 2017; Rozwandowicz et al., 2018).

The widespread detection of CPE is an emerging issue with potentially serious public health implications; further, the distribution of the most common carbapenemase genes in Enterobacteriaceae occurs in a country- and region-specific manner (Logan and Weinstein, 2017). In Japan, IMP is the most predominant type of carbapenemase among clinical CPE isolates (Koyano et al., 2013; Ohno et al., 2017; Yamamoto et al., 2017). VIM, OXA-48-like, KPC, and NDM carbapenemases are detected at low frequencies in Japan, whereas KPC and NDM are predominant

in China and OXA-48-like and KPC are the predominant types of carbapenemases in Europe and United States, respectively.

Most CRE/CPE infections occur in hospitals, with major outbreaks at long-term care facilities and affecting patients with severe medical conditions under long stays for clinical management (Grabowski et al., 2017). There are a number of factors that predispose individuals to infections by CRE and other multi-drug resistant Enterobacteriaceae, including extended-spectrum β-lactamase (ESBL)-producers. Indeed, healthy carriers of CTX-M-type ESBL-harboring bacteria represent major public health concerns, because the carriage rates are on the rise, particularly in South East Asia and Eastern Mediterranean regions. Further, carriers from these regions could potentially spread these bacteria to other communities (Woerther et al., 2013).

Exposure to AMR bacteria can cause serious infections in patients with risk factors such as empirical antimicrobials, advanced age, immune-suppression, admission to the intensive care unit, mechanical ventilation, transplantation, and prolonged hospital stay (Gasink et al., 2009). Early intervention, through the administration of effective antimicrobials to such high-risk group patients, must be achieved to prevent death. In addition, a recent systematic review identified a prevalence of 0–29.5% for community-associated CRE, suggesting that the early detection of CRE-carriers among hospitalized patients upon admission to long-term care facilities might help to prevent nosocomial outbreaks and control the limited distribution of such emerging public health threats (Kelly et al., 2017).

Generally, *Klebsiella*, *Escherichia coli*, *Enterobacter*, *and Citrobacter* have been reported as the main contributors to the nosocomial transmission of CPE (Hrabak et al., 2014; Goodman et al., 2016; Kwong et al., 2018). Other opportunistic pathogens among Enterobacteriaceae species can acquire carbapenemase genes through plasmid transmission from main CPE contributors. *Kluyvera* is a group of gram-negative rod-shaped bacteria and is a member of the Enterobacteriaceae family; the genus contains four species, namely *Kluyvera ascorbata*, *Kluyvera cryocrescens*, *Kluyvera georgiana*, and *Kluyvera intermedia*, which have all been found in humans (Farmer et al., 1981). *K. ascorbata* and *K. cryocrescens* were reported as potential pathogens that are associated with sepsis, bacteremia, catheter-associated urinary tract infections, pyelonephritis, and intraabdominal symptoms in immunocompromised hosts (Karadag Oncel et al., 2015; Yoshino et al., 2016).

Here, we report a novel IMP-6-producing isolate of *Metakosakonia* sp., namely strain MRY16-398, from a clinical specimen (ascites), and determined the genomic features of this carbapenemase-producing species.

MATERIALS AND METHODS

Ethics Approval

The study protocol was approved by the ethics committee of the National Institute of Infectious Diseases in Japan (Approval No. 642, 11/Dec/2015). It was conducted according to the principles of the Declaration of Helsinki, in compliance with the Law Concerning the Prevention of Infections and Medical Care for Patients of Infections of Japan; the ethical committee waived the need for written consent regarding the research of bacteria isolates; the personal data related to the clinical information were anonymized, and we do not request written consent for all patients suffering from bacterial infections.

Bacterial Strains and Identification

Upon admitting a patient complaining of acute abdominal pain, abdominal computed tomography (CT) scanning showed a diverticulum in the descending and sigmoid colon, resulting in the diagnosis of sigmoid colon diverticulitis. A summary of laboratory data for the patient is shown in **Supplementary Table S1**. Empiric antimicrobial treatment with cefmetazole (0.5 g twice per day) was administered, and the volume of ascites was decreasing at 5 days from onset.

The *Metakosakonia* sp. MRY16-398 strain was isolated from the ascites of a patient with sigmoid colon diverticulitis in 2015 in Japan. The isolate was identified as *Klebsiella oxytoca* at the hospital microbiology laboratory using BD Phoenix (Becton Dickinson) with low reliability. Further phenotypic tests were performed using API 20E (bioMérieux) and Lysine-Indole-Motility Medium (Nissui, Tokyo Japan). Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)-based identification was conducted with a MicroFlex LT mass spectrometer (Bruker Daltonik), and analyzed using MALDI Biotyper software (Bruker Daltonik).

Antimicrobial Susceptibility and CPE Screening Tests

Antimicrobial susceptibility was investigated by broth microdilution using the MicroScan Neg MIC 1J panel (Beckman Coulter) and Etest (bioMérieux) according to manufacturers' instructions (Clsi, 2018). Boronic acid, clavulanic acid, and sodium mercaptoacetic acid (SMA) were used as inhibitors for double-disk synergy tests (DDSTs) to identify AmpC-types and KPC-types, as well as extended-spectrum and metallo-β-lactamases, respectively. Carbapenemase production was assessed by performing a Carba NP test, as described previously (Nordmann et al., 2012). PCR testing was subsequently performed for potential CPE using a specific primer-pair for the following types of β-lactamase-encoding genes: bla_{IMP} (Shibata et al., 2003), bla_{VIM} (Shibata et al., 2003), $bla_{\mathrm{OXA-48-like}}$ (Poirel et al., 2004), bla_{KPC} (Bradford et al., 2004), and bla_{NDM} (Segawa et al., 2017).

Plasmid and Chromosome DNA Analysis With Short-Read Sequencing

Plasmid DNA was separated from chromosomal DNA by S1 nuclease-digestion followed by pulsed-field gel electrophoresis. Visible plasmid DNA and chromosomal DNA bands were extracted from the gel and purified using the ZR-96 Zymoclean gel DNA recovery kit (Zymo Research, Irvine, CA, United States). A DNA sequencing library was prepared using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, United States) and was sequenced using an Illumina MiSeq

and NextSeq 500 for plasmids and chromosomes, respectively. Sequencing reads (plasmid: 2 × 300-mer, 140 × median coverage; chromosome: 2 × 150-mer, 99 × median coverage) were assembled into contigs using the A5-MiSeq pipeline (Coil et al., 2015). Plasmid replicon typing was performed using the curated PlasmidFinder database at the CGE website[1] (Carattoli et al., 2014).

Whole-Genome Sequence (WGS) Analysis With Long-Read Sequencing

Genomic DNA from the isolated strain was purified by collecting cells from a 5-mL overnight culture grown in TSB broth. The cell pellet was resuspended in 500 μL of TE10 [10 mM tris (pH 8.0) and 10 mM EDTA] supplemented with 500 μL phenol/chloroform, and the cells were subsequently lysed by bead-beating for 10 min in ZR BashingBead lysis tubes (Zymo Research, Irvine, CA, United States) attached to a vortex adapter (MO BIO Laboratories, QIAGEN, Carlsbad, CA, United States). After centrifugation at 10,000 × *g* for 5 min; the upper phase was further purified using a Qiagen DNA purification kit (Qiagen, Germany).

The complete genome sequence of the strain was determined using a PacBio RSII sequencer for long-read sequencing (SMRT cell v3 using P6C4 chemistry; insert size, approximately 10 kb). Purified genomic DNA (∼2.0 μg) was used to prepare a SMRTbell library using a SMRTbell template prep kit 1.0 (PacBio, Menlo Park, CA, United States) according to manufacturer's instructions. The obtained raw polymerase reads were analyzed using the HGAP v3.0 pipeline based on Celera *de novo* assembler and Quiver polishing scripts (Chin et al., 2013).

De novo assembly was performed using HGAP 4 of SMRT Link Analysis v. 4.0.0.190159 and circulator version 1.5.3 (Chin et al., 2013). Error correction of tentative complete circular sequences was performed using Pilon version 1.18 with Illumina short reads (Walker et al., 2014). Annotation was performed using DFAST (Tanizawa et al., 2018) with databases as follows: DFAST default database, ResFinder database (Zankari et al., 2012), Bacterial Antimicrobial Resistance Reference Gene Database (PRJNA313047), and Virulence Factors Database (Chen et al., 2012). Circular representations of complete plasmid sequences were visualized using GView server (Petkau et al., 2010).

Comparative Genome Sequence Analysis

All publicly available draft genome sequences were searched based on 16S rRNA gene homology, comparing them to that of the MRY16-398 strain, and 41 entries were retrieved from the NCBI genome database (see **Supplementary Table S2**). Among those 42 strains, orthologous core-gene sets were extracted using a nucleotide homology search with a threshold ≥80%, resulting in the identification of 479 core-gene sets (see **Supplementary Table S2**). Using these core-gene sets, core-gene phylogeny was generated using the maximum-likelihood phylogenetic method with FastTree v2.1.10 (Price et al., 2010). To construct a pairwise amino acid homology distance matrix, all amino acid sequences were compared pairwise, against each other, for each genome using the BLASTP program, which was followed by the calculation of average identity scores and standard deviations (**Supplementary Table S3**).

Nucleotide Sequence Accession Numbers

The complete, annotated genomic sequence of *Metakosakonia* sp. MRY16-398 was deposited in a public database (accession numbers: chromosome, AP018756; pMRY16-398_1, AP018757; pMRY16-398_2, AP018758). The short- and long-read sequences for DNA-Seq were deposited in the DNA Data Bank of Japan (BioProject PRJDB7098, BioSample SSUB009772, DRA accession DRA007011).

RESULTS

Bacterial Identification of Metakosakonia sp. MRY16-398

A potential CPE, designated as strain MRY16-398, was isolated from the ascites after abdominocentesis. The isolate was identified as *Klebsiella oxytoca* at the hospital laboratory using BD Phoenix (Becton Dickinson) with low reliability, whereas API20E testing indicated the isolate should be a *Pantoea* sp. instead of *K. oxytoca*. This isolate was negative for lysine decarboxylate activity and showed weak motility, which indicated that the isolate was not *Klebsiella*. MALDI-TOF MS did not result in any bacterial species with a score higher than 2.000, which secures genus and probable species identification. The highest score value was 1.885 for *Klebsiella aerogenes*, followed by 1.789 for *K. oxytoca*.

Generally, 16S-rRNA gene sequencing is one of first tools used to determine the correct bacterial species of such novel CPE isolates, and thus we considered that WGS would be a more straightforward approach to characterize the species and plasmids involved in AMR.

The MRY16-398 isolate was observed to harbor bla_{IMP-6}, exhibited resistance to meropenem, and was positive based on the Carba NP test and DDST using SMA. Further antimicrobial susceptibility testing indicated that MRY16-398 exhibited remarkably reduced susceptibility to most β-lactam antibiotics (**Table 1**).

Whole-Genome Sequence Analysis of Metakosakonia sp. MRY16-398

Basic information regarding the complete chromosome and plasmid sequences of *Metakosakonia* sp. MRY16-398 is shown in **Table 2**. The strain possessed two plasmids, and pMRY16-398_2 was determined to be an IncN replicon plasmid, harboring multiple AMR-encoding genes including the bla_{IMP-6} carbapenemase-encoding gene (**Table 2**). The IMP-6 metallo-β-lactamase is an IMP variant with a $S_{214}G$ amino acid substitution in the catalytic domain of IMP-1, resulting in significantly diminished enzymatic activity toward imipenem

[1] https://cge.cbs.dtu.dk//services/PlasmidFinder/

TABLE 1 | Antimicrobial susceptibility testing.

Antimicrobial agent	MIC (μg/mL)/ antimicrobial susceptibility	MIC breakpoint (μg/mL)[a]		
		S	*I*	*R*
Piperacillin	>256/R	≤16	32–64	≥128
Amoxicillin-clavulanic acid	8/S	≤8/4	8–16	≥32/16
Piperacillin-tazobactam	2/S	≤16/4	32/4–64/4	≥128/4
Cefepime	128/R	≤2	4–8	≥16
Ceftazidime	128/R	≤4	8	≥16
Imipenem	0.75/S	≤1	2	≥4
Meropenem	16/R	≤1	2	≥4
Doripenem	8/R	≤1	2	≥4
Ertapenem	>32/R	≤0.5	1	≥2
Gentamicin	8/I	≤4	8	≥16
Tobramycin	16/R	≤4	8	≥16
Amikacin	2/S	≤16	32	≥64
Minocycline	8/I	≤4	8	≥16
Ciprofloxacin	0.5/S	≤1	2	≥4
Fosfomycin	128/I	≤64	128	≥256

[a]*CLSI guideline for MIC breakpoints of Enterobacteriaceae. S, Susceptible; I, Intermediate; R, Resistant.*

TABLE 2 | Whole genome information for *Metakosakonia* sp. MRY16-398.

Replicon	Nucleotide length (bp)	Gene coding	GC%	Inc type	AMR genes	GenBank ID
Chromosome	5,919,168	5,638	53.1	NA	ND	AP018756
pMRY16-398_1	224,544	239	52.8	IncFIB(K), IncFII	ND	AP018757
pMRY16-398_2	47,417	55	52.3	IncN	*aacA4′-3, aadA2,* $bla_{CTX-M-2}$, bla_{IMP-6}, *sul1, tet*(A)	AP018758

NA, not available; ND, not detected.

but not meropenem (Oelschlaeger et al., 2005). Thus, MRY16-398 showed susceptibility to imipenem, but resistance to other carbapenems (**Table 1**). The *aacA4′-3* gene encoding aminoglycoside-3″-adenylyltransferase, and the *aadA2* gene encoding streptomycin 3″-adenylyltransferase are involved in resistance to aminoglycosides. The *tet*(A) gene could be involved in reduced susceptibility to minocycline. Comparative analyses of the MRY16-398 genome sequence including the pMRY16-398_2 plasmid are described in the following section.

Orthologous Gene Phylogenetic Analysis of *Metakosakonia* sp. MRY16-398

To determine the potential bacterial species of the MRY16-398 strain, we performed orthologous gene phylogenetic analysis using 41 publicly available bacterial genome sequences (on 2017/03/14), including draft genomes (see the strain list in **Supplementary Table S2**). Among those 42 strains including MRY16-398, 479 orthologous gene sets were extracted at ≥80% nucleotide identity, and phylogeny and matrix distance clearly suggested that MRY16-398 was closely related to the bacterial species [*Kluyvera*] *intestini* str. GT-16 (Tetz et al., 2017), which was isolated from the stomach of a patient with gastric cancer (**Figure 1**). Recently, the taxonomic nomenclature for [*Kluyvera*] *intestini* str. GT-16 has been re-classified into a new proposed genera, namely *Metakosakonia*, which includes *M. massiliensis* JC163 (Alnajar and Gupta, 2017). As well as the proposal, this study demonstrated GT-16 strain shows the closest lineage to *Metakosakonia,* and having a distinct lineage from the main *Kluyvera* species (*K. georgiana* and *K. intermedia*) (**Figure 1**). Thus, MRY16-398 was found to be a clearly distinct lineage from *Kluyvera, Pantoea*, and other well-characterized genera of the Enterobacteriaceae family (**Figure 1**), indicating that this clinical isolate is a novel species. Here, we tentatively classified MRY16-398 as *Metakosakonia* sp.

Structural Comparison of pMRY16-398_2-Associated IncN Plasmids

S1-PFGE suggested that MRY16-398 carries two plasmids (**Figure 2A**), and the size of both plasmids corresponded to sequencing results as well as whole genome sequencing (**Table 2**). An analysis of conserved genes in the pMRY16-398_2 plasmid indicated that horizontally acquired AMR genes [class 1 integron, $bla_{CTX-M-2}$, and *tet*(A)] are variable in each plasmid, although IncN backbone regions remained well conserved (**Figure 2B**). The class 1 integron has been classified as In722 (*intI1*, *aacA4'-3*, bla_{IMP-6}, *aadA2*, and

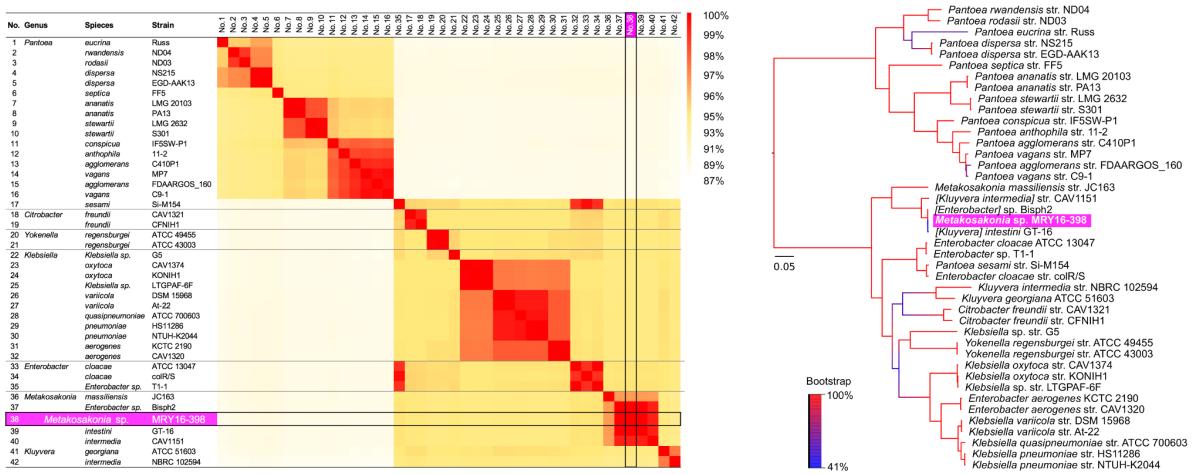

FIGURE 1 | Pairwise homology sequence analysis using 479 core-gene sets from 42 strains related to MRY16-398. Maximum-likelihood phylogenetic analysis with 1,000× bootstrapping and the generation of a pairwise homology matrix were performed for the core-gene set listed in **Supplementary Table S2**. The MRY16-398 strain is highlighted with a bright purple background. The phylogeny and matrix distance clearly suggested that MRY16-398 is closely related to the bacterial species [*Kluyvera*] *intestini* str. GT-16 (Tetz et al., 2017), and distinct from other Enterobacteriaceae. Detailed homology % values for the matrix distances can be seen in **Supplementary Table S3.**

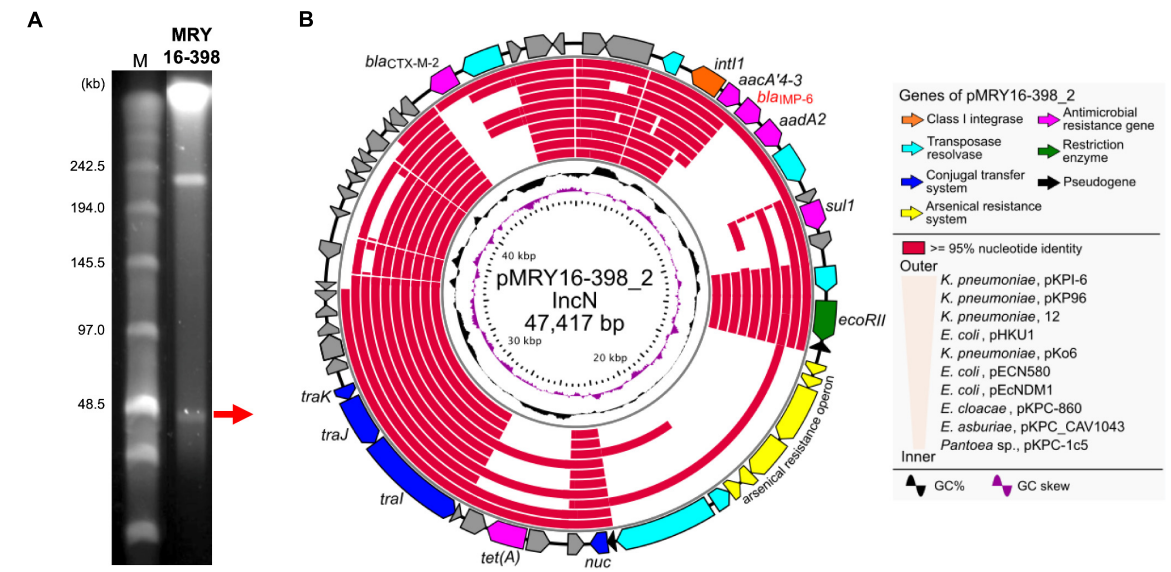

FIGURE 2 | Representation of conserved gene analysis of a plasmid from *Metakosakonia* sp. MRY16-398. **(A)** Plasmids were identified by pulsed-field gel electrophoresis using an S1 nuclease-treated genomic DNA plug. **(B)** Circular representation of the plasmid pMRY16-398_2 carrying bla_{IMP-6}, and conserved gene analysis, with comparative information for other indicated similar plasmids (**Supplementary Table S4**). From inward, slot 1, GC skew; slot 2, GC content; slot 3 to 12, source of IncN plasmids (see **Supplementary Table S4**), slot 13 and 14, open reading frames.

*gc*ISKpn22) in the INTEGRALL database[2] (Moura et al., 2009). pMRY16-398_2 shared an identical ARG profile and organization with pKPI-6 from *Klebsiella pneumoniae* KPI-6 (**Figure 2B**).

Pairwise alignment clearly showed that some genes involved in the conjugal transfer system have been excised and replaced with arsenical resistance proteins (Ars system) (Diorio et al., 1995) via an IS*6100*-mediated homologous recombination event (**Figure 3**). A mating transconjugation experiment to recipient *E. coli* showed negative plasmid transmission with pMRY16-398_2, although positive transmission was observed with a certain IncN plasmid harboring a full set of *tra* genes (data not shown). In addition to multiple AMR genes, pMRY16-398_2 appears to have lost its self-conjugation transfer ability to other bacteria, whereas it acquired arsenic resistance. This likely led to an increase in the persistence and fitness of the novel bacterial

[2]http://integrall.bio.ua.pt/

species, which is an opportunistic pathogen, in the presence of high concentrations of disinfectants in the hospital environment.

Additional Potential AMR Genes

A search for ARGs revealed an additional potential class A β-lactamase (MRY16398_50310), with 76% amino acid similarity to the TEM-1A variant, in the chromosomal DNA of MRY16-398 (**Figure 4**). A maximum-likelihood phylogeny among TEM-1A-related class A β-lactamases suggested that MRY16398_50310 is closely related to those of *Kluyvera* spp.

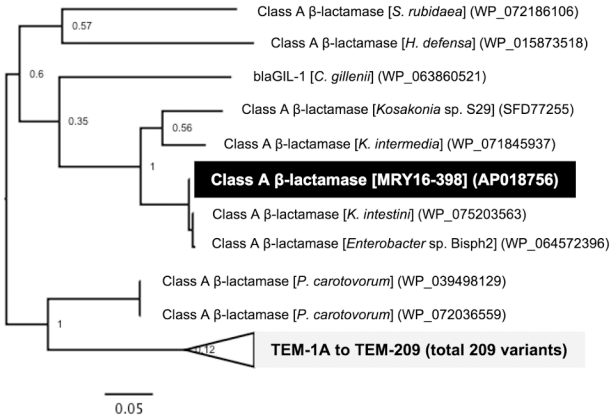

FIGURE 4 | Class A β-lactamase-encoding genes in the chromosome of *Metakosakonia* sp. MRY16-398. A maximum-likelihood phylogeny among TEM-1A-related class A β-lactamases suggesting that MRY16398_50310 encodes a possible class A β-lactamase. Bootstrapping values are shown at the nodes.

DISCUSSION

In this study, we isolated an IMP-6-producing novel Enterobacteriaceae species, *Metakosakonia* sp. strain MRY16-398, from the ascites of a diverticulitis patient. Nosocomial CPE outbreaks are generally caused by virulent pathogens; however, avirulent bacteria can cause opportunistic infection as apparent pathogens upon acquiring a notable resistance determinant. Such rare cases of avirulent or novel bacteria species have are not often documented as case reports, because conventional testing for bacterial identification are not always correct for novel species, as shown in this study. WGS comprised a straightforward approach to characterize the overall features of this isolate and its plasmids involved in AMR, and this genome sequence will be helpful for further characterization of infections caused by *Metakosakonia* sp.

A few studies related to the *Metakosakonia* genus have been reported thus far, and the most genetically related genus *Kluyvera* represents an informative reference for further discussion. *Kluyvera* spp. strains have been reported as potential pathogens in immunocompromised hosts; in addition, the *Kluyvera* genus is one source of CTX-M genes, which are thought to be the most common and important extended-spectrum β-lactamase-encoding genes (Humeniuk et al., 2002). For example, KLUA-producing *Kluyvera ascorbata* can survive for a long time in environments such as sewage and the human gut and promote drug resistance-associated gene transfer (Farmer et al., 1981). Based on a report of AMR in *Kluyvera*-related species, bla_{GES-5} positive, carbapenem-resistant *K. intermedia* were isolated from a hospital environment (Ribeiro et al., 2014). Further, KPC-2-producing *K. ascorbata* has been reported in a case of biliary tract infection (Wang et al., 2018). A *K. ascorbata* isolate positive for the colistin resistance gene, *mcr-1*, was identified from hospital sewage in China (Zhao and Zong, 2016). Such opportunistic pathogens including *Kluyvera* represent important multi-drug resistant bacteria in clinical settings and other environmental sources.

Here, we isolated a novel species, *Metakosakonia* sp. MRY16-398, and revealed the horizontal acquisition of the bla_{IMP-6} plasmid in this novel species that is rarely associated with the clinical settings. Such novel opportunistic pathogens might act as a potential reservoir/source of clinically relevant antibiotic

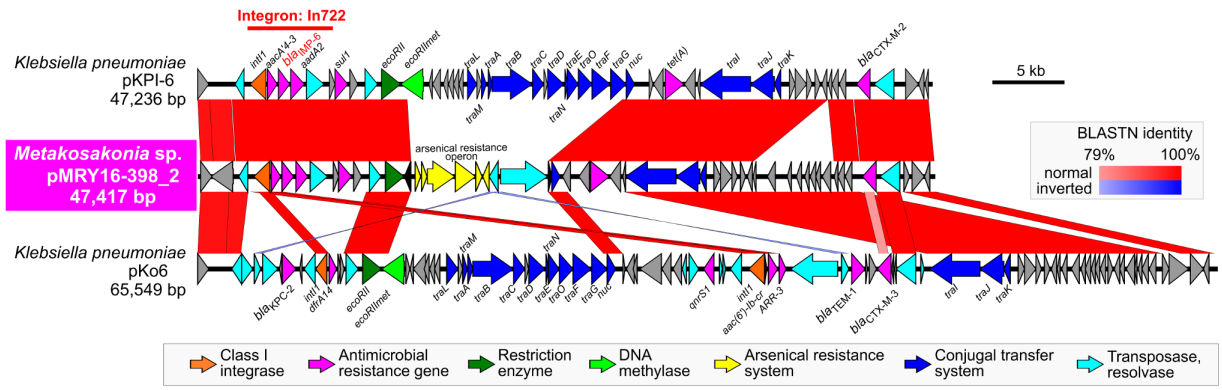

FIGURE 3 | Structural comparison of the bla_{IMP-6}-positive IncN plasmids. pMRY16-398_2 was aligned with IncN plasmid pKPI-6 (GenBank ID: AB616660) from *Klebsiella pneumoniae* KPI-6 isolated in 2007 in Japan. Homologous regions between the two are shown as red boxes with the color gradient corresponding to % homology. These plasmids carry the class 1 integron, In722 (*intI1*, *aacA4'-3*, bla_{IMP-6}, *aadA2*, and *gc*ISKpn22), classified in the INTEGRALL database (http://integrall.bio.ua.pt/) (Moura et al., 2009), and additional $bla_{CTX-M-2}$ and *tet* (A).

resistance genes. In conclusion, these findings highlight the fact that bacterial identification is a crucial primary step when an isolate exhibits markedly reduced susceptibility as CPE. Moreover, continuous and comprehensive monitoring including WGS should be conducted for the detection of CPE even in nonpathogenic bacteria isolated from the clinical settings.

DATA AVAILABILITY STATEMENT

The complete, annotated genomic sequence of [*Kluyvera*] *intestini* MRY16-398 was deposited in a public database (Accession Nos. chromosome, AP018756; pMRY16-398_1, AP018757; pMRY16-398_2, AP018758). The short- and long-read sequences for DNA-Seq were deposited in the DNA Data Bank of Japan (BioProject PRJDB7098, BioSample SSUB009772, DRA accession DRA007011).

AUTHOR CONTRIBUTIONS

TT and AT contributed to the isolation of the IMP-6 positive *Metakosakonia* sp. strain MRY16-398. MM performed S1-PFGE analysis to detect individual plasmids. MM and SS performed antimicrobial susceptibility testing and DNA preparation for whole genome sequencing. TS and MK performed genome sequencing and the comparative genome analysis. MH performed mating transconjugation experiments. MK wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02853/full#supplementary-material

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REVIEW
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Carbapenem-Resistant Enterobacteriaceae Infections: Taiwan Aspects

*Shio-Shin Jean[1,2], Nan-Yao Lee[3], Hung-Jen Tang[4,5], Min-Chi Lu[6], Wen-Chien Ko[3] and Po-Ren Hsueh[7,8]**

[1] Department of Emergency, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, [2] Department of Emergency Medicine and Department of Emergency and Critical Care Medicine, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan, [3] Department of Internal Medicine, National Cheng Kung University Medical College and Hospital, Tainan, Taiwan, [4] Department of Medicine, Chi Mei Medical Center, Tainan, Taiwan, [5] Department of Health and Nutrition, Chia Nan University of Pharmacy and Science, Tainan, Taiwan, [6] Department of Microbiology and Immunology, School of Medicine, China Medical University, Taichung, Taiwan, [7] Department of Laboratory Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan, [8] Department Internal Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan

Carbapenem-resistant Enterobacteriaceae (CRE), a major resistance concern emerging during the last decade because of significantly compromising the efficacy of carbapenem agents, has currently become an important focus of infection control. Many investigations have shown a high association of CRE infections with high case-fatality rates. In Taiwan, a few surveys observed that a significant proportion (29–47%) of the CR-*Klebsiella pneumoniae* isolates harbored a plasmidic allele encoding *K. pneumoniae* carbapenemases (KPC, especially KPC-2). A significant increase in the number of oxacillinase (OXA)-48-like carbapenemases among CR-*K. pneumoniae* isolates was observed between 2012 and 2015. By striking contrast, isolates of CR-*Escherichia coli* and CR-*Enterobacter* species in Taiwan had a much lower percentage of carbapenemase production than CR-*K. pneumoniae* isolates. This differs from isolates found in China as well as in the India subcontinent. Apart from the hospital setting, CRE was also cultured from the inpatients from communities or long-term care facilities (LTCF). Therefore, implementation of regular CRE screening of LTCF residents, strict disinfectant use in nursing homes and hospital settings, and appropriate control of antibiotic prescriptions is suggested to alleviate the spread of clinical CRE isolates in Taiwan. Although there are some promising new antibiotics against CRE, such as ceftazidime-avibactam, meropenem-vaborbactam, aztreonam-avibactam and cefiderocol, these agents are not available in Taiwan currently. Therefore, in order to effectively decrease case-fatality rates among patients with the infections owing to carbapenemase-producing CRE isolates, combination antibiotic schemes, including colistin (or amikacin) and/or tigecycline in combination with an anti-pseudomonal carbapenem agent, remain the mainstay for treating clinical CRE infections.

Keywords: carbapenem-resistant Enterobacteriaceae, carbapenemase, *Klebsiella pneumoniae*, *Escherichia coli*, long-term care facility, tigecycline, colistin, avibactam

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Edited by:
Yi-Wei Tang,
Memorial Sloan Kettering Cancer Center, United States

Reviewed by:
Hasan Ejaz,
Al Jouf University, Saudi Arabia
Zhiyong Zong,
Sichuan University, China

***Correspondence:**
Po-Ren Hsueh
hsporen@ntu.edu.tw

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INTRODUCTION

Carbapenem-resistant Enterobacteriaceae (CRE), drawing great attention because of their serious resistance spectra and outbreak episodes in the northeastern United States since about two decades ago, have shown a high potential of rapid disseminations (Bratu et al., 2005a). Infections caused by CRE with production of various carbapenemases have become a major global worrisome concern due to their association with high (≥30%) case-fatality rates in the current antibiotic pipeline (Tumbarello et al., 2012; Tzouvelekis et al., 2012; Navarro-San Francisco et al., 2013; Jean et al., 2015). In addition, McConville et al. conducted an observational study investigating CRE colonization (by rectal swabs) with subsequent impact on clinical outcomes for patients requiring admission to an intensive care unit (ICU) (McConville et al., 2017). By multivariate analysis, they found that CRE colonization also independently predicted development of a further systemic CRE infection at 30 days [adjusted odds ratio (aOR), 10.8; 95% confidence interval (CI), 2.8–41.9, $P = 0.0006$]. In 2017, the World Health Organization ranked CRE one of the first antibiotic-resistant "critical priority pathogens" (World Health Organization [WHO], 2017). Nevertheless, among these CRE isolates, the carbapenem non-susceptibility phenotypes are attributed to production of carbapenemase(s), or more likely, production of extended-spectrum β-lactamase (ESBL) plus AmpC β-lactamase with dysfunctional entry routes (i.e., porin change) of carbapenems, integrons and insertion sequence common region 1 (ISCR1) carrying various resistance genes, and/or efflux pumps, etc. (Doumith et al., 2009; Yang et al., 2012; Rui et al., 2018). To alleviate the resistance burden, close monitoring of CRE prevalence rates, as well as investigation of carbapenemase types, has become the main foci for infection control in most countries (Gupta et al., 2011).

Between 2004 and 2013, the prevalence rates of CRE among all clinical Enterobacteriaceae isolates have gradually risen (up to about 7%) in medical centers and major regional teaching hospitals throughout Taiwan. However, official surveillance data, conducted by Taiwan Centers for Disease Control (Taiwan CDC), clearly showed the annual prevalence rates of overall CRE isolates collected from ICUs rose from 3.7% in 2008 to 15.3% in 2017 (data until the third quarter) (Taiwan Nosocomial Infection Surveillance [TNIS], 2017). In addition, among *Escherichia coli* isolates acquired at ICUs, CRE rates rose from 1.2% in 2008 to 4.0% in 2017 at medical centers (bed number, >1000) (until the third quarter), while CRE rates in major regional teaching hospitals (bed number, 500–1000) rose from 1.0% in 2008 to 2.8% in 2017. These data might reflect the differences in CRE burden between hospitals of various care levels. By striking contrast, during the same period, significantly higher CRE rates for ICU-acquired *Klebsiella pneumoniae* isolates were observed in medical centers (from 6.1 to 28.2%) and in major regional teaching hospitals (from 3.7 to 24.8%) than those of ICU-acquired *E. coli* (Taiwan Nosocomial Infection Surveillance [TNIS], 2017). It was determined by multivariate logistic regression analyses that clinicians in Taiwan (aOR, 1.73; 95% CI, 1.03–2.92) and in the US (aOR, 1.89; 95% CI, 1.05–3.39) are more likely to prescribe carbapenem antibiotics to treat the potential ESBL-producing Enterobacteriaceae infections instead of β-lactam combination agents compared to other countries (Harris et al., 2017). Clinical overuse of carbapenem agents unavoidably result in increased drug resistance (Tumbarello et al., 2012; Sheu et al., 2018).

STUDY DESIGNS

We searched and reviewed literature from the 2002–2018 PubMed database containing important keywords, including "carbapenem-resistant Enterobacteriaceae," "carbapenemase-producing Enterobacteriaceae," "prevalence rates," "mortality," "case-fatality," "Taiwan," "Centers for Disease Control," "*Klebsiella pneumoniae,*" "*Klebsiella pneumoniae* carbapenemase (KPC)," "New Delhi metallo-β-lactamase (NDM)," "oxacillinase-48 (OXA-48)-like," "*Escherichia coli,*" "*Enterobacter* species," "resistance mechanisms," "community-acquired," "long-term care facility," "Clinical and Laboratory Standards Institute," "European Committee on Antimicrobial Susceptibility Testing," "monotherapy," "combination therapy," "tigecycline," "colistin," and "novel antibiotics."

PREVALENCE AND MORTALITY RATES OF CARBAPENEMASE-PRODUCING ENTEROBACTERIACEAE (CPE) AMONG CRE

In Pakistan, spread of special sequence types (STs; ST15 and ST48) of multidrug-resistant (MDR) *Klebsiella* species (most were ESBL-producing *K. quasipneumoniae*) isolates was observed since 2010 at one tertiary-care hospital (Ejaz et al., 2017). Similarly, among 83 carbapenem-resistant *K. pneumoniae* isolates studied in Brazil, a clinical isolate of KPC-2 and OKP-B-6 β-lactamase-producing *K. quasipneumoniae* subsp. *similipneumoniae* was also reported. (Nicolás et al., 2018). By stark contrast, global dissemination of CPE (especially *K. pneumoniae* isolates) in fact has been occurring at an alarming pace for many years (Logan and Weinstein, 2017). In Taiwan, most CP-*K. pneumoniae* isolates are the KPC producers (Jean et al., 2015). The bla_{KPC} genes mostly reside on transferable plasmids which contain transposase, resolvase, and mobile transposons (Tn*4401*), thereby posing a formidable threat to infection control (Nordmann et al., 2009). As compared to other Enterobacteriaceae species, it is noteworthy that these mobile transposons are only detected among few epidemic clones of KPC producers of *K. pneumoniae* with distinct STs (predominantly ST258 in US and Israel, while ST11 in China and Taiwan) (Naas et al., 2008). The ST11 clone was the most prevalent ESBL-producing clone (particularly CTX-M-15, CTX-M-14, and SHV-5 types) of *K. pneumoniae* isolates in many Asian countries (Lee et al., 2011; Ma et al., 2013a).

Regarding the molecular analyses of carbapenemase types in CRE in Taiwan, a multicenter study first examined imipenem-resistant *K. pneumoniae* isolates collected from 13 Taiwanese hospitals from 2002 through 2009 (Ma et al., 2013a). This study revealed that a majority (84.6%) of *K. pneumoniae* strains (mainly

ST11 clone) with > 2 mg/L minimum inhibitory concentrations (MICs) to imipenem harbored bla_{IMP-8}, plus various genes encoding ESBLs in combination with loss of porins (mainly OmpK35) (Ma et al., 2013a). In 2012, a small-scale Taiwanese study, conducted by Chang et al., investigated 66 patients with CRE (comprising *K. pneumoniae* and *E. coli*) infections (n = 46) or colonization (n = 20). It showed that the CPE prevalence rate was 28.8% (n = 19; 14 of which harbored bla_{KPC-2}), and a 30-day mortality rate was 50% (23/46) among patients with CRE infections (Chang et al., 2015). The CPE prevalence of Chang's study was different from that [11.5%; most were metallo-β-lactamase (MβL) producers] of the study conducted by Javed et al. (2016) in Pakistan during 2013–2014. In addition, this mortality rate was higher compared to that (40.8%) of the other CRE study (susceptibility evaluated by the disk diffusion method, and data interpretation in accordance with the 2009 criteria) at a medical center located in the middle-western part of Taiwan during 2010–2011 (Huang et al., 2014). In Chang's study, nearly one-half [47.8% (22/46)] of the infections originated in the lower respiratory tract. Furthermore, patients with a co-morbidity of diabetes mellitus, initially presenting with shock or high scores of the Acute Physiology and Chronic Health Evaluation (>23 points), or receiving non-susceptible antibiotic regimen therapy (regardless of single or combination drug therapy) for > 48 h

TABLE 1 | Studies of carbapenem-resistant Enterobacteriaceae in Taiwan and other countries.

Study periods	CRE case numbers, or CRE isolates under survey	Carbapenemase production, %	Outcomes	Reference
2010–2012	1,135 carbapenem non-susceptible Enterobacteriaceae isolates (from various clinical sources)	5% (57/1135)	NA	Wang et al., 2015
2012	66 (including 46 patients with diverse CRE infections and 20 patients with CRE colonization)	28.8% (19/66)	In-hospital mortality, 50% (23/46) for patients with CRE infections	Chang et al., 2015
2012–2013	105 (including 49 patients with various CRE infections, and 56 patients with CRE colonization)	29.5% (31/105)	Overall in-hospital mortality, 43.8% (46/105); for 49 cases with CRE infections, 63.3% (31/49)	Wu et al., 2016
2012–2015	1,457 CR-*K. pneumoniae* isolates	31.4% (457/1457)	NA	Chiu et al., 2018
2017	85 bloodstream CRE isolates, comprising *Escherichia coli* (n = 14) as well as *K. pneumoniae* (n = 71)	41.2% (35/85); 46.5% (33/71) for CR-*K. pneumoniae* isolates	NA	Jean et al., 2018b
2015	70 CRE isolates (from various clinical sources), collected from patients in Brazil	80% (56/70); all harbored bla_{KPC}	NA	Lorenzoni et al., 2017
2016–2017	22 CRE isolates, collected from urine samples of patients in the United Kingdom	45.4% (10/22)	NA	Woodford et al., 2017
2017	287 clinical CRE isolates, collected from patients in Thailand	77.7% (223/287), a majority of them harbored bla_{NDM-1}	NA	Laolerd et al., 2018

CRE, carbapenem-resistant Enterobacteriaceae. NA, non-applicable.

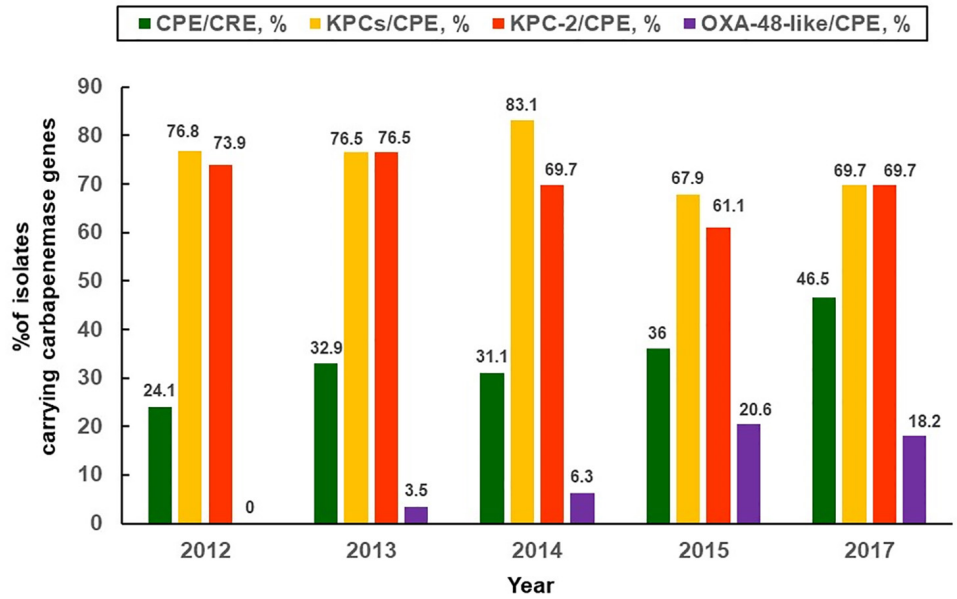

FIGURE 1 | Distribution of carbapenemases. Annual proportions of overall and different types of carbapenemase producers (*Klebsiella pneumoniae* carbapenemase, oxacillinsase-48-like) among carbapenem-resistant *K. pneumoniae* isolates collected between 2012 and 2017 in Taiwan (Chiu et al., 2018; Jean et al., 2018b).

had significantly higher case-fatality rates ($P < 0.05$) than the other factors by the univariate analysis (Chang et al., 2015). Of note, patients who acquired CRE infections owing to KPC (dominant carbapenemase type)-producing Enterobacteriaceae also had a trend toward more fatal outcomes than those without KPC ($P = 0.14$) (Chang et al., 2015). An additional CRE study conducted at a single medical center of northern Taiwan during 2012–2013 showed that a CRE isolate with an imipenem MIC $\geq$ 16 mg/L independently predicted 14-day mortality among patients regardless if the isolate was from infection or colonization (Wu et al., 2016). In the survey published by Wu et al., KPC-2 was the dominant (87.1%) carbapenemase among CPE (29.5% of overall CRE), and the in-hospital mortality rate among patients with CR-*K. pneumoniae* was 43.8% (Wu et al., 2016). Wang et al. examined 1135 clinical isolates of various Enterobacteriaceae species collected from four major hospitals in Taiwan between 2010 and 2012. Fifty-seven isolates were carbapenemase-producing Enterobacteriaceae (CPE) isolates (5%), 54.4% of the CPE isolates co-harbored bla_{ESBL} alleles. Furthermore, Wang et al. found that *Enterobacter cloacae* isolates predominantly harbored the $bla_{\mathrm{IMP-8}}$ allele (26/27), while isolates of CP-*K. pneumoniae* mainly harbored $bla_{\mathrm{KPC-2}}$ alleles (16/17; ST11 clone predominated) (Wang et al., 2015). In addition, among Taiwanese CRE isolates surveyed, approximately one-half (45.8%) harbored various bla_{ESBL} genes (80% were the $bla_{\mathrm{CTX-M}}$ types), while 62.0% harbored alleles encoding various plasmid-mediated AmpC β-lactamases (70.6% were bla_{DHA} and 22.3% were bla_{CMY}) (Wang et al., 2015). The high rates of *K. pneumoniae* isolates co-harboring ESBL and/or AmpC-encoding alleles among CRE corresponded well with those of an Asia-Pacific study with respect to Enterobacteriaceae isolates responsible for complicated intra-abdominal infections (cIAI) and complicated urinary tract infections (cUTI) from 2008 through 2014 (Jean et al., 2017). According to the above findings, instead of carbapenemase production, ESBL with concomitant AmpC production and membrane impermeability caused by porin loss obviously more likely confers *in vitro* non-susceptibility to carbapenem agents (especially ertapenem) among CRE in Taiwan and the Asia-Pacific region. These results are similar to that found in Hong Kong (Ho et al., 2016).

Despite non-CPE isolates account for a majority of CRE in Taiwan, an up-surging CPE trend [three-fourths (74.5%) of CPE harboring $bla_{\mathrm{KPC-2}}$ and were ST11 clone, followed by CPE harboring $bla_{\mathrm{VIM-1}}$ allele (12.7%), etc.] was observed by Chiu et al. (2013). The CPE rates have increased from 0% in 2010 to 22.3% in 2012 among Taiwanese CR-*K. pneumoniae* isolates (showing > 1 mg/L of MICs to imipenem or meropenem) (Chiu et al., 2013). Furthermore, it is noteworthy that the prevalence rates of CPE [36.4%; mostly related to various bla_{KPC} (75.9%), followed by $bla_{\mathrm{OXA-48}}$-like (8.8%), $bla_{\mathrm{VIM-1}}$ (7.9%), and $bla_{\mathrm{IMP-8}}$ (5.7%), etc] among the studied CR-*K. pneumoniae* isolates (approximately 99% were non-susceptible to imipenem, and 85.8% were collected from medical centers) were estimated to have 1.5-fold increase from 2012 through 2015. Most significantly, an increasing trend was observed in the middle-western part of Taiwan (Chiu et al., 2018). Data regarding important CRE studies in Taiwan and other countries are shown in **Table 1**. There were big variations in the rates of CPE among clinical CRE isolates in Taiwan as compared to those from other countries (Lorenzoni et al., 2017; Woodford et al., 2017; Laolerd et al., 2018). It is notable that persistently high KPC-2 rates (73.9% in 2012, and 61.1% in 2015) and emergence of KPC-17 as well as KPC-34 since 2014 were observed (Chiu et al., 2018). As the *K. pneumoniae* of ST11 clone accounted for

86.9% of overall CP-*K. pneumoniae* isolates (Chiu et al., 2018), heightened infection control maneuvers are absolutely warranted for Taiwanese medical centers. **Figure 1** illustrates the annual percentages of overall carbapenemase producers, overall KPCs and KPC-2 producers, as well as OXA-48-like producers among clinical CR-*K. pneumoniae* isolates (mainly non-susceptible to imipenem and ertapenem) collected between 2012 and 2015 in Taiwan (Chiu et al., 2018; Jean et al., 2018b).

Carbapenem-resistant Enterobacteriaceae that only harbor bla_{OXA-48}-like allele theoretically exhibit non-susceptibility to most carbapenem agents while sparing most β-lactam agents. However, most of the bla_{OXA-48}-like-harboring CRE (especially *K. pneumoniae*) isolates are actually also the ESBL and/or AmpC co-producers (Saïdani et al., 2012; Girlich et al., 2014). Consequently, many OXA-48-like-producing CRE strains show *in vitro* extensively drug-resistant phenotypes. In 2014, an OXA-48-like carbapenemase-producing *E. coli* strain, which concomitantly harbored $bla_{CTX-M-1}$, was cultured from pus sample of a Cambodian patient who resided in Taiwan (Jao et al., 2017). An additional 2010 study from India regarding CRE, some of which were CPE harboring bla_{NDM}, bla_{VIM} or $bla_{OXA-181}$-like alleles, revealed that the case-fatality rate was 56.7% (Mariappan et al., 2017). A sharp rise in the annual prevalence rate of OXA-48-like production among Taiwanese CPE isolates between 2012 and 2015 was also observed (Chiu et al., 2018) (**Figure 1**). Spread of bla_{NDM} alleles occurred rapidly in the Indian subcontinent during the last decade (Kumarasamy et al., 2010), and also in southeastern Asia (Vietnam and the Philippines) (Jean et al., 2017). Nevertheless, reports regarding the NDM-producing Enterobacteriaceae colonization in one patient (3 *K. pneumoniae* isolates harboring bla_{NDM-1}, probably acquired from India) (Wu et al., 2010) and infection in the other patient (1 *Klebsiella oxytoca* isolate harboring bla_{NDM-1}, acquired from China) (Lai et al., 2011) in Taiwan were rare (Jean et al., 2017).

COMMUNITY AND LONG-TERM CARE FACILITY (LTCF)-ACQUIRED CRE

Few studies have addressed the percentage of community acquisition of clinical CRE infections in the PubMed database. However, a few investigations in Taiwan have addressed this important topic. A clinical study, conducted at a medical center located in southern Taiwan in 2015, showed approximately 30% (23/78) of cases, with predominance of elderly female patients and infections originating in the urologic system, acquired CRE in a community setting (Tang et al., 2016). The percentage of community-acquired CRE was higher than that [12% (14/117)] of another Taiwanese study conducted at a large medical center in 2010 ($P = 0.002$) (Lai et al., 2013), and also higher than that [5.6% (17/305)] of an US-CRE study (Thaden et al., 2014). In the latter study, 91% of overall CRE isolates were *K. pneumoniae*, while 59% were cultured from urine between 2008 and 2012 ($P < 0.0001$) (Thaden et al., 2014). In addition, it was observed that many residents from the LTCF in Taiwan were also the carriers of CRE, most (24/27) were *K. pneumoniae* (Chuang, 2015; Mao et al., 2018). This was similar to the study conducted by Lee C.M. et al. (2017) who observed that a significant proportion of *K. pneumoniae* strains cultured from rectal swabs of LTCF residents were CRE (38.9 and 88.9% showed *in vitro* non-susceptibility to ertapenem and imipenem, respectively). Therefore, in Taiwan, regular screening for CRE carriage (urine, sputum, and rectal swabs) among the LTCF residents to implement subsequent contact isolation precaution, hand hygiene, strict implementation of environmental disinfection (including equipment of LTCF), and adequate education to caregivers and family about the importance of infection control are beneficial to decrease CRE carriage rates of LTCF residents. All of these measures are crucial for preventing CRE spread at LTCF (Lee C.M. et al. (2017); Lai et al., 2018; Liu et al., 2018).

SURVEILLANCE DATA OF ANTIMICROBIAL RESISTANCE IN TAIWAN IN 2017

To fully explore the evolutionary trends of the annual resistance burden, the Taiwan CDC conducted a consecutive 4-year survey of antimicrobial resistance (AMR) regarding important clinical bacterial pathogens since 2017 (Jean et al., 2018b). Among 673 bloodstream *K. pneumoniae* isolates collected from 16 major teaching hospitals throughout Taiwan in 2017, 71 (10.5%) displayed non-susceptibility to at least one carbapenem agent. In addition, a total of 92 (13.8%) *K. pneumoniae* isolates exhibited ESBL phenotypes that were unrelated to KPC and/or NDM production [with a 92% non-susceptible (NS) rate to piperacillin-tazobactam, 100% to ceftazidime, and 74% to cefepime]. Phenotypic ESBL isolates had high NS rates to ertapenem (40.2%), tigecycline [7.6% based on the US Food and Drug Administration (FDA) criteria, and 16.3% based on the criteria recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in 2018 (European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2018)], amikacin (13.0%), as well as ceftolozane-tazobactam [70.7% based on criteria recommended by Clinical, and Laboratory Standards Institute [CLSI], 2018, and 80.4% based on the EUCAST 2018 criteria]. Of note, among *K. pneumoniae* blood isolates validated as CPE by multiplex PCR tests, 24 (3.6%) harbored bla_{KPC} allele (1 isolate showed susceptibility to all tested carbapenem agents), eight (33.3%) of the KPC-producing *K. pneumoniae* isolates were acquired from a community setting. In addition, 7 (1.0%) *K. pneumoniae* isolates harbored bla_{OXA-48}-like alleles (1 isolate showed susceptibility to all tested carbapenem agents, while 6 exhibited ESBL phenotypes), 1 (0.15%) and 4 (0.6%) isolates harbored bla_{NDM} and *mcr*-1 gene, respectively. The rate of KPC-producing isolates [32.4% (23/71)] among Taiwanese CR-*K. pneumoniae* isolates in 2017 was significantly higher than that (2.8%) in the 2010-2012 Taiwanese study (Wang et al., 2015). Furthermore, out of the total KPC-producing *K. pneumoniae* isolates ($n = 24$), there were two (8.3%) showing non-susceptibility to ceftazidime-avibactam, 5 (20.8%) showing non-susceptibility to colistin, and 4 (16.7%) displaying non-susceptibility to tigecycline based on

the EUCAST 2018 criteria (Jean et al., 2018b, in Infect Drug Resist).

CARBAPENEM RESISTANCE MECHANISMS AMONG CR-*E. coli* ISOLATES

As compared to a much higher CPE rate among clinical isolates of *K. pneumoniae*, two Taiwanese studies concluded that co-existence of a plasmidic AmpC β-lactamase (DHA-1, CMY-2) in combination with loss of an outer membrane porin (OmpC/F) is the main mechanism responsible for non-susceptibility to carbapenems for CR-*E. coli* (Chia et al., 2009; Ma et al., 2013b). This finding differs from that in Mainland China [89% of CR-*E. coli* isolates (n = 164) harbored bla_{NDM} (n = 81) or bla_{KPC} (n = 65) alleles] (Zhang et al., 2017), and in India (most CR-*E. coli* harbored bla_{NDM} with or without $bla_{\mathrm{OXA-48}}$-like alleles) from 2011 through 2013 (Mohanty et al., 2017). Surveillance data from the Taiwan CDC-AMR 2017 showed a total of 686 *E. coli* blood isolates were collected. Among them, only 14 (2.0%) isolates showed *in vitro* non-susceptibility to ertapenem. Of note,

TABLE 2 | Clinical efficacy of different antibiotic regimens on patients with clinical infections due to isolates of carbapenemase-producing Enterobacteriaceae.

Study periods	Carbapenemase production	Antibiotic regimens	Clinical outcomes	Reference
2005–2009	41 patients, with KPC-producing *K. pneumoniae* bloodstream infections	Carbapenem + colistin, or tigecycline	28-day mortality rates, 13% for combination group vs. 67% for colistin or tigecycline monotherapy	Qureshi et al., 2012
2001–2011 (review of MEDLINE database)	Diverse infections, due to isolates of KPC-producing *K. pneumoniae*	Polymyxin + carbapenem, tigecycline, or aminoglycoside	Treatment failure rates, 25% vs. 49% for combination therapy vs. monotherapy group (73% in polymyxin, and 60% in carbapenem monotherapy group)	Lee and Burgess, 2012
2010–2011	125 patients, with KPC-*K. pneumoniae* bloodstream infections	Meropenem + tigecycline + colistin (regimen of (triple combination)	Mortality rates, 13% for triple combination therapy vs. 42% monotherapy group	Tumbarello et al., 2012
2001–2010	Patients with CPE septicemia, including primarily bacteremia (n = 244), and pneumonia (n = 32), etc. Among CPE isolates, 158 KPC + 140 MβL producers predominantly	Carbapenem + colistin/or aminoglycoside	Mortality rates, 6% vs. > 23% for $\geq$2 *in vitro* active drugs vs. non-susceptible drugs	Tzouvelekis et al., 2012
2009–2010	205 patients, with bloodstream CPE (*K. pneumoniae*) infections due to 163 KPC $\pm$ VIM producers, and 42 VIM producers	Two *in vitro* active drugs	28-day mortality rates, 27.2% (with MICs of *K. pneumoniae* to imipenem, meropenem, or doripenem $\leq$ 8 mg/L) plus a second *in vitro* active agent vs. 44.4% for monotherapy group	Daikos et al., 2014
2007–2014 (data were pooled from 20 clinical studies)	414 patients, with diverse infections due to CPE, mainly harboring KPC or VIM alleles	Heterogeneous regimens, including the carbapenem-containing vs. carbapenem-sparing schemes	Mortality rates, 18.8% for carbapenem-containing therapy group vs. 30.7% for carbapenem-sparing therapy group.	Tzouvelekis et al., 2014

KPC, Klebsiella pneumoniae carbapenemase. CPE, carbapenemase-producing Enterobacteriaceae. MβL, metallo-β-lactamase. VIM, Verona integron-encoded metallo-β-lactamases.

5 (0.7%) isolates harbored the *mcr*-1 gene, similar to the one found in Mainland China (Quan et al., 2017), while the other two harbored a bla_{KPC} and bla_{NDM-1} allele, respectively.

CARBAPENEM RESISTANCE MECHANISMS AMONG CR-*Enterobacter* spp. ISOLATES

Fewer studies have examined mechanisms of non-susceptibility to carbapenem agents among isolates of *Enterobacter* species than those for *K. pneumoniae* in the PubMed database. A study focusing on ertapenem-resistant *Enterobacter cloacae* isolates (MIC > 2 mg/L, in accordance with the CLSI 2009 criteria) collected in 2007 was conducted by Yang et al. (2012) in Taiwan. Analysis of porin expression, detection of efflux pumps and β-lactamase(s), as well as susceptibility tests were performed. Only a few isolates [3/53 (5.7%)] harbored bla_{IMP-8}, while porin change (30–40%) and efflux pump(s) (≥70%) in combination with ESBL or AmpC significantly contributed to ertapenem non-susceptibility for Taiwanese ertapenem-resistant *E. cloacae* isolates. Because of geographic variations, production of different types of carbapenemases among the carbapenem (imipenem, ertapenem)-NS *E. cloacae* isolates was observed in India (VIM-2, VIM-6, and NDM-1) (Khajuria et al., 2014) and in Israel (KPC-2) (Marchaim et al., 2008).

MONOTHERAPY VS. COMBINATION THERAPY FOR CRE INFECTION

Patients with CPE infections are undoubtedly at an extremely high risk for inappropriate antibiotic therapy (Tumbarello et al., 2012; Chang et al., 2015). As stated earlier, tigecycline and colistin showed good *in vitro* susceptibility results against most CRE isolates and KPC or MβL-producing CPE isolates (Bratu et al., 2005b; Castanheira et al., 2008). As stated in the Taiwan CDC-AMR 2017 data, the tigecycline/colistin MIC values of one *E. coli* and one *K. pneumoniae* isolate harboring the bla_{NDM} allele were 0.06/0.25 mg/L and 0.25/0.25 mg/L, respectively. Nevertheless, to maximize the clinical effectiveness of colistin against impending resistant Gram-negative bacteria (GNB; i.e., MIC of colistin is 2 mg/L), a study showed that both the loading, as well as maintenance dose should be increased (Garonzik et al., 2011). In addition, tigecycline was validated as a bacteriostatic agent with suboptimal concentrations in bloodstream (<2 mg/L) after administration of the standard-dose regimen (Yahav et al., 2011). Consequently, monotherapy with tigecycline or colistin is not recommended in the treatment of severe GNB infections owing to high clinical failure and superinfection rates. Bass et al. found that combination therapy of multiple agents that have appropriate *in vitro* activities for ≥ 48 h was associated with improved survival rates (OR, 0.19; 95% CI, 0.06 to 0.56; $P < 0.01$) for critically ill patients with CR-GNB bacteremia (regardless of Enterobacteriaceae species or non-fermentative GNB) (Bass et al., 2015; Lee C.H et al., 2017). Many combination therapies were also been shown to result in favorable outcomes in patients with CP-GNB. For example, a combination of doripenem with colistin was reported to have an excellent *in vitro* synergistic effect against CP-*K. pneumoniae* isolates (Jernigan et al., 2012; Rodríguez-Baño et al., 2018). In addition, a tigecycline-colistin combination was also shown to be superior to colistin monotherapy in decreasing future resistance to colistin (Lee et al., 2009).

In Taiwan, data comparing therapeutic efficacy between different antibiotic regimens against CRE or CPE were scarce (Lee et al., 2011; Ku et al., 2017). Although the rate of tigecycline non-susceptibility among Taiwanese CRE (*E. coli* plus *K. pneumoniae*) blood isolates in 2017 was not high [3.5% (3/85) based on FDA criteria and 12.9% (11/85) based on the EUCAST 2018 criteria], a Taiwanese study aiming specifically at 16 tigecycline-resistant *K. pneumoniae* strains elucidated that *ramR* deficiency and/or widespread mutated *tet*(A) are the main mechanisms conferring non-susceptibility to the tetracycline-class agents for these *K. pneumoniae* isolates (Chiu et al., 2017). In addition, acquisition of fosfomycin-resistant genes, *fosA* subtypes and *fosC2*, which were mainly transmitted by plasmids and/or transposons, was found in many of the Enterobacteriaceae species in Eastern Asian countries, including Taiwan (Yang et al., 2017). In addition, although amikacin, gentamicin and fosfomycin exhibited acceptable *in vitro* susceptibility rates against some CPE species (KPC and VIM) (Endimiani et al., 2010; Lorenzoni et al., 2017), these agents are recommended as mere adjunctive options against critical CP-GNB infections because of rapid induction of resistance after monotherapy (Tseng et al., 2017; Yang et al., 2017). By contrast, against the non-CP-CR-*K. pneumoniae* strains (with changes of out-membrane porins) collected from a medical center of southern Taiwan during 2008–2012, Ku et al. used the sub-inhibitory concentrations (1/2 × MIC) of antibiotics under evaluation and time-kill studies to analyze *in vitro* synergism between different combination regimens. As compared to tigecycline plus fosfomycin and colistin plus fosfomycin, Ku et al. observed that tigecycline in combination with colistin showed much better *in vitro* synergism, as well as bactericidal efficacy (Ku et al., 2017).

CLINICAL EVIDENCE OF COMBINED ANTIMICROBIAL SCHEMES AGAINST CPE INFECTIONS

Although the above *in vitro* synergy data supported the use of combination regimens, well-designed clinical randomized control trials that strictly investigated which antimicrobial schemes are effective against KPC-producing Enterobacteriaceae isolates were few. Nevertheless, there were many retrospective studies in favor of the use of various combination therapy regimens against clinical CPE infections. None of these studies were investigated in Taiwan. The recommended antibiotic regimens included anti-pseudomonal carbapenem plus colistin or tigecycline, polymyxin plus tigecycline or

aminoglycoside, or meropenem in combination with tigecycline and colistin. Important data regarding therapeutic efficacy of different antibiotic regimens (various combination schemes vs. monotherapy) for patients with CPE infections are illustrated in **Table 2** (Lee and Burgess, 2012; Qureshi et al., 2012; Tumbarello et al., 2012; Tzouvelekis et al., 2012, 2014; Daikos et al., 2014; Food and Drug Administration [FDA], 2018; Rodríguez-Baño et al., 2018).

OTHER ANTIMICROBIAL AGENTS AGAINST CPE INFECTIONS

Apart from tigecycline and colistin against CPE, previous studies showed that avibactam, a bridged diazabicyclo octanone, exhibited good *in vitro* activity against GNB with serine β-lactamases (especially against the KPC producers) when combined with ceftazidime, but it is inactive against class B β-lactamases (Bonnefoy et al., 2004; Tzouvelekis et al., 2012; van Duin and Bonomo, 2016). In the Taiwan CDC-AMR 2017 survey, the MICs for ceftazidime-avibactam for one *E. coli* and one *K. pneumoniae* isolate that harbored the bla_{NDM} allele were both > 64 mg/L. Consequently, the Taiwanese 2017 data were consistent with previous studies. Ceftazidime-avibactam has been approved by the US-FDA in treatment of CRE-related cIAI and cUTI. In addition, meropenem-vaborbactam has an equivalent potency to ceftazidime-avibactam against class A carbapenemases (typified by KPC), but also has limited activity against class B along with oxacillinase carbapenemases (Castanheira et al., 2017; Chew et al., 2018). This drug was also approved in treatment of CRE-related cUTI recently. By contrast, aztreonam-avibactam displays good *in vitro* susceptibility in dual-carbapenemase (KPC, NDM)-producing CRE isolates (Chew et al., 2018). In addition, cefiderocol, a new siderophore cephalosporin, also shows potentially good *in vitro* activity against both KPC, as well as NDM-producing Enterobacteriaceae isolates (Hackel et al., 2018). The clinical trials comparing clinical efficacy of aztreonam-avibactam and cefiderocol with other best available antibiotics are undergone. The other future promising agents against CPE include imipenem-relebactam, plazomicin, and eravacycline (Rodríguez-Baño et al., 2018).

ANTIBIOTIC TREATMENT FOR NON-CARBAPENEMASE-PRODUCING-CRE INFECTIONS

Compared to ample databases regarding treatment of CPE, limited data were available regarding treatment recommendations for clinical non-CP-CRE isolates. Nevertheless, Tamma et al. observed MIC values for imipenem and meropenem in the subgroup of CPE isolates (>90% were KPC producers) were significantly higher than those of the non-CP-CRE subgroup. Furthermore, the overall 14-day mortality rate was 4-fold higher among patients with CPE bacteremia than those of the non-CP-CRE group (Tamma et al., 2017). Recently, we investigated detection of CPE predictors among ertapenem-NS Enterobacteriaceae isolates causing IAI collected from patients hospitalized in Asia-Pacific countries during 2008–2014 (Jean et al.,2017). This Asia-Pacific IAI-CRE study showed that imipenem non-susceptibility (i.e., MIC > 2 mg/L) and culturing ertapenem-NS Enterobacteriaceae isolates from the peritoneal space were highly likely as CPE (dominated by the bla_{NDM} and bla_{IMP}-harboring isolates, rather than KPC producers; Jean et al., 2018a, in Infect Drug Resist). Moreover, the non-CP-ertapenem-NS IAI Enterobacteriaceae isolates were more likely to have cefepime MICs of > 8 mg/L, which might be valuable in distinguishing it from the CPE. However, the clinical application of the dose adjustment of an anti-pseudomonal carbapenem agent to effectively treat the non-CP ertapenem-NS Enterobacteriaceae isolates needs further investigation in clinical scenarios.

SUMMARY

In Taiwan, the main research gap is that there are no sufficient clinical studies exploring the significant risk factors with respect to acquisition of CRE isolates among hospitalized or LTCF patients. Of note, the annual CPE proportion has been on a sharp rise among clinical CRE isolates since 2012, particularly for CR-*K. pneumoniae* isolates. To effectively limit the spread of CPE (especially ST11 *K. pneumoniae* clone) in clinical settings, close monitoring of this worrisome MDR trend is warranted at all major teaching hospitals, as well as nursing homes. In addition, although ceftazidime-avibactam and meropenem-vaborbactam show excellent efficacy against some CPE, it is not currently available in Taiwan. Combination therapy schemes, such as colistin and/or tigecycline plus an anti-pseudomonal carbapenem agent, are still the preferred treatment for CRE and CPE infections in Taiwan.

AUTHOR CONTRIBUTIONS

S-SJ, N-YL, and P-RH collected and analyzed the data. S-SJ participated in the writing of the manuscript. S-SJ, N-YL, H-JT, M-CL, W-CK, and P-RH read and approved the final version of the manuscript.

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Genetic Diversity of Carbapenem-Resistant *Enterobacteriaceae* (CRE) Clinical Isolates From a Tertiary Hospital in Eastern China

Minhui Miao[1,2†], Huiyan Wen[1†], Ping Xu[3†], Siqiang Niu[4], Jingnan Lv[1], Xiaofang Xie[1], José R. Mediavilla[5], Yi-Wei Tang[6], Barry N. Kreiswirth[5], Xia Zhang[7], Haifang Zhang[1]*, Hong Du[1]* and Liang Chen[5]*

[1] Department of Clinical Laboratory, The Second Affiliated Hospital of Soochow University, Suzhou, China, [2] Department of Clinical Laboratory, Jiangyin Hospital of Traditional Chinese Medicine, Jiangyin, China, [3] Department of Clinical Laboratory, The Fifth People's Hospital of Suzhou, Suzhou, China, [4] Department of Laboratory Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China, [5] Public Health Research Institute Tuberculosis Center, New Jersey Medical School, Rutgers University, Newark, NJ, United States, [6] Memorial Sloan Kettering Cancer Center, New York, NY, United States, [7] Department of Clinical Laboratory, The North District of Affiliated Suzhou Hospital, Nanjing Medical University, Suzhou, China

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***Correspondence:**
Hong Du
hong_du@126.com;
hongdu@suda.edu.cn
Haifang Zhang
haifangzhang@sina.com
Xia Zhang
xiazhangsz@qq.com

† These authors have contributed equally to this work

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The prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) is increasing globally, with different molecular mechanisms described. Here we studied the molecular mechanisms of carbapenem resistance, including clonal and plasmid dissemination, of 67 CRE isolates collected between 2012 and 2016 from a tertiary hospital in Eastern China, an CRE endemic region. Species identification and susceptibility testing were performed using the BD Phoenix Automated Microbiology System. Isolates were characterized by PCR (for carbapenemases, ESBLs, AmpC and porin genes), multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and conjugation transfer experiments. Selected bla_{KPC-2} -harboring plasmids were subjected to next-generation sequencing using the Illumina Miseq platform. Among the 67 CRE isolates, 42 *Klebsiella pneumoniae*, 10 *Serratia marcescens*, 6 *Enterobacter cloacae*, 2 *Raoultella ornithinolytica*, 2 *K. oxytoca*, 1 *K. aerogenes*, and 4 *Escherichia coli* isolates were identified. Six different carbapenemases were detected, including bla_{KPC-2} (n = 45), bla_{KPC-3} (n = 1), bla_{NDM-1} (n = 6), bla_{NDM-5} (n = 1), bla_{IMP-4} (n = 2), and bla_{VIM-1} (n = 2); bla_{OXA-48}-like genes were not detected. One *E. cloacae* strain possessed both bla_{NDM-1} and bla_{KPC-3}, while two *E. cloacae* isolates harbored bla_{NDM-1} and bla_{VIM-1}. ESBLs (CTX-M, SHV, and TEM) and/or AmpC (CMY, DHA, and ACT/MIR) genes were also identified in 59 isolates, including 13 strains that lacked carbapenemases. Several insertions or stop codon mutations were found within porin genes of *K. pneumoniae*, *E. coli* and *S. marcescens* isolates, both with and without carbapenemases. The 42 *K. pneumoniae* isolates belonged to 12 different sequence types (ST), with ST11 being the most common, while the 6 *E. cloacae* isolates comprised 4 different STs. The 10 *S. marcescens* all shared the same PFGE pulsotype, suggestive of clonal

spread. Complete plasmid sequencing and PCR screening revealed both intra-strain and inter-species spread of a common bla_{KPC-2}-harboring plasmid in our hospital. Taken together, our study revealed extensive genetic diversity among CRE isolates form a single Chinese hospital. CRE isolates circulating in the hospital differ significantly in their species, STs, porin genes, carbapenemase genes, and their plasmid content, highlighting the complex dissemination of CRE in this endemic region.

Keywords: carbapenem-resistant *Enterobacteriaceae*, carbapenemase, resistance mechanism, genetic diversity, plasmid

INTRODUCTION

Enterobacteriaceae are among the most common pathogenic Gram-negative bacteria (GNB), causing various community- and healthcare-acquired infections. Nowadays, multidrug resistant GNB (MDR-GNB) are increasingly described in clinical settings, and carbapenems are regarded as the most effective antibiotic therapy for infections caused by MDR-GNB. However, as a result of clinical use of carbapenems since the late 1980s, the occurrence of carbapenem-resistant *Enterobacteriaceae* (CRE) has been increasingly reported worldwide, including in China (Gupta et al., 2011; Nordmann and Poirel, 2014). According to reports by the China Antimicrobial Resistance Surveillance System (CARSS), the detection rate of CRE in China increased from 2005 to 2014, demonstrating a continuous upward trend and suggesting a worsening situation (Hu et al., 2016).

Carbapenem resistance in *Enterobacteriaceae* can arise through distinct molecular mechanisms, mainly via the production of carbapenemases, but also as a consequence of outer membrane porin dysfunction coupled with hyper-production of AmpC cephalosporinases or extended-spectrum β-lactamases (ESBLs) (Bush and Jacoby, 2010; Bush and Fisher, 2011). Carbapenemases are a group of β-lactamases that are capable of hydrolyzing carbapenem antibiotics, in addition to cephalosporins and other β-lactam antimicrobials. Three major class of carbapenemases are widespread globally in clinical CRE isolates, including class A (mainly KPC), class B (VIM, NDM, and IMP) and class D (OXA-48 and its variants, OXA-162 and OXA-181, etc.) (Ambler, 1980; Hall et al., 2003; Nordmann and Poirel, 2014). Notably, carbapenemase genes are primarily carried by large conjugative plasmids, thereby facilitating horizontal transfer of carbapenem resistance among different bacterial strains and species. As mentioned above, another common mechanism of carbapenem resistance involves the combination of porin dysfunction with hyper-production of AmpC (e.g., CMY, DHA, and ACT) or ESBLs (e.g., TEM, SHV, and CTX-M) (Logan and Weinstein, 2017). The lack of the production of porins can preclude diffusion of antibiotics through bacterial membranes, along with the action of ESBL and AmpC enzymes, thereby producing the phenotype of carbapenem resistance in *Enterobacteriaceae* (Paterson and Bonomo, 2005; Jacoby, 2009; Bush and Jacoby, 2010). Unlike carbapenemases, porin dysfunction-associated resistance is not able to spread through horizontal transfer, but may disseminate via clonal expansion.

Dissemination of CRE usually demonstrates geographical and temporal variation in specific global regions. Here we characterized CRE clinical strains collected from a tertiary hospital in eastern China between August 2012 and August 2016. The genetic relatedness, antimicrobial susceptibility, and carbapenem-resistance mechanisms of these CRE isolates were examined in detail.

MATERIALS AND METHODS

Identification of Carbapenem-Resistant *Enterobacteriaceae* Isolates

Sixty-seven unique (one isolate per patient) CRE clinical isolates were retrospectively collected from the Second Affiliated Hospital of Soochow University between August 2012 and August 2016. In this study, carbapenem resistance was defined as resistance to meropenem or imipenem based on 2016 Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2016). The isolates were collected from various sources, including sputum (n = 48), urine (n = 14), blood (n = 2), catheter (n = 1), ascites (n = 1), and drainage fluid (n = 1). Species identification was performed using the Phoenix 100 Automated Microbiology System (Becton-Dickinson, United States), and confirmed by 16S rRNA sequencing (Weisburg et al., 1991). This study was approved by the institutional review board (IRB) of The Second Affiliated Hospital of Soochow University. The clinical isolates were retrospectively collected, and patient data were not included in this study.

Antimicrobial Susceptibility Testing

The minimal inhibitory concentrations (MICs) of the CRE strains were performed using the Phoenix 100 Automated Microbiology System and interpreted according to CLSI criteria (CLSI, 2016). A total of 18 antibiotics belonging to eight classes of antimicrobials were tested, including carbapenems (imipenem and meropenem), penicillins (ampicillin), β-lactam/β-lactamase inhibitor complexes (amoxicillin-clavulanate, ampicillin-sulbactam, and piperacillin-tazobactam), cephalosporins (cefazolin, cefuroxime, ceftazidime, and cefepime), monocyclic β-lactams (aztreonam), aminoglycosides (gentamicin and amikacin), fluoroquinolones (ciprofloxacin and levofloxacin), folate metabolic pathway inhibitors (trimethoprim-sulfamethoxazole), colistin and tigecycline.

Detection of Carbapenemases, ESBLs, AmpC, and Porin Genes

Polymerase chain reaction (PCR) was performed to investigate the presence of carbapenemase-encoding genes, including bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP} and bla_{OXA-48}. Simultaneously, we examined ESBLs (CTX-M, SHV, and TEM), AmpC cephalosporinases (CMY, ACT, and DHA), and mutation in porin encoding genes (OmpK35/OmpF, OmpK36/OmpC), using PCR followed by Sanger sequencing. Oligonucleotide primers used for screening the above genes have been reported previously (Mammeri et al., 2010; Bokaeian et al., 2015; Candan and Aksoz, 2015; Sugawara et al., 2016).

Multilocus Sequence Typing (MLST)

Multilocus sequence typing (MLST) was conducted to investigate the genetic relationships of different CRE isolates. PCR followed by Sanger sequencing was used to detect conserved housekeeping genes in distinct species, including *Klebsiella* spp. (*gapA, infB, mdh, pgi, phoE, rpoB*, and *tonB*), *E. coli* (*adk, fumC, gyrB, icd, mdh, purA, and recA*), and *E. cloacae* (*dnaA, fusA, gyrB, leuS, pyrG, rplB, and rpoB*). Allelic profiles and sequence types (STs) were determined according to species-specific MLST databases[1]. Primers used for MLST were described in previous reports (Diancourt et al., 2005; Wirth et al., 2006; Miyoshi-Akiyama et al., 2013; Herzog et al., 2014).

Pulsed-Field Gel Electrophoresis (PFGE)

Raoultella *ornithinolytica* and Serratia *marcescens* strains (for which no MLST schemes Are available) Were further investigated by PFGE using a CHEF Mapper Power Module instrument (Bio-Rad, United States). In brief, genomic DNA Was digested With *Xba* I, and then electrophoresed Under the following conditions: voltage 6 V/cm, running time 18–19 h, temperature 14°C, and pulse times of 5–40 s (*R. ornithinolytica*) and 5–20 s (*S. marcescens*). *Salmonella* strain H9812 Was used as a control strain and size marker. Clonal relatedness Between strains Was evaluated based on the criteria proposed by Tenover et al. (1995).

Plasmid Sequencing and Screening

Conjugation transfer experiments were performed with selected bla_{KPC-2}-harboring strains and rifampicin-resistant *E. coli* EC600. Experiments were carried out using mixed broth culture method as described previously (Chen et al., 2014a). Transconjugants were identified by detecting resistance genes using PCR. Plasmid DNA from *E. coli* EC600 transconjugants harboring single plasmids was extracted using a Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA, United States), and sequenced using the Illumina Miseq system (Illumina, United States) (Du et al., 2016). Sequencing reads were assembled *de novo* into contigs using SPAdes (Bankevich et al., 2012), then manually inspected using Geneious 9.1[2]; and gaps were closed by PCR and Sanger sequencing.

A PCR mapping strategy was developed to detect a common bla_{KPC-2}-harboring pSZF_KPC/p628-KPC-like plasmid sequenced in this study. The scheme includes six individual PCR reactions. PCR-I was designed to target the region spanning IncFII replicon gene *repA* and its downstream DNA methylase gene, while PCR-V was designed to target the junction of second replicon gene *repB* and its upstream *parA*. PCR-II, III, and PCR-IV were designed to target the bla_{KPC-2} neighboring regions of Δ*rep-klcA*, *klcA*-bla_{KPC-2}, and bla_{KPC-2}-IS*26*, respectively (**Figure 1**). PCR-VI was used to detect the *traX-finO* junction in pSZF_KPC/p628-KPC-like plasmids. The oligonucleotide primer target regions for identification of pSZF_KPC/p628-KPC-like plasmids are shown in **Figure 1**, and primer sequences are listed in **Table 1**.

[1]http://pubmlst.org/

[2]http://www.geneious.com/

RESULTS

Species and Antimicrobial Susceptibility

A total of 67 non-duplicate CRE isolates were collected from our hospital from August 2012 to August 2016, consisting of 42 *K. pneumoniae*, 2 *K. oxytoca*, 1 *K. aerogenes*, 10 *S. marcescens*, 6 *E. cloacae*, 2 *R. ornithinolytica,* and 4 *E. coli*. The results of antimicrobial susceptibility testing are shown in **Table 2**. All isolates were resistant to ampicillin, cefazolin, cefuroxime, imipenem, meropenem, and amoxicillin/clavulanate, and exhibited high resistance rates to most of the other β-lactam antibiotics tested. The most active compounds against all isolates were colistin (97.0% susceptible), tigecycline (94.0% susceptible), trimethoprim/sulfamethoxazole (56.7% susceptible) and amikacin (50.7% susceptible).

Detection of Carbapenemase Genes

In this study, 54 (80.6%) of the 67 CREs were found to harbor at least one carbapenemase gene (**Table 3**). However, the distribution of carbapenemases among different species varied significantly, while the frequencies of carbapenemase-producing *Enterobacteriaceae* (CPE) in different species were 100%, 88.1%, 50.0%, and 50.0% in *E. coli*, *K. pneumoniae*, *E. cloacae* and *S. marcescens*, respectively.

Various carbapenemases were identified among the 54 CPE isolates, including bla_{KPC-2} ($n = 45$), bla_{KPC-3} ($n = 1$), bla_{NDM-1} ($n = 6$), bla_{NDM-5} ($n = 1$), bla_{IMP-4} ($n = 2$), and bla_{VIM-1} ($n = 2$). No strains were found to carry bla_{OXA-48}-like genes. Among these, KPC was the most predominant carbapenemase (80.7%), and was primarily found in *K. pneumoniae* (37/42, 88.1%). Three *E. cloacae* strains were found to co-harbor two carbapenemase-encoding genes, with one strain harboring both bla_{KPC-3} and bla_{NDM-1}, while the other two harboring both bla_{NDM-1} and bla_{VIM-1}.

Other Mechanisms Associated With Carbapenem Resistance

As described above, PCR failed to identify any carbapenemases among 13 out of 67 CRE strains, including 5 *K. pneumoniae*, 3 *E. cloacae* and 5 *S. marcescens*, suggesting that other mechanisms may have contributed to the phenotypic carbapenem resistance

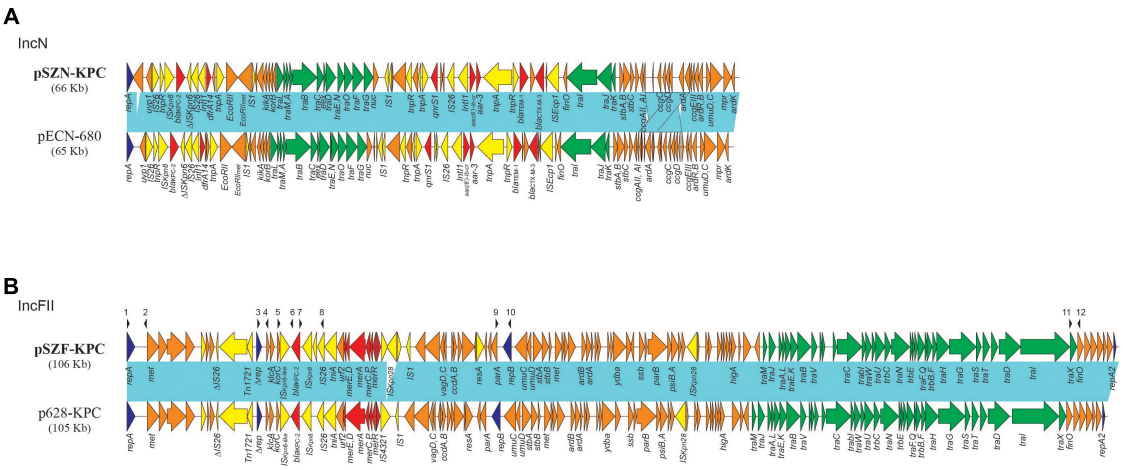

FIGURE 1 | Comparative analysis of **(A)** IncN and **(B)** IncFII bla_{KPC-2}–like harboring plasmids. Light blue shading denotes shared regions of homology with >99% identities. ORFs are portrayed by arrows and colored according to predicted gene function: orange arrows indicate plasmid scaffold regions; green arrows denote genes associated with the *tra* locus; dark blue arrows indicate replication-associated genes; Red arrows denote antimicrobial and mercury resistance genes; and yellow arrows indicate accessory genes. Small black arrowheads above the plasmids indicate the locations of primers used for PCR screening (primer sequences are shown in **Table 1**).

TABLE 1 | Oligonucleotide primers used to screen pSZF_KPC/p628-KPC-like plasmids.

PCRs	No.[a]	Name	Sequences	Size (bps)	Targets
PCR-I	1	repA-F1	GGGAACAACTACACGCGACT	1447	Junction between IncFII *repA* and DNA methylase gene
	2	repA-R1	GTTTTGCCCATGCTCAACTT		
PCR-II	3	Δrep-F	TGAGACAAGTCCCTCCCCTA	1138	Junction between Δ*rep* and *klcA*
	4	klcA-R	GCCCTTTCATTTGCTGGTAA		
PCR-III	5	korC-F	GGTGAGCAAAACCAACCCTA	1417	Junction between *korC* and bla_{KPC-2}
	6	KPC-R	ACAAGGATGACAAGCACAGC		
PCR-IV	7	KPC-F	CGAGTTTAGCGAATGGTTCC	2030	Junction between bla_{KPC-2} and downstream IS*26*
	8	IS26-R	CGCCTGGTAAGCAGAGTTTT		
PCR-V	9	parA-F	GCCCAGTGACATCAGATACG	870	Junction between *parA* and *repB*
	10	repB-R	TAAACTGGCCCTCAAGCAGT		
PCR-VI	11	traX-F	CCAGGTGTCGTTTATGCTCA	563	Junction between *traX* and *finO*
	12	finO-R	GGTTTTCGTTTCAGGCTCAG		

[a] *The primer locations are illustrated in* ***Figure 1****.*

among these isolates. We therefore examined ESBL-encoding genes (bla_{TEM}, bla_{SHV}, and bla_{CTX-M}), AmpC-encoding genes (bla_{CMY}, bla_{DHA}, and bla_{ACT}) and outer membrane porin genes. ESBL and AmpC genes were tested in all isolates, while outer membrane porin genes were examined in *K. pneumoniae* (*ompK35* and *ompK36*), as well as in *E. coli*, *E. cloacae*, and *S. marcescens* (*ompF* and *ompC*).

Fifty-nine isolates were found to carry at least one ESBL and/or AmpC gene, including 13 non-carbapenemase-producing strains. Specifically, 51 (76.1%) strains were found to carry bla_{CTX-M} genes, including $bla_{CTX-M-65}$ (n = 19), $bla_{CTX-M-14}$ (n = 19), $bla_{CTX-M-9}$ (n = 7), $bla_{CTX-M-15}$ (n = 3), and $bla_{CTX-M-3}$ (n = 3). These were found in several species, including *K. pneumoniae* (n = 32), *S. marcescens* (n = 10), *E. cloacae* (n = 6), *E. coli* (n = 2), and *R. ornighinolytica* (n = 1). In addition, 30 strains were positive for bla_{SHV-12}, most of which were *K. pneumoniae* (26/30, 86.7%), while 21 *K. pneumoniae* harbored bla_{DHA-1}, 6 *E. cloacae* possessed bla_{ACT}, and two *E. coli* were positive for bla_{CMY-2}.

Outer membrane porin gene sequence analysis showed that 27 *K. pneumoniae* isolates harbored *ompK35* and/or *ompK36* mutations. *ompK35* mutations (n = 26) were exclusively due to premature stop codons, while *ompK36* mutations included glycine-aspartic acid (GD) insertions at amino acid positions 134–135 (n = 23), IS*10* insertions (n = 2), and stop codons (n = 1). Five non-carbapenemase-producing *K. pneumoniae* CRE contained at least one outer membrane porin gene mutant (*ompK35* or *ompK36*) while also harboring ESBL genes bla_{CTX-M} or bla_{SHV-12}, which likely explains the carbapenem resistance among these isolates. Sequence analysis of *ompF* and *ompC* genes in *S. marcescens* showed that they all possess mutated *ompF*, with premature stop codons at amino acid position 72. One non-carbapenemase-producing *E. cloacae* isolate was also found to carry an *ompF* mutation (stop codon), in addition to bla_{ACT}

and $bla_{CTX-M-9}$. However, two non-carbapenemase-producing *E. cloacae* CRE isolates did not display mutations in either *ompF* or *ompC*, although they were found to harbor ESBL genes (either $bla_{CTX-M-14}$ or $bla_{CTX-M-3}$). We suspect additional mechanisms, such as efflux pumps or penicillin-binding protein modifications, may contribute to carbapenem resistance in the latter two isolates. Therefore, carbapenem resistance among 11 out of 13 non-carbapenemase-producing CRE isolates may be

TABLE 2 | Susceptibility of CRE isolates against different antimicrobial agents.

Antimicrobial agents*	All isolates (*n* = 67)	*Klebsiella pneumonia* (*n* = 42)	*Klebsiella oxytoca* (*n* = 2)	*Enterobacter cloacae* (*n* = 6)	*Enterobacter coli* (*n* = 4)	*Klebsiella aerogenes* (*n* = 1)	*Raoultella ornithinolytica* (*n* = 2)	*Serratia marcescens* (*n* = 10)
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
AMP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
CZO	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
CXM	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
CAZ	4 (6)	1 (2.4)	0 (0)	1 (16.7)	0 (0)	0 (0)	1 (50)	0 (0)
FEP	3 (4.5)	2 (4.8)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)
AMC	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
SAM	1 (1.5)	1 (2.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
TZP	4 (6)	1 (2.4)	1 (50)	1 (16.7)	0 (0)	1 (100)	0 (0)	0 (0)
ATM	5 (7.5)	1 (2.4)	2 (100)	1 (16.7)	1 (25)	0 (0)	0 (0)	0 (0)
IPM	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
MEM	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
GEN	10 (15.0)	5 (11.9)	1 (50)	1 (16.7)	1 (25)	0 (0)	2 (100)	0 (0)
AMK	34 (50.7)	15 (35.7)	2 (100)	3 (50.0)	2 (50)	1 (100)	1 (50)	10 (100)
CIP	6 (9.0)	4 (9.5)	0 (0)	1 (16.7)	0 (0)	0 (0)	1 (50)	0 (0)
LEV	11 (16.4)	7 (16.7)	1 (50)	1 (16.7)	0 (0)	0 (0)	2 (100)	0 (0)
SXT	38 (56.7)	23 (54.8)	2 (100)	2 (33.3)	1 (25)	1 (100)	0 (0)	9 (90)
TGC	63 (94.0)	38 (90.5)	2 (100)	6 (100)	4 (100)	1 (100)	2 (100)	10 (100)
CL	65 (97.0)	40 (95.2)	2 (100)	6 (100)	4 (100)	1 (100)	2 (100)	10 (100)

**IPM, imipenem; MEM, meropenem; AMP, ampicillin; AMC, amoxicillin-clavulanate; SAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam; CZO, cefazolin; CXM, cefuroxime; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; LEV, levofloxacin; SXT, trimethoprim-sulfamethoxazole; TGC, tigecycline; CL, colistin. n (%), n = number of isolates that were susceptible; % = percentage of isolates susceptible.*

TABLE 3 | Molecular characteristics of CRE clinical isolates.

Species	Number	Carbapenemases (n, %)	ESBLs and AmpC (n, %)*	Mutations of encoding Porin (n, %)*	STs (n, %)
K. pneumoniae	42	KPC-2 (37, 88.1%)	CTX-M-9 (6, 14.3%), CTX-M-14 (7, 16.7%), CTX-M-65 (19, 45.2%), SHV-12 (26, 61.9%), DHA-1 (21, 50.0%)	*ompK35* (26, 61.9%), *ompK36* (26, 61.9%)	ST11 (25, 59.5%), ST774 (3, 7.1%), ST1107 (3, 7.1%), ST12 (2, 4.8%), ST45 (2, 4.8%), ST8 (1, 2.4%), ST36 (1, 2.4%), ST211 (1, 2.4%), ST218 (1, 2.4%), ST395 (1, 2.4%), ST655 (1, 2.4%), ST697 (1, 2.4%)
K. oxytoca	2	NDM-1 (1, 50%), IMP-4 (1, 50%)	–	N/A	ST135 (1, 50%), ST180 (1, 50%)
K. aerogenes	1	KPC-2 (1, 100%)	–	N/A	N/A
R. ornithinolytica	2	KPC-2 (1, 50%), IMP-4 (1, 50%)	CTX-M-15 (1, 50%), SHV-12 (1, 50%)	N/A	N/A
S. marcescens	10	KPC-2 (5, 50%)	CTX-M-14 (10, 100%)	*ompF* (10, 100%)	N/A
E. coli	4	KPC-2 (1, 25%), NDM-1 (2, 50%), NDM-5 (1, 25%)	CTX-M-15 (2, 50%), SHV-12 (1, 25%), CMY-2 (2, 50%)	*ompF* (1, 25%)	ST167 (1, 25%), ST1488 (1, 25%), ST3234 (1, 25%), ST354 (1, 25%)
E. cloacae	6	KPC-3 (1, 16.7%), NDM-1 (3, 50%), VIM-1 (2, 33.3%)	ACT (6, 100%), CTX-M-3 (3, 50%), CTX-M-9 (1, 16.7%), CTX-M-14 (2, 33.3%), SHV-12 (2, 33.3%)	*ompF* (1, 16.7%)	ST231 (3, 50%), ST120 (1,16.7%), ST97 (1, 16.7%), ST421 (1, 16.7%)

**–, negative; N/A, not available or not performed.*

explained by the combination of porin gene mutants and the presence of ESBL or AmpC-encoding genes, while the resistance mechanisms in two non-carbapenemase-producing *E. cloacae* CRE isolates remain to be determined. Meanwhile, among the 54 CPE isolates, 28 (51.9%) also carry at least one outer membrane porin mutant.

Distribution of MLST Sequence Types and PFGE Patterns

Multilocus sequence typing results showed that 42 *K. pneumoniae* belonged to 12 different STs, with ST11 being the most common (25/42, 59.5%). All ST11 isolates possessed the same OmpK35 stop codon, while 23 of them harbored the 134–135 GD OmpK36 mutant. The two *K. oxytoca* isolates belonged to ST135 and ST180, while the 4 *E. coli* strains were assigned to ST1488, ST3234, ST167, and ST354. The six *E. cloacae* also comprised 4 STs, including ST231 (n = 3), ST120 (n = 1), ST421 (n = 1), and ST97 (n = 1). Two *R. ornithinolytica* and 10 *S. marcescens* strains were further analyzed by *Xba* I-PFGE. The results showed that all 10 *S. marcescens* strains shared the same PFGE pattern, suggesting clonal spread (**Supplementary Figure 1**). In addition, the ten *S. marcescens* isolates were collected within 7 months in 2013 from three closed wards (respiratory, neurology, and renal wards) and mostly from respiratory samples (n = 9), suggestive of the likelihood of a small *S. marcescens* outbreak. As aforementioned, all *S. marcescens* harbored the same OmpF mutant, and among them, 5 were also found to carry bla_{KPC-2}. By contrast, the two *R. ornithinolytica* isolates displayed unrelated PFGE patterns (data not shown).

Sequencing and Screening of bla_{KPC-2}-Harboring Plasmids

Since KPC-2 is the most common carbapenemase found in our hospital, we sought to determine the complete sequences of bla_{KPC-2} -harboring plasmids spreading through our institution. We selected 4 bla_{KPC-2}-harboring strains, including two *K. pneumoniae* (ST8 and ST11), one *E. coli* (ST3234), and one *S. marcescens* for plasmid conjugation and complete plasmid sequencing.

The bla_{KPC-2}-harboring plasmid (subsequently named pSZN_KPC) isolated from *K. pneumoniae* ST8 belonged to incompatibility group N. Plasmid pSZN_KPC is 65,604 bp in length, with an average G+C content of 53.3 %, and contains 88 predicted open reading frames (ORFs) (**Figure 1**). A BLAST search of the pSZN_KPC plasmid sequence against the GenBank database[3] showed that pSZN_KPC exhibits a high degree of identity to the previously published IncN plasmid pECN580 (accession no. KF914891) from an *E. coli* strain found in China (Chen et al., 2014a), with 100% query coverage and overall 99% nucleotide identity. The major difference is that the region harboring antirestriction protein gene *ardA* in plasmid pSZN_KPC was located downstream of gene *ccgCD* (**Figure 1**), likely as a consequence of recombination.

The bla_{KPC-2}-harboring plasmids from *K. pneumoniae* ST11 strain Kp715, *E. coli* ST3234 strain Ec732, and *S. marcescens* strain Sm703 were highly similar (each differed by only 4 SNPs), and belonged to the same IncFII incompatibility group. This plasmid (subsequently named pSZF_KPC) is 106,201 bp in length with a G+C content of 53.2 %, and harbors 122 predicted ORFs. Comparative sequence analysis for plasmid pSZF_KPC showed that it is highly similar (100% query coverage and >99% nucleotide identity) to p628-KPC (accession no. KP987218) from a *K. pneumoniae* isolate collected in our hospital in 2010 (Wang et al., 2015). Compared to p628-KPC, the main difference in pSZF_KPC is that there is an additional IS*Kpn18* element, with IS*4321* located downstream of the *mcr* operon (**Figure 1**). The sequences of pSZN_KPC and pSZF_KPC have been deposited in GenBank under the accession numbers MH917122 and MH917123, respectively.

The plasmid sequence results described above suggest that pSZF_KPC/p628-KPC-like plasmids have been spreading in our hospital since at least 2010, undergoing horizontal transmission into different species. We therefore developed a set of PCR assays to screen for the presence of pSZF_KPC/p628-KPC-like plasmids among our 67 CRE isolates. The PCR results showed that 27 (64.2%) of the 42 bla_{KPC-2} positive strains harbored pSZF_KPC/p628-KPC-like plasmids. The 27 strains encompassed different species and STs, including *K. pneumoniae* ST11 (n = 14), ST774 (n = 3), ST1107 (n = 2), ST211 (n = 1), ST655 (n = 1), and ST218 (n = 1); *S. marcescens* (n = 4); and *E. coli* ST3234 (n = 1). These pSZF_KPC/p628-KPC-like plasmid-harboring isolates were collected in 8 different wards, spanning from 2012 to 2016, indicating the frequent horizontal transfer of this common plasmid among *Enterobacteriaceae* in our hospital.

DISCUSSION

At the present time, KPC, NDM and OXA-48 are the most common carbapenemases worldwide (Nordmann and Poirel, 2014; Sugawara et al., 2016). KPCs are most frequently identified in *K. pneumoniae* from the United States, China, Colombia, Israel, Greece, and Italy, while NDMs are primarily found in *K. pneumoniae*, *E. coli* and *Enterobacter spp.* from the Indian subcontinent, and OXA-48-like carbapenemases in *K. pneumoniae* and *E. coli* from North Africa and Turkey (Nordmann and Poirel, 2014). In addition, the spread of CPEs has been associated with several high-risk clones. One notable example involves the global spread of KPCs, which has been largely associated with *K. pneumoniae* clonal group 258 (CG258) strains, of which ST258 is the most predominant KPC-producing *K. pneumoniae* clone in North America, while ST11 is most common in East Asia, especially China (Patel and Bonomo, 2013; Chen et al., 2014b; Zhang et al., 2017).

China, in particular eastern China, is regarded as one of the primary global endemic regions for CRE (Zhang et al., 2017). In this study, we phenotypically and genetically characterized the CRE isolates collected from an eastern Chinese hospital, and investigated the molecular mechanisms underlying carbapenem resistance. Our study revealed several interesting findings.

[3]http://blast.ncbi.nlm.nih.gov/Blast.cgi

Firstly, the CRE isolates were recovered from seven different *Enterobacteriaceae* species. Although carbapenem resistance has been frequently identified in *K. pneumoniae*, *Enterobacter* spp., and *E. coli*, it is fairly uncommon in other species such as *R. ornithinolytica* and *S. marcescens*. *R. ornithinolytica* is mostly recovered from the environment and rarely causes severe infections in humans; nevertheless several reports have described the emergence of carbapenem-resistant *R. ornithinolytica* in China (Zhou et al., 2013; Qin et al., 2014; Yang et al., 2018). Notably, in this study we identified two carbapenem-resistant *R. ornithinolytica*, harboring bla_{KPC-2} and bla_{IMP-4}, respectively, suggesting that different carbapenemase plasmids have spread into *R. ornithinolytica.* In contrast, carbapenem resistance in *S. marcescens* has been historically associated with a specific group of carbapenemases, SMEs. However, in this study, none of the ten *S. marcescens* strains were found to carry bla_{SME}, and the observed carbapenem resistance was likely due to the mutations of OmpK35 porin encoding genes, as well as production of CTX-M-14 and KPC-2. Interestingly, only five of the ten *S. marcescens* strains were found to harbor bla_{KPC-2}, although PFGE results suggested that the spread of carbapenem-resistant *S. marcescens* was largely clonal. We suspect it is likely that the *S. marcescens* OmpK35 mutant further acquired a bla_{KPC-2} plasmid (e.g., pSZF_KPC/p628-KPC-like).

In this study, *K. pneumoniae* was the most common CRE species, accounting for 62.7% (42/67) of all CRE isolates. Among these, ST11, a member of the epidemic CG258 clone, was the predominant ST (59.5%, 25/42), which is consistent with the molecular epidemiology described in other regions of China (Zhou et al., 2013; Zhang et al., 2017; Yang et al., 2018). However, in our study carbapenem-resistant *K. pneumoniae* were found in 12 different STs, including some STs rarely associated with carbapenem resistance (e.g., ST774 and ST1107). Our plasmid screening results showed that the diversity of STs was largely due to the frequent transfer of a common bla_{KPC-2} vector into different *K. pneumoniae* genetic backgrounds.

Secondly, our study revealed diverse molecular mechanisms of carbapenem resistance within our hospital, albeit with carbapenemase production the primary cause. Diverse types of carbapenemases, including KPC-2, KPC-3, NDM-1, NDM-5, IMP-4, and VIM-1, were identified, whereas in most Chinese hospitals, usually only KPC and/or NDM carbapenemases are prevalent (Hu et al., 2014). It is noteworthy that four different classes of carbapenemases were found in our hospital, suggesting that different carbapenemase producing plasmids/strains are spreading locally. Notably, however, ∼20% strains were non-carbapenemase producers. It is therefore likely that besides carbapenemases, other mechanisms such as porins defects and production of ESBLs (TEM, SHV, and CTX-M) or AmpC β-lactamases (CMY, DHA, and ACT) significantly contributed to carbapenem resistance in our hospital. Meanwhile, it is worth noting that 51.9% (28/54) of carbapenemase-producing isolates also harbored additional porin gene mutations, potentially rendering antimicrobial treatment more challenging in comparison to strains without porin mutations (Clancy et al., 2013).

Lastly, we identified a widespread bla_{KPC-2}-harboring plasmid vector within our institution. Several pSZF_KPC/p628-KPC-like plasmids were identified within distinct *K. pneumoniae* clones (STs), as well as in different species (*K. pneumoniae*, *S. marcescens* and *E. coli*). Our PCR-based screening of bla_{KPC-2}-positive *K. pneumoniae* isolates revealed that this plasmid is widely disseminated in our hospital and was found in nearly two third (22/37) of KPC-2 positive isolates, highlighting the importance of plasmid horizontal transfer in the dissemination of KPC. More importantly, this plasmid was identified in different *K. pneumoniae* STs, as well as other species, suggesting that intra-strain and inter-species transfer of this plasmid have significantly contributed to the spread of carbapenem resistance in our hospital.

In conclusion, our study revealed extensive genetic diversity among CRE isolates from a single Chinese hospital. Both clonal expansion (e.g., *K. pneumoniae* ST11 and *S. marcescens*) and plasmid horizontal transfer (i.e., pSZF_KPC/p628-KPC-like plasmids) were identified. Different carbapenemases classes and outer membrane porin defects were found in several species. CRE isolates circulating in our hospital differ significantly in their species, STs, porin genes, carbapenemase genes, and plasmid content, highlighting complex dissemination of CRE within our hospital. Further studies are required to understand the factors underlying the genetic diversity of CRE in our hospital, in order to effectively control further spread of carbapenem resistance.

AUTHOR CONTRIBUTIONS

MM, HW, and PX contributed to work, data analysis, and manuscript preparation. SN, JL, JM, Y-WT, and BK prepared the manuscript. XX contributed to work and data analysis. XZ and HZ analyzed the data. HD and LC contributed to study design, data analysis, and manuscript preparation.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Infections Caused by Carbapenem-Resistant *Enterobacteriaceae*: An Update on Therapeutic Options

Chau-Chyun Sheu[1,2], Ya-Ting Chang[2,3], Shang-Yi Lin[2,3], Yen-Hsu Chen[2,4,5] and Po-Ren Hsueh[6,7]**

[1] Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, [2] School of Medicine, Sepsis Research Institute, Graduate Institute of Medicine, Graduate Institute of Clinical Medicine, Center of Dengue Fever Control and Research, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, [3] Division of Infectious Disease, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, [4] Department of Internal Medicine, Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung, Taiwan, [5] Department of Biological Science and Technology, College of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan, [6] Department of Laboratory Medicine, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan, [7] Department of Internal Medicine, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan*

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Liang Chen, Public Health Research Institute (PHRI), United States

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Elizabeth Diago Navarro, Stony Brook University, United States
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***Correspondence:**
Yen-Hsu Chen
infchen@gmail.com
Po-Ren Hsueh
hsporen@ntu.edu.tw

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Carbapenems are considered as last-resort antibiotics for the treatment of infections caused by multidrug-resistant Gram-negative bacteria. With the increasing use of carbapenems in clinical practice, the emergence of carbapenem-resistant pathogens now poses a great threat to human health. Currently, antibiotic options for the treatment of carbapenem-resistant *Enterobacteriaceae* (CRE) are very limited, with polymyxins, tigecycline, fosfomycin, and aminoglycosides as the mainstays of therapy. The need for new and effective anti-CRE therapies is urgent. Here, we describe the current understanding of issues related to CRE and review combination therapeutic strategies for CRE infections, including high-dose tigecycline, high-dose prolonged-infusion of carbapenem, and double carbapenem therapy. We also review the newly available antibiotics which have potential in the future treatment of CRE infections: ceftazidime/avibactam, which is active against KPC and OXA-48 producers; meropenem/vaborbactam, which is active against KPC producers; plazomicin, which is a next-generation aminoglycoside with *in vitro* activity against CRE; and eravacycline, which is a tetracycline class antibacterial with *in vitro* activity against CRE. Although direct evidence for CRE treatment is still lacking and the development of resistance is a concern, these new antibiotics provide additional therapeutic options for CRE infections. Finally, we review other potential anti-CRE antibiotics in development: imipenem/relebactam and cefiderocol. Currently, high-dose and combination strategies that may include the new β-lactam/β-lactamase inhibitors should be considered in severe CRE infections to maximize treatment success. In the future, when more treatment options are available, therapy for CRE infections should be individualized and based on molecular phenotypes of resistance, susceptibility profiles, disease severity, and patient characteristics. More high-quality studies are needed to guide effective treatment for infections caused by CRE.

Keywords: avibactam, carbapenems, carbapenemase, carbapenem-resistant *Enterobacteriaceae*, combination therapy, relebactam, vaborbactam

INTRODUCTION

The increasing prevalence of bacterial resistance to antibiotics is a critical public health problem. Infections caused by antibiotic-resistant bacteria are associated with significant morbidity and mortality worldwide. Many previous efforts to combat multidrug-resistant (MDR) bacteria were focused on methicillin-resistant *Staphylococcus aureus* (MRSA). In recent years, several new therapeutic options for MRSA have become available (David et al., 2017). Currently the major threat of antibiotic-resistant bacteria is from MDR Gram-negative organisms, particularly those which have developed resistance to carbapenem. Along with carbapenem-resistant *Acinetobacter baumannii* (CRAB) and carbapenem-resistant *Pseudomonas aeruginosa* (CRPA), carbapenem-resistant *Enterobacteriaceae* (CRE) are among the top tier of the WHO list of antibiotic-resistant "priority pathogens" that pose the greatest threat to human health (Willyard, 2017).

Enterobacteriaceae are common pathogens causing a variety of severe infections, including bloodstream infections (BSIs), community-acquired pneumonia (CAP), hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), complicated urinary tract infections (cUTIs), and complicated intra-abdominal infections (cIAIs). Therefore, antibiotic resistance in these bacteria has significant clinical and socioeconomic impacts (Lee et al., 2017; Rodriguez-Bano et al., 2018; Ting et al., 2018). As the prevalence of infections caused by extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* is increasing, the medical community has been forced to use carbapenem as a first-line empirical treatment. The increasing use of carbapenem for possible ESBL infections has led to a more serious problem: the emergence of carbapenemase-producing *Enterobacteriaceae* (CPE) (Sheu et al., 2018).

Carbapenem is a β-lactam antibiotic which inhibits transpeptidases (penicillin-binding proteins) and prevents peptidoglycan synthesis, leading to lytic cell death (Kohanski et al., 2010). The resistance of CRE to carbapenems is generally based on two mechanisms: carbapenemase production or the combination of structural mutations with the production of other β-lactamases, such as AmpC cephalosporinase (AmpC) and ESBL (Tzouvelekis et al., 2012; Munoz-Price et al., 2013; Goodman et al., 2016; Tamma and Simner, 2018). The classification and characteristics of major carbapenemases in CRE are summarized in **Table 1**. The three major classes of carbapenemases are Ambler Class A *Klebsiella pneumoniae* carbapenemase (KPC); Class B metallo-β-lactamases (MBLs) such as New Delhi MBL (NDM), Verona integrin-encoded MBL (VIM), and imipenemase (IMP); and Class D oxacillinases (OXA)-type enzymes such as OXA-48-like carbapenemases. These carbapenemases exhibit variable levels of carbapenem resistance through their carbapenem-hydrolyzing activity. For instance, a certain proportion of VIM and IMP-producing *K. pneumoniae* have been observed to have low carbapenem minimum inhibitory concentrations (MICs) in studied isolates (Yan et al., 2001; Psichogiou et al., 2008; Daikos et al., 2009). On the other hand, NDM carbapenemase seemed to exhibit higher carbapenem MICs (Kumarasamy et al., 2010) while KPC-producing isolates demonstrated wide variations in carbapenem MICs in different geographic regions (Endimiani et al., 2009a; Daikos and Markogiannakis, 2011; Qi et al., 2011). Some carbapenem-producing *Enterobacteriaceae* (CPE) are even susceptible to carbapenems themselves and this is particularly observed in OXA-48 producers (Dautzenberg et al., 2014; Navarro-San Francisco et al., 2013). Some CPE may also coproduce AmpC or ESBLs. The impact of the co-production of these enzymes on treatment and outcomes remains unclear.

To date, the treatment options for CRE infections remain very limited. Polymyxins (colistin or polymyxin B) and tigecycline have been historically considered as drugs of choice for infections caused by CRE. However, resistance to these antibiotics is increasing (Capone et al., 2013; Giacobbe et al., 2015; Yang et al., 2018). In addition to polymyxins and tigecycline, fosfomycin and aminoglycosides are occasionally used (Tang et al., 2016; Tseng et al., 2017; Rodriguez-Bano et al., 2018). Carbapenems still play a role in the treatment of CRE infections, particularly when used in the treatment of CRE with lower MICs, either in higher doses, in combination with other active anti-CRE agents, or through double-carbapenem therapy (DCT). Older antibiotics such as minocycline, doxycycline, trimethoprim/sulfamethoxazole, and chloramphenicol may be effective for some CRE isolates (Falagas et al., 2011; Livermore et al., 2011). Recently, novel β-lactamase inhibitor combinations have provided new therapeutic options for CRE infections. However, these new β-lactamase inhibitors are not active against all carbapenemases. Avibactam inhibits both Class A KPC and Class D OXA-48 (Zasowski et al., 2015), while vaborbactam and relebactam inhibits only Class A KPC (Petty et al., 2018; Zhanel et al., 2018) (**Table 1**).

Table 2 summarizes the currently available antimicrobial agents and their recommended doses for treatment of CRE infections. Because the therapeutic options are limited, all potentially active drugs should be tested *in vitro*. It is recommended to select anti-CRE agents according to *in vitro* susceptibility data, clinical severity, and all other available information. Higher doses may be necessary for severe infections, including pneumonia and septic shock (Rodriguez-Bano et al., 2018). Many anti-CRE agents have recently been reviewed (Ni et al., 2016; Thaden et al., 2017; Trecarichi and Tumbarello, 2017; Zavascki et al., 2017; Rodriguez-Bano et al., 2018). We therefore focus the present review on several potential combination therapeutic strategies and new antibiotics (**Table 3**).

COMBINATION THERAPEUTIC STRATEGIES

Many studies have investigated the benefits of various combinations of antimicrobial agents for the treatment of CRE, *in vitro* or *in vivo* (Ku et al., 2017). However, it has been difficult to draw conclusions due to the diversity of study designs and resistance mechanisms. Among the studies that found combination therapy to contribute to lower mortality rates than monotherapy (Qureshi et al., 2012; Tumbarello et al., 2012, 2015; Tofas et al., 2016; Trecarichi et al., 2016; Gutierrez-Gutierrez et al., 2017; Machuca et al., 2017; Papadimitriou-Olivgeris

TABLE 1 | Classification and characteristics of major carbapenemases in *Enterobacteriaceae*.

Carbapenemase	KPC	MBLs (NDM, VIM, IMP)	OXA-48
Ambler molecular class	A	B	D
Substrates of hydrolysis	All β-lactams	All β-lactams except for aztreonam	Penicillins and carbapenems
Inhibited by classic β-lactamase inhibitors	Minimally	No	No
Inhibited by avibactam	Yes	No	Yes
Inhibited by vaborbactam	Yes	No	No
Inhibited by relebactam	Yes	No	No
Common species in *Enterobacteriaceae*	*K. pneumoniae, E. coli, Enterobacter* spp.	NDM: *K. pneumoniae, E. coli* VIM: *K. pneumoniae* IMP: *K. pneumoniae*	*K. pneumoniae*

KPC, Klebsiella pneumoniae carbapenemase; MBL, metallo-β-lactamase; NDM, New Delhi metallo-β-lactamase; VIM, Verona integrin-encoded metallo-β-lactamase; IMP, imipenemase; OXA, oxacillinase.

TABLE 2 | Antimicrobial agents used for carbapenem-resistant *Enterobacteriaceae* infections.

Antimicrobial agents	Recommended dose for CRE infections[a]	Comments
Meropenem	2 g every 8 h by prolonged infusion for isolates with MICs of 2–8 mg/L	May not be effective for isolates with MIC > 8 mg/L
Ertapenem	Consider 2 g every 24 h	Used in double-carbapenem therapy
Colistin	Loading dose of 9 MU, followed by 9 MU/day in 2–3 divided doses	
Polymyxin B	Loading dose of 2–2.5 mg/kg, followed by 5 mg/kg/day in 2 divided doses	
Tigecycline	Loading dose of 100 mg, followed by 50 mg every 12 h	Consider loading dose of 200 mg, followed by 100 mg every 12 h for severe infections
Eravacycline	1 mg/kg every 12 h	Approved by FDA in August 2018 for the treatment of cIAI. Activity against carbapenem-resistant *Enterobacteriaceae* has been demonstrated *In vitro*. Clinical data in CRE infections are still lacking
Gentamicin Tobramycin	5–7 mg/kg/day	Used in combination therapy. Consider a higher dose of 10–15 mg/kg/day for severe infections without other options. Risk of toxicity may increase. TDM is recommended
Amikacin	15–20 mg/kg/day	Used in combination therapy. Consider a higher dose of 25–30 mg/kg/day for severe infections without other options. Risk of toxicity may increase. TDM is recommended
Plazomicin	15 mg/kg/day	Approved by FDA in June 2018 for the treatment of cUTI including pyelonephritis. Activity against ESBL- and carbapenemase-producing *Enterobacteriaceae* has been demonstrated *In vitro*. Clinical data in CRE infections are still lacking
Fosfomycin	4 g every 6 h to 8 g every 8 h	Used in combination therapy
Aztreonam	1–2 g every 8 h	MBL producers are susceptible if not ESBL or AmpC producers
Ceftazidime	1–2 g every 8 h	OXA-48 producers are susceptible if not ESBL or AmpC producers
Ceftazidime/avibactam	2.5 g (2 g/0.5 g) every 8 h	KPC and OXA-48 producers are frequently susceptible
Meropenem/vaborbactam	2 g (1 g/1 g) every 8 h	KPC producers are frequently susceptible

cIAI, complicated intraabdominal infection; cUTI, complicated urinary tract infection; ESBL, extended-spectrum β–lactamase; KPC, Klebsiella pneumoniae carbapenemase; MBL, metallo-β-lactamase; MIC, minimum inhibitory concentration; OXA, oxacillinase; TDM, therapeutic drug monitoring.
Adapted from Rodriguez-Bano et al. (2018).
[a]*For patients with normal renal function.*

et al., 2017), the two largest retrospective studies to date concordantly identified the protective effects to be significant in populations with high disease severity (Tumbarello et al., 2015; Gutierrez-Gutierrez et al., 2017). In the multicenter Italian cohort, with 661 episodes of BSI and non-BSI caused by KPC-producing *K. pneumoniae*, Tumbarello et al. (2015) compared clinical outcomes between 307 patients receiving monotherapy (colistin in 121, tigecycline in 116, gentamicin in 70) and 354 patients receiving combination therapy (receiving 2 or more *in vitro*-active drugs, with meropenem in all cases). They found significantly decreased mortality rates with combination therapy among patients with BSI (OR, 0.45; 95% CI, 0.29–0.68), lower respiratory tract infections (OR, 0.35; 95% CI, 0.11–0.99), high APACHE III scores (OR, 0.55; 95% CI, 0.37–0.80), septic shock (OR, 0.18; 95% CI, 0.05–0.53), and among isolates with a meropenem MIC of ≤8 mg/L (OR, 0.57; 95% CI, 0.32–1.03) (Tumbarello et al., 2015). The INCREMENT project, which included monomicrobial BSIs due to CPE from a total of 437 patients worldwide (26 tertiary hospitals in 10 countries), is by far the largest retrospective international cohort study. The

TABLE 3 | Potential combination therapeutic strategies and new antibiotics for the treatment of carbapenem-resistant *Enterobacteriaceae* infections.

Combination therapeutic strategies
High-dose tigecycline
High-dose prolonged-infusion of carbapenem
Double-carbapenem therapy
New antibiotics
Ceftazidime/avibactam
Meropenem/vaborbactam
Plazomicin
Eravacycline
New antibiotics in development
Imipenem/cilastatin and relebactam
Cefiderocol

subgroup analysis concluded that combination therapy, defined as receiving more than one *in vitro*-active antimicrobial, did not improve survival except in patients with a high mortality score (Gutierrez-Gutierrez et al., 2017).

The highly heterogeneous methodologies between studies and the fact that most data were derived from isolates of CRKP, preclude optimal synthesis using the currently available evidence. Several systematic reviews have proposed viewpoints regarding combination therapy. Falagas et al. (2014) reviewed 20 non-randomized studies, comprising 692 patients, and proposed that combination therapy may be considered for severely ill patients. Tzouvelekis et al. (2012) performed a systematic review that included 34 studies and suggested that carbapenem-containing combinations contribute to higher treatment success rates. Polymyxin-based and tigecycline-based combination therapies were reported to significantly decrease 30-day mortality when compared with respective monotherapy by systematic reviews (Ni et al., 2015, 2016). Zusman et al. (2017) performed a meta-analysis to compare polymyxin-based combination therapy and monotherapy. The subgroup analysis for CRE comprised of *K. pneumoniae* BSI and included seven studies with a total of 285 patients. The meta-analysis favored combination therapy (potentially double-coverage) and demonstrated an OR of 2.09 (95% CI, 1.21–3.6; I^2 = 0%), but with low-quality evidence (Zusman et al., 2017). The most recent meta-analysis, performed by Martin et al. (2018), included 22 studies describing CRE infections. Seven studies were extracted for comparison between combination therapy and monotherapy. Four of the studies included patients with BSIs and three with mixed infections. The results showed a significantly higher risk of overall mortality among patients treated with monotherapy (OR, 2.19; 95% CI, 1.00–4.80), with a high heterogeneity (I^2 = 84.2%; QP = 0.003) (Martin et al., 2018).

The first and only RCT for the treatment of carbapenem-resistant Gram-negative bacteria was recently published, and is therefore not included in any of the above-mentioned systematic reviews or meta-analyses. The open-label RCT compared the outcomes of colistin monotherapy vs. combination therapy with high-dose and prolonged infusion meropenem (2 g every 8 h, infused over 3 h) (Paul et al., 2018). A total of 406 patients were enrolled, with pneumonia and bacteremia comprising 87% of the infections. Most infections were caused by *A. baumannii* (77%), while *Enterobacteriaceae* only contributed to 18% (73/406) of all infections. Most *Enterobacteriaceae* infections were BSIs (77%), with *K. pneumoniae* being the main pathogen (89%). In the *post hoc* subgroup analysis of *Enterobacteriaceae* infections, there were no significant differences in clinical outcomes between colistin monotherapy and combination therapy with meropenem. However, combination therapy seemed to be associated with a lower clinical failure rate (46% vs. 68%, $P = 0.185$) and a lower 28-day mortality (21% vs. 35%, $P = 0.235$). There is another ongoing RCT (NCT01597973) investigating colistin monotherapy vs. combination with carbapenem in the treatment of bacteremia or pneumonia caused by extensively drug-resistant Gram-negative bacteria. The trial is estimated to be completed in 2021.

Because of the suboptimal quality of the available data, it is not yet possible to make solid recommendations regarding combination therapy in CRE infections. However, there is a growing body of evidence supporting the use of combination therapy, particularly in critically ill patients.

High-Dose Tigecycline

Besides carbapenems, the most commonly studied high-dose regimen is tigecycline, owing to its non-nephrotoxic nature compared to other potentially active antimicrobial agents for CRE, such as polymyxins and aminoglycosides. A high-dose colistin regimen has been more extensively investigated for its efficacy against carbapenem-resistant *A. baumannii* infections and seldom for CRE (Gibson et al., 2016; Cheng et al., 2018).

A high-dose tigecycline regimen consists of a 200 mg loading dose and a maintenance dose of 100 mg every 12 h, while a standard-dose regimen consists of a loading dose of 100 mg and a maintenance dose of 50 mg every 12 h. One study assessed the efficacy of tigecycline for carbapenem-producing *K. pneumoniae* (CPKP) by using 164 non-duplicate clinical strains of CPKP isolated from HAP and incorporating a Monte Carlo simulation into a pharmacokinetic/pharmacodynamics (PK/PD) model. The study revealed that a higher cumulative fraction of response, indicating better clinical efficacy, can be gained by doubling the tigecycline dose (90.2% vs. 71.2%) (Trecarichi et al., 2016).

Two small retrospective studies conducted by Sbrana et al. (2013) and Balandin Moreno et al. (2014) included 26 episodes of KPC-producing *K. pneumoniae* and 16 episodes of VIM-1-producing *K. pneumoniae* infections from a trauma-referral ICU and a multidisciplinary ICU, respectively. In the study by Sbrana et al. (2013), high-dose tigecycline was administered in 25/26 infection episodes in combination with gentamicin (19/26) and colistin (12/26). Fosfomycin was used as a third antibiotic in 13/26 episodes. In the study by Balandin Moreno et al. (2014), high-dose tigecycline was administered in 10/16 infection episodes and standard-dose regimen in 6/16 episodes. Fourteen (14/16) episodes were treated with combination therapy, including colistin in 8/16, carbapenem in 5/16, ciprofloxacin in 2/16, piperacillin/tazobactam in 1/16, and amikacin in 1/16. Sbrana et al. (2013) suggested a favorable outcome by the double- or triple-combination with high-dose tigecycline, with a 30-day

crude mortality rate of 14%. On the other hand, Balandin Moreno et al. (2014) found no significant differences in the mortality rates between high-dose and standard-dose tigecycline. Di Carlo et al. (2013) compared standard-dose to high-dose tigecycline with the combination of colistin in 30 postoperative abdominal surgery ICU patients who had at least two positive blood cultures for KPC-producing *K. pneumoniae*. They observed a significantly lower mortality rate in the high-dose tigecycline group. In terms of infection sources, De Pascale et al. (2014) found high-dose tigecycline to be the only independent predictor of clinical cure in the VAP subgroup of critically ill patients with MDR bacterial infections (total 63 patients, 28 isolates of CRAB and 27 isolates of carbapenem-resistant *K. pneumoniae* [CRKP]). For BSIs, a retrospective cohort study of 40 patients with nosocomial CPKP BSI showed no significant differences in in-hospital mortality between 23 patients undergoing high-dose tigecycline-based combination therapy and 17 patients undergoing standard-dose tigecycline therapy (52.2% vs. 76.5%, $P = 0.117$) (Geng et al., 2018). One systemic review encompassed 25 studies reporting the efficacy and/or safety of tigecycline-based regimens for treating CRE infections, while the subgroup meta-analysis found a much lower ICU mortality with high-dose tigecycline than standard-dose tigecycline (OR, 12.48; 95% CI, 2.06–75.43; $P = 0.006$) (Ni et al., 2016).

In summary, high-dose tigecycline-based combination therapy may be considered in critically ill patients with CRE infections and limited treatment options, either from the PK/PD viewpoint or clinical observations. However, the infection source should be cautiously evaluated since better outcomes were observed in trauma (Sbrana et al., 2013) or postoperative abdominal surgery (Di Carlo et al., 2013) patients, but not in patients with BSI (Geng et al., 2018), which is compatible with the common consensus that tigecycline is extensively distributed beyond the plasma volume and concentrates into tissues.

High-Dose and Prolonged-Infusion of Carbapenems

The fact that wide disparities of carbapenem MICs exist, even among CPE isolates, complicates the discourse for the role of carbapenems in the treatment of CRE or CPE. Several studies have investigated the efficacy of carbapenems against CPKP in animal models and suggested that with a higher dose of carbapenems it is possible to attain reliable reductions in bacterial density in isolates with lower carbapenem MICs (Daikos et al., 2007; Bulik and Nicolau, 2010b; Bulik et al., 2010a; Souli et al., 2011). Daikos and Markogiannakis (2011) proposed, based on several animal infection model studies, that high-dose, prolonged-infusion carbapenems can achieve bactericidal effects in immunocompetent animals infected by KPC-producing *K. pneumoniae* isolates with MICs up to 8 mg/L. In addition, Daikos and Markogiannakis (2011) also analyzed 22 clinical studies (mostly case series) and found: (1) the therapeutic efficacy of carbapenems increases from 29% for an MIC of >8 mg/L, to 69% for an MIC $\leq$ 4 mg/L, which is similar to patients infected with non-CPKP (73%); (2) among the 138 patients treated by combination regimens, the mortality rate was lowest in patients who received carbapenem-containing combinations and were infected with isolates of MIC $\leq$ 4 mg/L (OR, 5.3; 95% CI, 1.5–18.9). More recent retrospective cohort studies echoed the observation that a combination regimen containing carbapenem is associated with significantly higher survival rates in CPKP BSI isolates (Daikos et al., 2014) and KPC-producing *K. pneumoniae* isolates with a meropenem MIC $\leq$ 8 mg/L (Tumbarello et al., 2015).

In addition to carbapenem-containing combinations, the strategy of high-dose (2 g every 8 h) carbapenem with prolonged infusion (over 3 h) was also found to be associated with better outcomes in CPKP infections (Tumbarello et al., 2012, 2015; Daikos et al., 2014). Moreover, Giannella et al. (2018) evaluated the efficacy of high-dose carbapenem-based combination therapy among 595 patients with CRKP BSI and studied the benefits of high-dose carbapenem in strains with meropenem MIC $\geq$ 16 mg/L, which comprised 77% of all isolates in the study. These clinical observations are in line with PK/PD studies showing that a high-dose prolonged-infusion carbapenem regimen can reach attainable targets in isolates with meropenem MICs up to 32–64 mg/L, though failure was observed with very high MICs of 256–1024 mg/L (Del Bono et al., 2017; Giannella et al., 2018).

Based on the current evidence, using a high-dose prolonged-infusion carbapenem-containing combination regimen for the treatment of CRE isolates with carbapenem MIC $\leq$ 8 mg/L may be considered when there are no other treatment options. It should also be noted that since most conclusions from the above-mentioned studies were derived from CPKP or CRKP isolates, the extrapolation of high-dose prolonged-infusion of carbapenem-containing combination therapy to other CRE with different resistance mechanisms requires further investigation.

Double-Carbapenem Therapy

The most well-investigated DCT is the combination of ertapenem (with a standard infusion time of 30–60 min) prior to a prolonged infusion of meropenem or doripenem over 3–4 h, with high-dose meropenem of 2 g every 8 h being most commonly applied. This regimen originated from the revolutionary approach proposed by Bulik and Nicolau (2011), as a salvage option for CPKP. The study validated enhanced activities for both ertapenem and doripenem in combination, using an *in vitro* chemostat and *in vivo* murine thigh infection model (Bulik and Nicolau, 2011). The rationale for this combination came from the hypothesis that ertapenem might play a sacrificial role, being preferentially hydrolyzed due to its greater affinity to KPC (Anderson et al., 2007), permitting the concomitant administration of carbapenem to sustain a high concentration. Some *in vitro* studies show a beneficial effect of lower MICs of meropenem (MIC $\leq$ 128 mg/L) (Oliva et al., 2017b) or doripenem (MIC $\leq$ 16 mg/L) (Wiskirchen et al., 2013) with regard to ertapenem-based DCT. Nevertheless, ertapenem-based DCT is not the only combination that demonstrates synergism (Poirel et al., 2016; Fredborg et al., 2017). One *in vitro* study reported variable synergistic patterns among KPC and OXA-48 producers, while no synergism was observed for the NDM-producing strain (Poirel et al., 2016).

Despite the diversity of the above-mentioned observations, nearly all reported cases and clinical studies adopted an ertapenem-based DCT with recommended or high-dose

doripenem/meropenem. The short stability of the intravenous imipenem preparation that hinders prolonged infusion may be one of the reasons for such a phenomenon (Mashni et al., 2018). Souli et al. (2017) conducted the largest observational cohort study to date in terms of exclusive DCT as a salvage therapy. The study included 27 patients with CPKP infections, mostly with cUTI (59.3%) and BSI (48.2%) and reported a high clinical success rate of 77.8%. It is noteworthy that the subgroup of pandrug-resistant infections also had a successful clinical and microbiological outcome of 78.5% (11/14). Among critically ill patients with severe sepsis or septic shock, a successful outcome was noted in 81.8% (9/11) (Souli et al., 2017). Oliva et al. (2017a) and Venugopalan et al. (2017), both conducted observational comparator studies to compare the efficacy of DCT. Venugopalan et al. (2017) enrolled 36 patients with CRKP bacteremia, including 18 patients receiving doripenem and ertapenem (DCT group), and 18 patients receiving doripenem and colistin (control group). They found the DCT group had a significantly improved clinical cure rate of 72% (13/18, vs. control group of 39%, 7/18; $P = 0.049$) and a lower 30-day mortality of 31%, with a trend toward statistical significance (vs. 61%, $P = 0.087$) (Venugopalan et al., 2017). Oliva et al. (2017a) enrolled 32 patients with CRKP infections, including 18 patients receiving ertapenem and meropenem, and 14 patients receiving ertapenem, meropenem, and colistin. They found the combination of colistin and DCT obtained rapid bactericidal activity up to 24 h in the *in vitro* analysis. However, there were no significant differences regarding the early response or 60-day mortality between the two groups. Therefore, the author concluded that the addition of colistin to DCT should be considered in severe cases with septic shock at presentation, then withdrawn after clinical stabilization with a stable switch to the less nephrotoxic regimen of DCT (Oliva et al., 2017a). A randomized controlled trial (RCT) addressing this topic has not yet been performed. The only matched case-control study to date included 48 patients with a DCT-containing combination (daily dose of meropenem and ertapenem up to 6 and 2 g, respectively), matched with 96 controls of DCT-sparing regimens. The other concomitant antibiotics comprised colistin (9 MU every 12 h), gentamicin (5–7 mg/kg daily) and high dose tigecycline. The 28-day mortality was significantly higher in the DCT-sparing arm (47.9% vs. 29.2%, $P = 0.04$). In multivariate analysis, the DCT-containing regimen was associated with a reduction in 28-day mortality (OR, 0.43; 95% CI, 0.23–0.79) (De Pascale et al., 2017). Mashni et al. (2018) performed a critical review of the current studies investigating DCT for CPKP infections, which contained eight case reports and six clinical studies (a total of 171 patients). Most patients were critically ill, and all were treated with ertapenem followed by a prolonged infusion of meropenem or doripenem. Clinical and microbiological successes were reported in approximately 70% of the patients and mortality in 24%. Adverse events, most frequently seizures, sodium disorders, and gastrointestinal symptoms, were reported in 16 patients, without the requirement for treatment interruption (Mashni et al., 2018).

Double-carbapenem therapy seems promising based on current reports, though the majority of infections involved in the studies were caused by Class A carbapenemase or KPC-producing *K. pneumoniae*. The efficacy of DCT against MBLs, such as NDM, would require further investigation.

NEW ANTIBIOTICS

Ceftazidime/Avibactam

Ceftazidime/avibactam (CAZ/AVI, AvyCaz®, Allergan Inc., Jersey City, NJ, United States) is a new β-lactam/β-lactamase inhibitor combination recently approved for the treatment of cIAIs and cUTIs in the United States in February 2015 (Kaye and Pogue, 2015), and for the treatment of HAP and VAP in January 2018. Unlike most β-lactamase inhibitors, avibactam is not a β-lactam. Avibactam is a novel synthetic non-β-lactam (diazabicyclooctane)/β-lactamase inhibitor that inhibits a wide range of β-lactamases, including Ambler Class A (GEM, SHV, CTX-M, and KPC), Class C (AmpC), and some Class D (OXA-48) β-lactamases (de Jonge et al., 2016). It does not inhibit Class B MBLs (IMP, VIM, VEB, and NDM) (Syue et al., 2016; Wong and van Duin, 2017). The addition of avibactam restores ceftazidime activity against various *Enterobacteriaceae* and *P. aeruginosa*, therefore expanding the activity spectrum of ceftazidime to MDR Gram-negative bacteria.

In vitro studies of CAZ/AVI showed adequate efficiency against CRE isolates (Castanheira et al., 2015; Dupont et al., 2016). However, clinical data on the efficacy of CAZ/AVI in severe infections caused by CRE are still lacking. At the time of writing this review, only one prospective, multicenter, observational study (van Duin et al., 2018) and a few case series and cohort studies describing the use of CAZ/AVI for treating CRE infections had been published (Shields et al., 2016, 2017b; Castón et al., 2017; King et al., 2017; Krapp et al., 2017; Temkin et al., 2017). Three studies have compared treatment with CAZ/AVI and other agents for CRE infections (**Table 4**) (Castón et al., 2017; Shields et al., 2017b; van Duin et al., 2018). The first study enrolled hematologic patients with CRE bacteremia in Spain and Israel (Castón et al., 2017). Compared to patients treated with other agents ($n = 23$), the patients receiving CAZ/AVI ($n = 8$) had a higher 14-day clinical cure rate in univariate analysis (85.7% [6/8] vs. 34.8% [8/23], $P = 0.031$). However, there was a lack of statistical significance in multivariate analysis due to the small number of cases. Another study of patients with CRE bacteremia showed that 13 patients treated with CAZ/AVI had a higher clinical success rates, compared to 96 patients treated with other regimens (85% [11/13] vs. 40.6% [39/96], $P = 0.003$) (Shields et al., 2017b). Although a better clinical response was consistent in multivariate analysis, these results were limited by small case numbers with CAZ/AVI treatment and a potential bias in the selection of therapy. Finally, the first prospective cohort study to compare the clinical outcomes for patients with CRE infections was recently published (van Duin et al., 2018). This observational study compared 38 patients treated with CAZ/AVI to 99 patients treated with colistin for KPC-producing CRE infections; combination therapy was used in 63 and 94% of patients treated with CAZ/AVI and colistin, respectively. Primary BSI was the most common infection foci (46%), followed by

TABLE 4 | Efficacy of ceftazidime/avibactam for treatment of carbapenem-resistant *Enterobacteriaceae* infections.

Reference	Study place, year	Study design	Patients, number	Infection foci	Organism(s)/ β-lactamase types	Antibiotic(s)	Clinical outcomes	Remarks
van Duin et al., 2018	United States, 8 sites, 2011–2015	Prospective	137	BSI (n = 63; 46%) and pneumonia (n = 30, 22%)	97% (133/137) were *K. pneumoniae*	CAZ/AVI (n = 38) vs. colistin (n = 99)	Adjusted all-cause mortality was significantly lower in the CAZ/AVI group (9% vs. 32%, P = 0.001)	Prospective, observational study on the use of CAZ/AVI compared to colistin specifically for infections due to CRE, including 30 (22%) patients with pneumonia
Castón et al., 2017	Spain, Israel, multicenter, 2012–2016	Retrospective	31, all with hematologic malignancy	Primary BSI (n = 14, 45.2%)	80.6% (25/31) were *K. pneumoniae*	CAZ/AVI (n = 8) vs. others (n = 23)	14-day clinical cure rate was higher in the CAZ/AVI group (85.7% [6/8] vs. 34.8% [8/23], P = 0.031)	Small case numbers; no difference in crude mortality
Shields et al., 2017b	United States, single site, 2009-2017	Retrospective	109	Secondary bacteremia resulted from abdominal (46%, 50/109)	97% (106/109) of *K. pneumoniae* harbored bla_{KPC}	CAZ/AVI (n = 13) vs. others (n = 96)	CAZ/AVI group had higher clinical success rates (85% [11/13] vs. 40.6% [39/96], P = 0.003)	Small case numbers with CAZ/AVI treatment; bias in selection of therapy

CAZ/AVI, ceftazidime/avibactam; BSI, bloodstream infection.

HAP (22%). Adjusted all-cause mortality was significantly lower in the CAZ/AVI group (absolute difference 23%; 95% CI, 9% to 35%; P = 0.001). A multicenter, retrospective study reviewed 60 patients receiving CAZ/AVI for CRE infections (King et al., 2017). The authors reported an overall in-hospital mortality rate of 32%, a microbiological cure rate of 53%, and a clinical success rate of 65%. There was no significant difference in the in-hospital mortality rate between patients receiving CAZ/AVI monotherapy vs. CAZ/AVI combination therapy (30% [10/33] vs. 33% [9/27], P = 1.0), and between patients with bacteremia vs. those without (39% [9/23] vs. 27% [10/37], P = 0.397). Shields et al. (2016) reported a case series in a single center including 37 cases of patients with CRE infections who received treatment with CAZ/AVI. The survival rate at 30 days was 76% (28/37). Clinical success was observed as 59% (22/37), with no significant difference between patients receiving monotherapy vs. combination therapy (58% [15/26] vs. 64% [7/11]). However, they also reported a CRE infection recurrence rate of 23% (5/22) among patients who had displayed clinical success, and an overall microbiologic failure rate of 27% (10/37). Temkin et al. (2017) reported a case series of 38 patients with CRE (n = 36) and *P. aeruginosa* (n = 2) infections treated with CAZ/AVI. Of these, 65.8% (25/38) of patients concurrently received other regimens. The overall clinical and/or microbiological cure rate was 73.7% (28/38), with 69.2% (9/13) in the monotherapy group and 76.0% (19/25) in the combination therapy group.

As described earlier, avibactam inhibits Ambler Class A (KPC) and Class D (OXA-48) but did not inhibit Class B MBLs (NDM, VIM, and IMP). Therefore, CAZ/AVI is not active against all CRE isolates (Falcone et al., 2018). In contrast, the monobactam antibiotic aztreonam (ATM) is stable against MBLs but is hydrolyzed by many other β-lactamases (ESBL, AmpC, and cephalosporinases) frequently co-produced by the MBL-producing strains (Marshall et al., 2017). The combination of CAZ/AVI and ATM has been proposed as a potential therapeutic strategy against infections caused by MBL-producing bacteria. Crandon and Nicolau (2013) tested the efficacy of CAZ/AVI and ATM using the neutropenic-mouse thigh infection model, and concluded that this combination represents an attractive treatment option for infections caused by MBL-producing strains that co-produce ESBLs or AmpC. Although studies have demonstrated good *in vitro* activity of CAZ/AVI and ATM against MBL-producing *Enterobacteriaceae* (Marshall et al., 2017; Wenzler et al., 2017), clinical data are still lacking.

These observational studies are subject to selection bias. Further RCTs are warranted to evaluate the effectiveness of CAZ/AVI, as well as CAZ/AVI and ATM combination, for the treatment of CRE infections. Whether CAZ/AVI combination therapy is more effective than monotherapy for deep-seat infections or high-risk patients also needs further evaluation. Moreover, the emergence of CAZ/AVI resistant strains during treatment has been reported. Shields et al. detected CAZ/AVI resistance due to mutations in the $bla_{rmKPC-3}$ gene in 3 of 10 microbiologic failures, following CAZ/AVI treatment for 10–19 days (Shields et al., 2016, 2017a). Clinicians should be aware of the possible emergence of resistance following treatment with CAZ/AVI.

Meropenem/Vaborbactam

Vaborbactam is a novel boron-containing serine-β lactamase inhibitor which confers activity against certain meropenem-resistant bacteria by inhibiting Ambler Class A and C serine carbapenemases, such as KPC (Castanheira et al., 2017). However, it has no *in vitro* activity against Class B metallo-β-lactamases producers (NDM or VIM) or Class D OXA-48 β-lactamases (Castanheira et al., 2016; Nelson et al., 2017). Meropenem/vaborbactam (MER/VAB) (Vabomere®, The Medicines Company, Parsippany, NJ, United States) is a novel carbapenem/β-lactamase inhibitor antimicrobial agent approved in August 2017 by the United States Food and Drug Administration (FDA) for the treatment of cUTIs, including pyelonephritis.

MER/VAB exhibited effective *in vitro* antibacterial activity against CRE isolates (Castanheira et al., 2017; Pfaller et al., 2018), with susceptibility rates ranging from 66.2 to 100% (Dhillon, 2018). The clinical data supporting MER/VAB for CRE infections is from the multicenter, randomized, open-label Tango II trial comparing the efficacy and safety of MER/VAB with the best available therapy (BAT) for the treatment of serious CRE infections (Wunderink et al., 2018). Of the 77 enrolled patients, 47 had confirmed CRE infections, including 22 BSI, 16 cUTI/pyelonephritis, 5 HAP/VAP, and 4 cIAI. The results showed statistical significance in favor of MER/VAB over BAT for a clinical cure (65.6% [21/32] vs. 33.3% [5/15], $P = 0.03$) and 28-day mortality (15.6% [5/32] vs. 33.3% [5/15], $P = 0.03$). Furthermore, MER/VAB was associated with decreased nephrotoxicity as compared with BAT (4.0% vs. 24%) (Wunderink et al., 2018). Data for MER/VAB on CRE infections are still accumulating. A study of MER/VAB vs. piperacillin/tazobactam on HAP/VAP (TANGO III, NCT02168946) is ongoing.

Plazomicin

Plazomicin is a next-generation aminoglycoside synthetically derived from sisomicin, which retains activity against bacteria containing aminoglycoside-modifying enzymes (Landman et al., 2010; Castanheira et al., 2018a). Plazomicin (Zemdri™, Achaogen, Inc., San Francisco, CA, United States) was approved in June 2018 by the FDA for the treatment of adults with cUTI including pyelonephritis who have limited or no alternative treatment options, with a recommended dose of 15 mg/kg every 24 h for normal renal function. The approval was based on two phase 3 clinical trials comparing the efficacy and safety of plazomicin with meropenem (NCT02486627, not published yet) and levofloxacin (NCT01096849, not published yet) for the treatment of cUTI and acute pyelonephritis. Plazomicin demonstrates a broad-spectrum activity against Gram-positive cocci and Gram-negative bacilli, including ESBL producers and CRE (Walkty et al., 2014; Karaiskos et al., 2015). Studies have shown that plazomicin is more potent than other aminoglycosides against KPC-producing *Enterobacteriaceae* (Endimiani et al., 2009b). A recent study evaluating the activity of plazomicin and comparators against clinical isolates showed that isolates carried 16S rRNA methyltransferases were resistant to all available aminoglycosides and had elevated plazomicin MICs (Castanheira et al., 2018b). The methyltransferase enzymes commonly found in MBL producers still render bacteria resistant to plazomicin.

The clinical data supporting plazomicin for the treatment of serious infections due to CRE is from a multicenter, randomized, open-label study comparing the efficacy and safety of plazomicin vs. colistin (both in combination with tigecycline or meropenem) (CARE trial, NCT01970371). Although the full report of this trial is not yet available, preliminary analysis shows that the plazomicin group had a significantly lower 28-day mortality compared to the colistin group (7.1% [1/14] vs. 40.0% [6/15]; difference, −32.9%; 95% CI, −60.1% to −4.0%) (McKinnell et al., 2017). In addition to a lower mortality rate, it was well-tolerated and was associated with a lower incidence of serum creatinine elevations. However, these data should be interpreted with caution due to the small sample size.

Eravacycline

Eravacycline, a synthetic fluorocycline antibacterial agent of the tetracycline class, has broad-spectrum antimicrobial activity against Gram-positive, Gram-negative, and anaerobic bacteria, exception for *P. aeruginosa* (Zhanel et al., 2016). Eravacycline (Xerava™, Tetraphase Pharmaceuticals, Inc., Watertown, MA, United States) was approved by the FDA in August 2018 for the treatment of cIAIs, based on two phase 3 clinical trials which demonstrated statistical non-inferiority of eravacycline to two commonly used comparators: ertapenem (IGNITE1 trial) (Solomkin et al., 2017) and meropenem (IGNITE4 trial, full data not published yet). The recommended dosage is 1 mg/kg intravenous infusion every 12 h. Of note, eravacycline is not indicated for the treatment of cUTI because clinical trials (NCT01978938 and NCT03032510) did not demonstrate the efficacy for the combined endpoints of clinical cure and microbiological success. Eravacycline has been shown to have effective *in vitro* activity against MDR pathogens (Sutcliffe et al., 2013; Livermore et al., 2016; Zhang et al., 2016), with twofold higher activity than tigecycline against CRE (Livermore et al., 2016). However, there is limited clinical data for the treatment efficacy of eravacycline in CRE infections.

POTENTIAL ANTIBIOTICS IN DEVELOPMENT

Imipenem/Cilastatin and Relebactam

Imipenem/cilastatin and relebactam (IMI/REL, Merck & Co. Inc., Kenilworth, NJ, United States) combines an approved carbapenem with a novel β-lactamase inhibitor. The chemical structure of relebactam is similar to avibactam (Watkins et al., 2013). Like avibactam, relebactam contains a diazabicyclooctane core, which covalently and reversibly binds Class A and C β-lactamases *in vitro*, with an inhibitory mechanism similar to that of avibactam (Blizzard et al., 2014). However, relebactam cannot inhibit Class D OXA-48 like avibactam (Petty et al., 2018; Zhanel et al., 2018).

Relebactam potentiates imipenem activity against imipenem-non-susceptible *Enterobacteriaceae* (Karlowsky et al., 2018). The clinical efficacy and safety of IMI/REL has been shown in the phase 2 studies for cUTIs (NCT01505634) (Sims et al., 2017) and cIAIs (NCT01506271) (Lucasti et al., 2016).

The RESTORE-IMI 1 study (NCT02452047) is a multicenter, randomized, double-blind, comparator-controlled trial, comparing the efficacy and safety of IMI/REL vs. colistin plus imipenem/cilastatin (COL + IMI) in patients with imipenem-non-susceptible bacterial infections. Patients with HAP/VAP, cIAI, or cUTI due to imipenem-non-susceptible pathogens, were randomized 2:1 to receive IMI/REL or COL + IMI. In this study, 31 of 47 randomized and treated patients met mMITT criteria. Favorable clinical responses at Day 28 were comparable in the IMI/REL and the COL + IMI arms (71.4% [15/21] vs. 70.0% [7/10]), and the 28-day all-cause mortality was lower in the IMI/REL arm compared to the COL + IMI arm (9.5% [2/21] vs. 30.0% [3/10]) (presented by Motsch et al. at the European Congress of Clinical Microbiology and Infectious Diseases; April 22, 2018; Madrid, Spain). In addition, treatment-emergent nephrotoxicity was lower with IMI/REL compared to COL + IMI (10.3% [3/29] vs. 56.3% [9/16], $P = 0.002$) (presented by Brown et al. at IDWeek, October 6, 2018; San Francisco, CA, United States).

Cefiderocol

Cefiderocol is the first siderophore antibiotic to advance into late-stage development. Cefiderocol, a novel siderophore cephalosporin, exhibits potent *in vitro* and *in vivo* activity against a variety of Gram-negative bacteria, including CRE (Saisho et al., 2018). Cefiderocol has a unique antibacterial mechanism in which its catechol side chain of binds to ferric acid, with the complex then being actively transported into bacteria via bacterial iron transporters (Ito et al., 2016). In addition, cefiderocol is also highly active against carbapenemase hydrolysis (Wright et al., 2017). Cefiderocol has demonstrated potent *in vitro* activity against CRE isolates, with 97.0% (991/1,022) of isolates demonstrating cefiderocol MICs of ≤4 mg/L (Hackel et al., 2018). A multicenter, randomized, open-label CREDIBLE-CR trial is ongoing to assess the efficacy of cefiderocol, compared to BAT, for severe infections caused by carbapenem-resistant Gram-negative pathogens (NCT02714595).

CONCLUSION

The increasing prevalence of CRE infections represents a major threat to human health. Effective antibiotics against CRE remain very limited, with polymyxins, tigecycline, fosfomycin, and aminoglycoside being the mainstays of anti-CRE therapy. With the high mortality of CRE infections and increasing resistance to available antibiotics, it is urgent for the medical community to develop new and effective therapeutic strategies. The first potential strategy is to increase the doses of anti-CRE agents. High-dose carbapenem, colistin, and tigecycline have been associated with better clinical outcomes in CRE infections. The second potential strategy is to combine these anti-CRE agents. Although large-scale observational studies and systematic reviews suggest that combination therapy may be beneficial to patients with severe CRE infections, the quality of evidence is low due to substantial heterogeneity in study design and patient population. A recent RCT showed no benefit of colistin combination therapy with meropenem when compared to colistin monotherapy. The debate on whether or not these agents should be used in combination will continue. DCT with ertapenem infusion prior to a high-dose meropenem or doripenem infusion has been adopted as a salvage therapy for critically ill patients with CRE infections. Studies suggest that a combination of colistin or tigecycline with DCT might be a reasonable strategy for severe CRE infections. However, the target patients and molecular phenotypes of CRE that would benefit from DCT require further investigation. The recent introduction of the novel β-lactamase inhibitors avibactam, vaborbactam, and relebactam, has provided additional therapeutic options for CRE infections. Of note, these new β-lactamase inhibitors are not active against all major carbapenemases. Avibactam inhibits KPC and OXA-48, while vaborbactam and relebactam inhibit only KPC. Expectantly, these novel β-lactamase inhibitor combinations will also provide carbapenem-sparing options for the treatment of MDR bacteria and help control the rapid emergence of carbapenemase-producing bacteria. The next-generation aminoglycoside plazomicin and the fully synthetic tetracycline antibiotic eravacycline, both recently approved by the FDA, may provide us alternative therapeutic options for CRE infections. The activity of plazomicin and eravacycline against CPE has been demonstrated *in vitro*. However, we need more clinical data to support their use in the treatment of serious infections due to CRE. Many other new antibiotics with potential anti-CRE activity are in various stages of development, including the novel β-lactamase inhibitor combination IMI/REL and the novel siderophore cephalosporin cefiderocol. Currently, high-dose and combination strategies that may include the new β-lactam/β-lactamase inhibitors should be considered for severe CRE infections to maximize treatment success.

PERSPECTIVE

In the future when more treatment options are available, anti-CRE therapy should be individualized and based on molecular phenotypes of resistance, susceptibility profiles, disease severity, and patient characteristics. More clinical trials are needed to provide high-quality evidence and guide the selection of effective anti-CRE strategies.

AUTHOR CONTRIBUTIONS

C-CS, Y-TC, and S-YL collected and analyzed the data, and wrote the manuscript. Y-HC and P-RH revised and edited the manuscript. C-CS, Y-TC, S-YL, Y-HC, and P-RH read and approved the final version of the manuscript.

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Dynamic Colonization of *Klebsiella pneumoniae* Isolates in Gastrointestinal Tract of Intensive Care Patients

Qiao-ling Sun[1], Danxia Gu[2], Qi Wang[1], Yanyan Hu[1], Lingbin Shu[1], Jie Hu[1], Rong Zhang[1] and Gong-Xiang Chen[1]**

[1] Department of Clinical Laboratory, Second Affiliated Hospital of Zhejiang University, School of Medicine, Hangzhou, China, [2] Key Laboratory of Tumor Molecular Diagnosis and Individualized Medicine of Zhejiang Province, Clinical Research Institute, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, China

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***Correspondence:**
Rong Zhang
zhang-rong@zju.edu.cn
Gong-Xiang Chen
chengongxiang@zju.edu.cn

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Gastrointestinal carriage is regarded as a major reservoir of *K. pneumoniae* infections, especially in intensive care patients. A total of 101 (95.3%) KPC-producing carbapenem-resistant *K. pneumoniae* (CRKP) isolates were identified among 106 CRKP isolates collected from stool samples of inpatients performing active rectal screening for carbapenem-resistant Enterobacteriaceae during hospitalization in the ICUs of a tertiary hospital between 2016 and 2017. Among them, six KPC-producing CRKP isolates from three patients (two isolates for each patient) were identified with distinct antibacterial susceptibility. Our findings showed that: (1) bla_{KPC-2} gene is predominant in CRKP strains isolated from the intensive care patients and can be incorporated into various plasmids that are transmissible among multiple bacterial hosts in the human gastrointestinal tract; (2) the human gastrointestinal tract has a capacity to dynamically colonize multiple clones of CRKP strains with varied plasmids, diverse antimicrobial resistance genes and virulence genes. *K. pneumoniae* colonization is an important step in progression to extraintestinal infection, which provides the rationale for establishing intervention measures to prevent subsequent infection. Thus, close surveillance on CRKP colonization, together with effective infection prevention and control measures, should be put into practice.

Keywords: CRKP, KPC-2, gastrointestinal carriage, dynamical colonization, multiple clones

INTRODUCTION

Klebsiella pneumoniae is a major opportunistic pathogen that can cause invasive hospital-acquired infections among immune-compromised patients especially for those from ICU ward with critical illness. Carbapenem is a first line therapy for the treatment of infections caused by multidrug-resistant *K. pneumoniae*. However, carbapenem-resistant *K. pneumoniae* (CRKP) has emerged as a public threat to cause serious infections with high mortality up to 33.24–50.06% (Xu et al., 2017). Recent data from the CHINET surveillance program showed that the prevalence of CRKP had dramatically increased from 3 to 17.9% since 2005 (Hu et al., 2016, 2017). A previous study on nationwide surveillance of clinical carbapenem-resistant Enterobacteriaceae (CRE) strains has

revealed that bla_{KPC-2} was the most prevalent genotype in China, accounting for 73% of CRKP isolates (Zhang et al., 2017).

Carbapenem-resistant Hypervirulent *K. pneumoniae* (CR-HvKP), especially ST11 KPC-producing strains, have been increasingly reported in recent years (Zhang R. et al., 2015; Zhang Y. et al., 2015; Gu et al., 2018). They often cause invasive, even life-threatening infections with high mortality among young and healthy populations. In a previous study, we reported an outbreak of ST11 CR-HvKP in the ICU ward; further study indicated that the acquisition of a virulence plasmid carrying *rmpA2* and aerobactin biosynthesis genes by classic ST11 CRKP strains played a critical role (Gu et al., 2018).

Gastrointestinal carriage has been regarded as a major reservoir of *K. pneumoniae* infections, especially in intensive care patients (Gorrie et al., 2017). A prospective study in 1971 indicated that 18.5% patients colonized with multidrug-resistant *K. pneumoniae* after hospital admission had higher risk to develop subsequent infection caused by identical bacteria within 21 days compared to those who did not become intestinal carriers (45% vs. 11%) (Martin and Bachman, 2018). A 2016 study reported similar colonization rates (23%) and increased risk of infection following colonization (5.2% in colonized vs. 1.3% in noncolonized) (Martin et al., 2016). The carriage rate of CRKP is reported to range from 8 to 9% in medical and surgical departments to 5% in intensive care units (Wiener-Well et al., 2010). CRKP colonization can persist and spread silently for years, even trigger a clonal outbreak in long-term care facilities while newly colonized patients can develop fatal infections (Martin and Bachman, 2018). Our previous study showed the evolution of tigecycline- and colistin-resistant CRKP has occurred *in vivo* under the antibiotic selection and CRKP could persistently colonize in the human gastrointestinal tract for 3 years even without antibiotic selection pressure (Zhang et al., 2018). Previous reports mainly focused on epidemiological data of CRKP carriage and associated risk factors (Papadimitriou-Olivgeris et al., 2012; Feldman et al., 2013; Papadimitriou-Olivgeris et al., 2013), in this study, we are going to investigate the molecular characterization of multiple clones of CRKP strains colonized in the gastrointestinal tract of intensive care inpatients, aiming at illustrating certain discipline underlying the colonization of CRKP.

MATERIALS AND METHODS

The Rectal CRKP Isolates From the Intensive Care Inpatients

A total of 106 *K. pneumoniae* isolates that exhibited carbapenem resistance phenotype (MIC value of meropenem $\geq$4 μg/ml) were identified from stool samples of inpatients performing active rectal screening for CRE during hospitalization in the ICUs of Second Affiliated Hospital of Zhejiang University, School of Medicine (Hangzhou, China) between 2016 and 2017. Identification of species was confirmed via a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany). The presence of carbapenem-resistant genes (bla_{NDM}, bla_{KPC}, bla_{IMP}, bla_{VIM}, and bla_{OXA-48}) was screened using PCR (Zhang et al., 2017). Positive products were validated with Sanger DNA sequencing. A total of 101 (95.3%) KPC-2-producing CRKP isolates were identified among 106 rectal CRKP isolates. Among them, six KPC-2-producing CRKP isolates from three patients (two isolates for each patient) were identified with distinct antibacterial susceptibility. In order to investigate the origin of the intestinal CRKP isolates and further look into the relationship between the rectal colonization and extraintestinal infections, we reviewed medical history of the three inpatients in detail and subjected the KPC-2-producing CRKP isolates collected during peri-hospitalization period to phenotypic and genotypic characterization.

Phenotypic Characterization

The antimicrobial susceptibilities of the isolates were determined using a broth microdilution procedure and the interpretations were in accordance with the guideline document M100-S26 established by Clinical and Laboratory Standards Institute (CLSI, 2016).

We did pulsed-field gel electrophoresis (PFGE), S1-PFGE, and Southern hybridization as previously reported (Huang et al., 2016). A dendrogram was generated from the homology matrix with a coefficient of 0.5% using the unweighted pair-group method using arithmetic averages ("UPGMA") to describe the relationships among PFGE profiles. Isolates were considered to belong to the same PFGE group if their Dice similarity index was $\geq$85%.

The virulence gene *rmpA2*, encoded on the virulence plasmid, was identified by PCR as previously described (Gu et al., 2018). All isolates were performed with the string test to identify the hypermucoviscous phenotype as described previously (Gu et al., 2018). As a test of virulence, we quantified virulence with *Galleria mellonella* larva models. The *G. mellonella* larva was injected into the hemocoel of each caterpillar via the last left proleg with 10 μl suspensions of a *K. pneumoniae* strain containing a final concentration of 10^6 CFU/mL, incubated at 37°C and observed every 12 h for 3 days. The effect of 1×10^6 CFU of each *K. pneumoniae* strain on survival was assessed in *G. mellonella*. HvKP strain *K. pneumoniae* 4 and strain PC *K. pneumoniae* 4, reported in a previous study (Gu et al., 2018), were treated as controls. HvKP strain *K. pneumoniae* 4 was a ST11 KPC-producing hypermucoviscous strain harboring various virulence genes (*rmpA2*, *iutA*, *iroE*, *kpn*, *ycfM*, *iucABCD*, *mrkABCDF*, and *fimA–H*), located on the virulence plasmid pLVPK. Strain PC *K. pneumoniae* 4 is a mutant strain of *K. pneumoniae* 4, of which the virulence plasmid has been removed in plasmid curing experiments. Strain PC *K. pneumoniae* 4 was negative for string test and demonstrated reduced virulence in *G. mellonella* models. Two control groups were performed: the first group was inoculated with PBS to monitor for killing due to physical trauma and attrition while the second received no injection. Eight larvae from each group were examined and all the experiments were performed in triplicates. Kaplan-Meier survival curves were plotted using GraphPad Prism version 7.00, the log rank (Mantel-Cox) test was used to analyze whether significant differences

($P < 0.05$) in the survival rates of the infected *G. mellonella* larvae were observed.

Whole Genome Sequencing and Bioinformatics Analysis

Genomic DNA was extracted from overnight cultures by using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, United States) and was subjected to whole genome sequencing using 150 bp pair-end strategies with the Illumina HiSeq X10 platform. Raw reads were trimmed and assembled to contigs using SPAdes v3.11.1 (Bankevich et al., 2012). Assembled genome sequences were submitted to the NCBI database with accession number QMKA00000000, QMKB00000000, QMKC00000000, QMKD00000000, QMKE00000000, and QMKF00000000. Genome sequences were annotated with the RAST tool (Overbeek et al., 2013) and Prokka (Seemann, 2014). Multilocus sequence types (MLSTs), virulence-associated genes encoding yersiniabactin, aerobactin, salmochelin and the regulators of mucoid phenotype were determined with Kleborate (Pichler et al., 2017). Serotyping was performed using Kaptive (Wyres et al., 2016). Acquired antibiotic resistance genes were identified with ResFinder 2.1 (Zankari et al., 2012). Heatmap of the antimicrobial resistance genes was generated using an in-house script.

Ethics Statement

The study was approved by the Ethics Committee of Second Affiliated Hospital of Zhejiang University, School of Medicine (2017-099). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Biosafety Statement

All concerns related to the safe and appropriate use of human-derived materials, infectious agents, or genetically modified organisms were approved by the Institutional Biosafety Committee of Second Affiliated Hospital of Zhejiang University, School of Medicine. All experiments were conducted under the guidelines from the Biological Agent Reference Sheet.

RESULTS

Three elderly patients aged 61–70 years were admitted to the ICU between August 2016 and February 2017. The three patients all developed diseases including bacterial pneumonia and bloodstream infections and underwent surgery, followed by antimicrobial treatment and mechanical ventilation, but they responded differently to antibiotic treatment. Only two patients, Patient 2 and 3, succeeded in infection control while Patient 1 experienced a persistent fever and pulmonary infection till against-advice discharge. Detailed information about the patients is available in the **Figures 1**, **2**. Active rectal screening for CRE was conducted for all the patients during hospital admission and peri-hospitalization period, and all the three patients were found to carry the KPC-producing CRKP strains in the gastrointestinal tract on admission and during stays at healthcare settings. Thereinto, two CRKP isolates from stool samples of each patient presented distinct antimicrobial susceptibility profiles. In order to study the origin of the intestinal CRKP carriage and the relationship between the rectal colonization and extraintestinal infections, three, five, and five more KPC-producing CRKP isolates from various types of specimens including stool were successively identified in Patient 1, Patient 2, and Patient 3 during peri-hospitalization period, respectively (**Figures 1**, **2**).

Acquisition of KPC-Producing CRKP Isolates From Stool Samples

Patient 1, a 71-year old woman, had previously been admitted to a local hospital for acute ventricular hemorrhage and was transferred to the Neurological intensive care unit (NICU) of our hospital on Aug 12, 2016. Three KPC-2-producing CRKP isolates (namely JZX2078, JZX2124, and JZX11-E8) were isolated. Isolate JZX2078 was recovered from stool sample after 1 week of admission, belonging to ST11 with unidentified serotype. It harbored three plasmids with sizes of ∼230, ∼210, and ∼80 Kb. The ∼210 Kb plasmid was a pLVPK-like virulence plasmid which bears *iucABCD*, *iroBCD*, and *rmpA2* genes. The ∼230 Kb plasmid was a pKP04VIM (KU318421.1)-like plasmid, while the ∼80 Kb one was a bla_{KPC-2}-bearing p69-2 (CP025458.1)-like plasmid (**Table 1**). The second isolate JZX2124 was also isolated from stool a week before discharge. It was identified as a K19, ST1 strain which harbored two multidrug resistance plasmids, the ∼130 Kb p11219-CTXM (MF133442.1)-like plasmid and the ∼170 Kb, bla_{KPC-2}-bearing pIT-06C07 (LT009688)-like plasmid (**Table 1**). Compared to isolate JZX2078 with 9 antibiotic resistance genes, isolate JZX2124 carried 12 resistance genes including *tet(A), oqxAB* genes and two different genes encoded for extended spectrum β-lactamases (ESBLs) as shown in **Figure 3**. And the third isolate JZX11-E8 was recovered from blood. With completely different antibacterial susceptibility profiles and distinct PFGE patterns (**Figure 2**), these three isolates were proved to originate from different clones.

Patient 2, a 66-year old man, was admitted to the Surgical Intensive Care Unit (SICU) for severe acute pancreatitis with acute respiratory distress syndrome on January 12th, 2017. Two weeks after admission, he was transferred to Surgical Ward preparing for a surgery of cholecystectomy and peritoneal lavage and drainage, immediately followed by transferring to the SICU and experiencing one more surgery of intraperitoneal hemostasis and peritoneal lavage and drainage. Two CRKP isolates, WJQ129 and WJQ196, were isolated from stool and sputum samples, respectively, during his staying in Surgical Ward, while three CRKP isolates (WJQ205, WJQ250, and WJQ298) were collected from stool, sputum, and drainage fluid samples, respectively, during his staying in SICU. With similar antimicrobial susceptibility profiles and PFGE patterns, isolates WJQ129 and WJQ196 shared high homology. The same situation was presented for isolates WJQ205, WJQ250, and WJQ298. However, as shown in **Figure 2**, isolates recovered from the patient during his earlier staying in Surgical Ward were completely distinct from those recovered during his later staying in SICU. It may be suggested that Patient 2

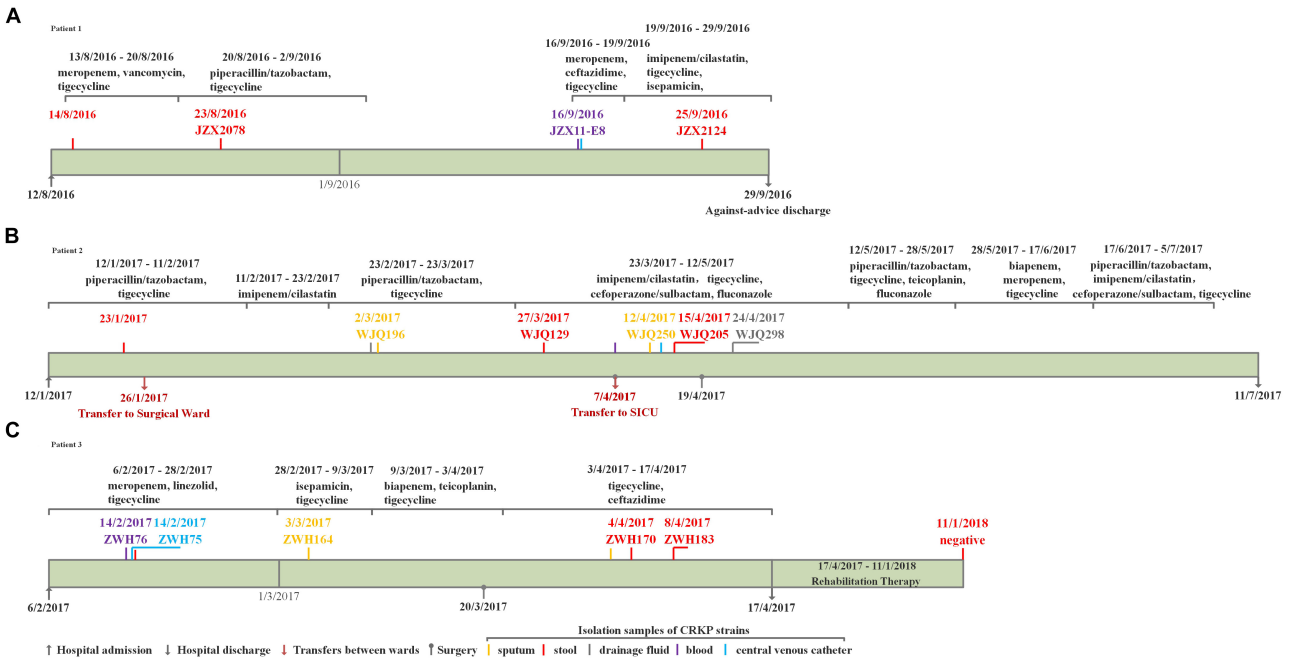
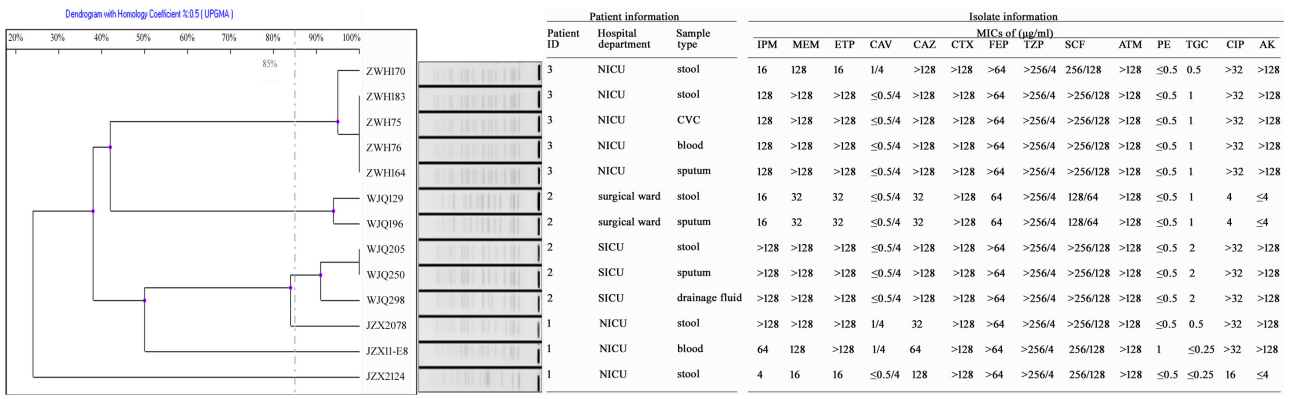


FIGURE 1 | The clinical course, therapeutic regimen, and outcomes of Patient 1 **(A)**, Patient 2 **(B)** and Patient 3 **(C)** who carried CRKP isolates.



Patient information			Isolate information											
Patient ID	Hospital department	Sample type	MICs of (μg/ml)											
			IPM	MEM	ETP	CAV	CAZ	CTX	FEP	TZP	SCF	ATM	PE	TGC	CIP	AK
3	NICU	stool	16	128	16	1/4	>128	>128	>64	>256/4	256/128	>128	≤0.5	0.5	>32	>128
3	NICU	stool	128	>128	>128	≤0.5/4	>128	>128	>64	>256/4	>256/128	>128	≤0.5	1	>32	>128
3	NICU	CVC	128	>128	>128	≤0.5/4	>128	>128	>64	>256/4	>256/128	>128	≤0.5	1	>32	>128
3	NICU	blood	128	>128	>128	≤0.5/4	>128	>128	>64	>256/4	>256/128	>128	≤0.5	1	>32	>128
3	NICU	sputum	128	>128	>128	≤0.5/4	>128	>128	>64	>256/4	>256/128	>128	≤0.5	1	>32	>128
2	surgical ward	stool	16	32	32	≤0.5/4	32	>128	64	>256/4	128/64	>128	≤0.5	1	4	≤4
2	surgical ward	sputum	16	32	32	≤0.5/4	32	>128	64	>256/4	128/64	>128	≤0.5	1	4	≤4
2	SICU	stool	>128	>128	>128	≤0.5/4	>128	>128	>64	>256/4	>256/128	>128	≤0.5	2	>32	>128
2	SICU	sputum	>128	>128	>128	≤0.5/4	>128	>128	>64	>256/4	>256/128	>128	≤0.5	2	>32	>128
2	SICU	drainage fluid	>128	>128	>128	≤0.5/4	>128	>128	>64	>256/4	>256/128	>128	≤0.5	2	>32	>128
1	NICU	stool	>128	>128	>128	1/4	32	>128	>64	>256/4	>256/128	>128	≤0.5	0.5	>32	>128
1	NICU	blood	64	128	>128	1/4	64	>128	>64	>256/4	256/128	>128	1	≤0.25	>32	>128
1	NICU	stool	4	16	16	≤0.5/4	128	>128	>64	>256/4	256/128	>128	≤0.5	≤0.25	16	≤4

FIGURE 2 | Antimicrobial susceptibility and PFGE profiles of 13 CRKP isolates collected from three inpatients. IPM, Imipenem; MEM, Meropenem; ETP, Ertapenem; CAV, Ceftazidime-avibactam; CAZ, Ceftazidime; CTX, Cefotaxime; FEP, Cefepime; TZP, Piperacillin-tazobactam; SCF, Cefoperazone-sulbactam; ATM, Aztreonam; PE, colistin; TGC, Tigecycline; CIP, Ciprofloxacin; AK, Amikacin; CVC, central venous catheter.

TABLE 1 | Sequence information for rectal CRKP isolates from the three inpatients.

Strain	Patient ID	Number of contigs >500 bp	Sequence length (bp)	G+C (%)	N50	MLST	Aerobactin	Salmochelin	Hypermucoidy	Serotype
JZX2078	1	139	5863800	56.78	151743	ST11	*iucABCD*	*iroBCD*	*rmpA2*	unidentified
JZX2124	1	425	6015115	56.7	202420	ST1	–	–	–	K19
WJQ129	2	149	5479176	57.13	217169	ST290	–	–	–	K21
WJQ205	2	231	5948640	56.71	99385	ST11	*iucABCD*	*iroBCD*	*rmpA2*	K64
ZWH170	3	159	5736283	57.02	129409	ST11	–	–	*rmpA*	K64
ZWH183	3	219	5852344	57.04	99385	ST11	*iucABCD*	–	*rmpA*, *rmpA2*	K64

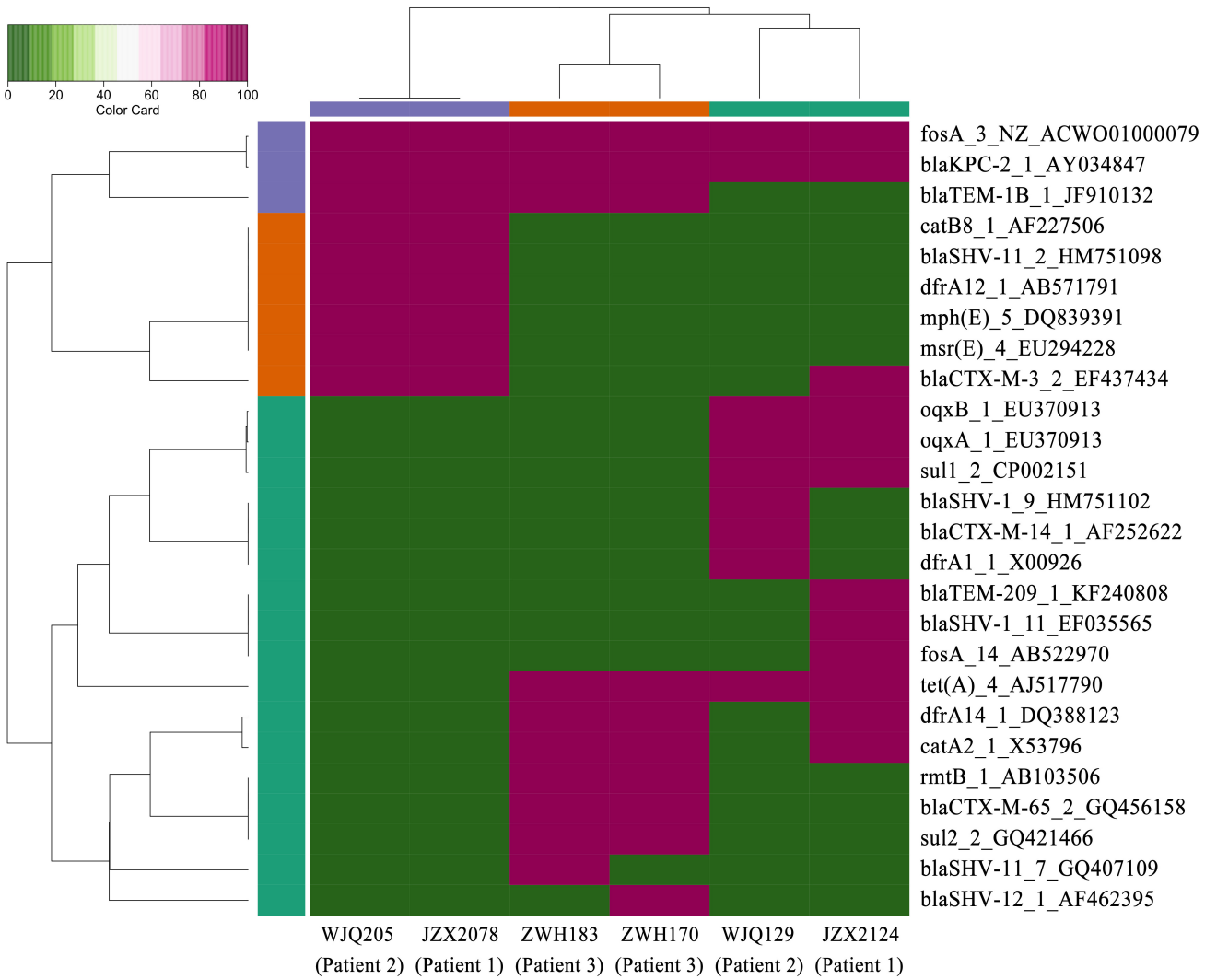


FIGURE 3 | Heatmap of antimicrobial resistance genes in the CRKP isolates from stool samples. Red and green boxes indicate the presence and absence of the corresponding antimicrobial resistance genes, respectively.

was successively infected by two different strains of KPC-producing *K. pneumoniae* isolated from different ICU wards. CRKP colonized in the gastrointestinal tract would easily cause extraintestinal infections once host defense system is weakened. Isolate WJQ129 was an ST290, K21 strain which harbored 9 antibiotic resistance genes (**Table 1**, **Figure 3**) and two plasmids with sizes of ∼230 and ∼90 Kb. The ∼230 Kb *K. pneumoniae* strain FDAARGOS_443 plasmid unnamed1 (CP023937.1)-like plasmid carries resistance genes *tet(A)*, *qnrS*, $bla_{CTX-M-14}$, and *dfrA1*. The ∼90 Kb p628-KPC (KP987218.1)-like plasmid carries bla_{KPC-2}. Isolate WJQ205 belonged to ST11, and identified as K64, carrying three plasmids (∼240, ∼210, and ∼80 Kb) (**Table 1**). The ∼210 Kb plasmid is a pLVPK-like virulence plasmid, carrying *iucABCD*, *iroBCD*, and *rmpA2* genes. The ∼240 and ∼80 Kb plasmids are pA324-IMP (MF344566)- and bla_{KPC-2}-bearing p69-2 (CP025458.1)-like plasmids, respectively.

Patient 3, a 61-year old woman, was admitted to our NICU for disorder of consciousness after a surgery of eliminating intracranial hematoma and bilateral external ventricular drainage in a local hospital. Five CRKP isolates were recovered from different samples belonging to two highly similar PFGE patterns. Interestingly, two homologous CRKP clones ZWH170 and ZWH183, collected from stool samples at short intervals, were both ST11, K64 serotype and harbored highly similar resistance genes, but showed different antibacterial susceptibility profiles (**Table 1** and **Figures 2**, **3**). Isolate ZWH170 carried plasmids with sizes of ∼138 and ∼78 Kb, while isolate ZWH183 carried one more plasmid which is around ∼105 Kb. The ∼138 Kb plasmid was a pKPC-CR-HvKP4-like resistance plasmid carrying bla_{KPC-2} and $bla_{CTX-M-65}$ genes. The ∼78 Kb plasmid was a p675920-2-like multidrug resistance plasmid carrying bla_{LAP-2}, *qnrS1*, *tet(A)*, and *sul2* genes. Additionally, the ∼105 Kb plasmid was a pLVPK-like virulence plasmid.

After half-year rehabilitation therapy in another hospital, the patient agreed to provide the stool sample for follow-up investigation. Surprisingly, CRKP was no longer detected from her stool samples.

Characterization of Virulence in CRKP Isolates

Six KPC-producing *K. pneumoniae* isolates (JZX2078, JZX2124, WJQ129, WJQ205, ZWH170, and ZWH183) from stool samples of three patients were selected for further virulence characterization. According to the presence of virulence plasmid, the six isolates were divided into two groups: *rmpA2*-positive strains (JZX2078, WJQ205, and ZWH183) and *rmpA2*-negative strains (JZX2124, WJQ129, and ZWH170). S1-PFGE and Southern hybridization of the marker gene of the virulence plasmid *rmpA2*, which was hybridized to the roughly 210 Kb and 105 Kb plasmids, confirmed the presence of the virulence plasmid in three of the ST11 KPC-producing CRKP isolates (JZX2078, WJQ205, and ZWH183). The ∼210 Kb pLVPK-like virulence plasmid, harboring *rmpA2*, *iroBCD*, and *iucABCD* genes, were identified in two ST11 CRKP strains isolated from different ICU wards. The ∼105 Kb virulence plasmid in isolate ZWH183, carrying the *rmpA2*, *iucABCD* genes was absent in isolate ZWH170, suggesting the attenuated virulence potential. The results of S1-PFGE and Southern hybridization were described in **Figure 4**.

All the six CRKP isolates from stool samples were negative for string test. Moreover, in *G. mellonella* larva models, the survival rate of larva infected with *rmpA2*-carrying CRKP strains (JZX2078, WJQ205, and ZWH183) ranged from 20 to 40% at 16 h after infection; as for *rmpA2*-negative CRKP strains (JZX2124, WJQ129, and ZWH170), 60–80% of the larva survived at 20 h (**Figure 5**). The control group infected with HvKP strain *K. pneumoniae* 4 resulted in 0% survival by 16 h, whilst 70% survival was reached in larva infected with PC *K. pneumoniae* 4 after 16 h. The survival rate of *G. mellonella* larvae infected with *rmpA2*-carrying CRKP strains (WJQ205 and ZHW183) was significantly lower than that infected with the counterpart *rmpA2*-negative CRKP strains (WJQ129 and ZWH170) ($P < 0.05$). However, there was no difference on the survival rate of *G. mellonella* larvae between the CRKP strains JZX2078 and JZX2124 ($P > 0.05$). The survival rate of *G. mellonella* larvae infected with *rmpA2*-carrying CRKP strains (WJQ205, ZHW183, and JZX2078) and *rmpA2*-negative CRKP strains (JZX2124, WJQ129, and ZWH170) showed

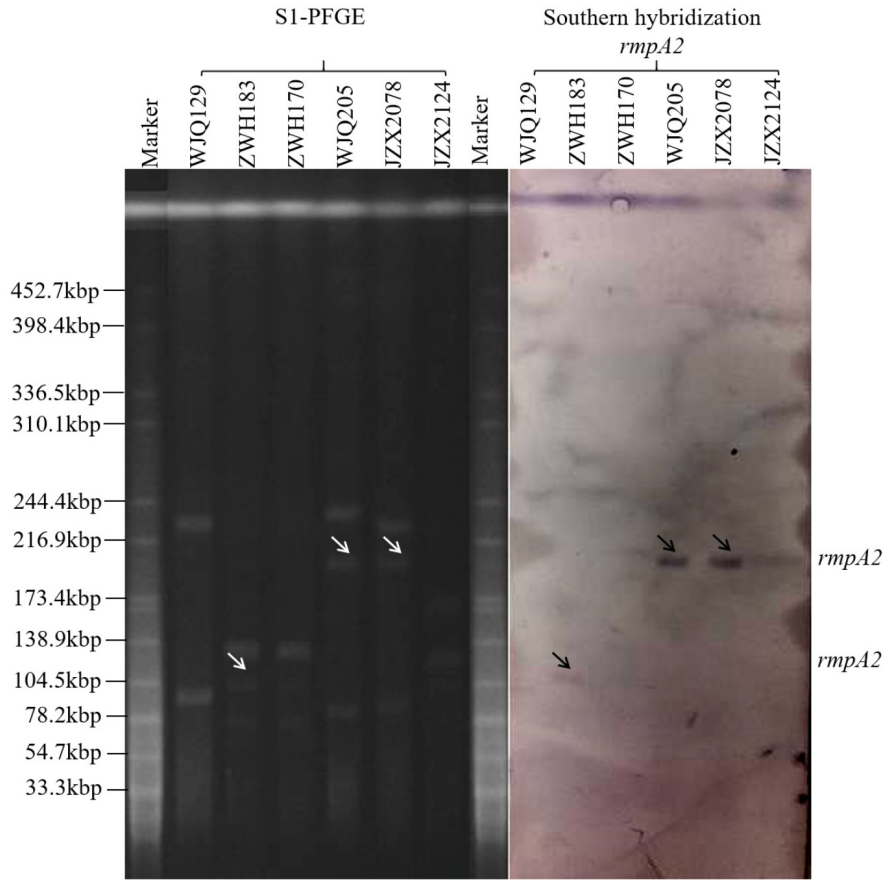


FIGURE 4 | Results of S1-PFGE and Southern hybridization of marker gene of the virulence plasmid *rmpA2* harbored by KPC-2-producing CRKP isolates recovered from stool samples of three patients.

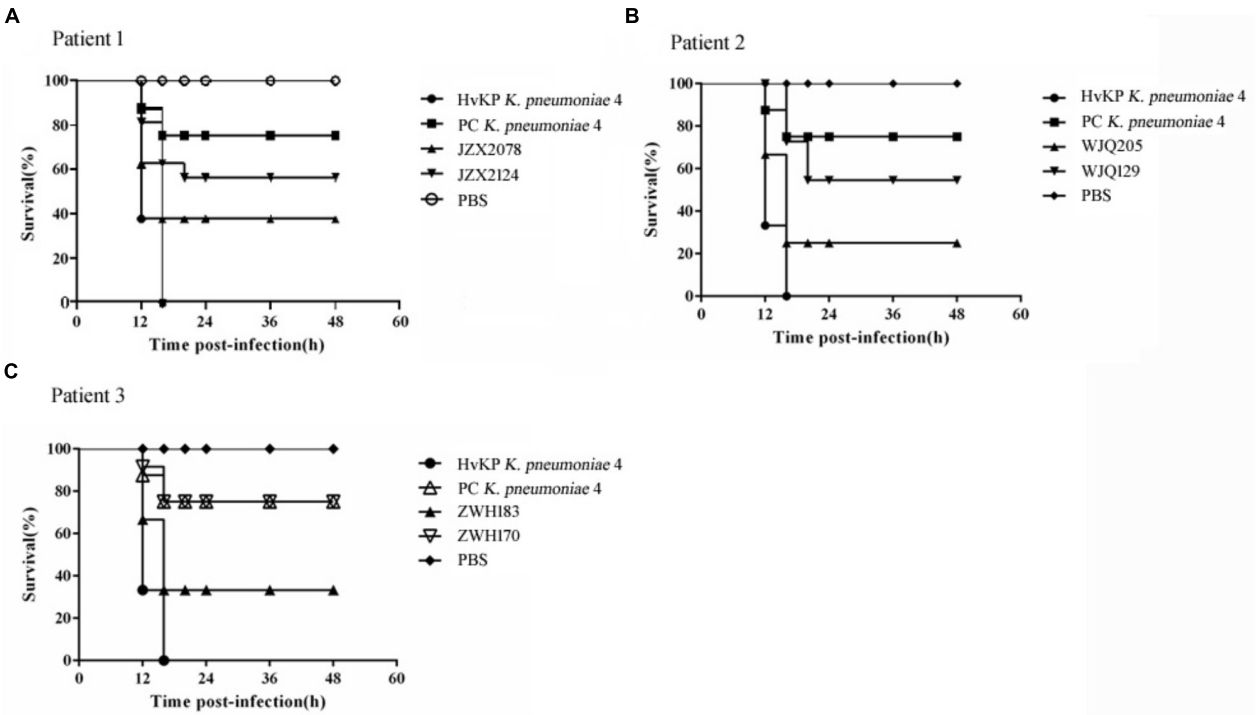


FIGURE 5 | Virulence potential of KPC-2-producing CRKP isolates from Patient 1 **(A)**, Patient 2 **(B)** and Patient 3 **(C)** in the *G. mellonella* infection models. The effect of 1×10^6 CFU of each *K. pneumoniae* strain on survival was assessed in *G. mellonella*. HvKP strain *K. pneumoniae* 4 was a ST11 KPC-producing hypermucoviscous strain harboring various virulence genes, located on the virulence plasmid pLVPK. Strain PC *K. pneumoniae* 4 is a mutant strain of *K. pneumoniae* 4, of which the virulence plasmid has been removed in plasmid curing experiments. Strain PC *K. pneumoniae* 4 was negative for string test and demonstrated reduced virulence in *G. mellonella* models.

statistical significance with that infected with HvKP ($P < 0.05$). Interestingly, the survival rate of *G. mellonella* larvae infected with all the *rmpA2*-carrying CRKP strains was significantly lower than that PC *K. pneumoniae* 4 ($P < 0.05$), while *rmpA2*-negative CRKP strains all exhibited no difference on the survival rate of *G. mellonella* larvae with PC *K. pneumoniae* 4 ($P > 0.05$).

DISCUSSION

Gastrointestinal colonization is regarded as a common and significant reservoir of *K. pneumoniae* in terms of risk of transmission and infection (Martin and Bachman, 2018). Previous studies have found that *K. pneumoniae* gastrointestinal colonization was significantly associated with subsequent infections in inpatients (odds ratio >4), and 5% of colonized patients developed infections. In addition, 80% concordance was showed between infecting and colonizing isolates of *K. pneumoniae* within infected patients (Martin et al., 2016; Gorrie et al., 2017; Martin and Bachman, 2018). In our study, each of Patient 2 and Patient 3 had a homologous strain in the gut and an invasive site according to the **Figure 2**, suggesting that CRKP in the gastrointestinal tract would readily cause extraintestinal infections once the host defense system is weakened. Thus, CRKP colonization in the gastrointestinal tract is of significant importance.

As was widely reported (Holt et al., 2015; Lam et al., 2018), *K. pneumoniae* isolates are often volatile with a wide spectrum of diversity. The human gut is always considered as a reservoir for antibiotic resistance genes, with various species and abundant genes; horizontal transfer of resistance genes is extremely active. CRKP isolates colonized in the gut can be even more diversified. In the current study, three KPC-producing CRKP isolates from Patient 1 were highly heterogeneous belonging to different clones. Interestingly, the two from her intestine demonstrated completely different antibacterial susceptibility, PFGE and plasmid profiles. Patient 2 was successively infected by two different strains of CRKP isolated from different ICU wards, which indicated that hospital-acquired CRKP strains among the ICU ward is responsible for the colonizing and infecting strains in inpatients, and multiple clones of CRKP strains might spread in the clinical setting. bla_{KPC-2} gene was the predominant genotype in 95.3% CRKP isolates and two bla_{KPC-2}-bearing plasmids belonged to two major types of ∼138 Kb and ∼80 Kb in size, suggesting that the bla_{KPC-2} gene can be incorporated into various extrachromosomal elements which are capable of horizontal transfer among multiple bacterial hosts in the human gastrointestinal tract.

According to a previous report (Feldman et al., 2013), 74% of the patients were identified with gastrointestinal carriage of CRKP 30 days after discharge from hospital, and when it came to

6 months, the proportion declined to <30%. Persistent carriage of CRKP was associated with several risk factors, including catheter use, long-term care facilities, recent acquisition (<4 months), and a low functional status (Feldman et al., 2013). Additionally, a hypothesis was posed that CRKP could colonize in host cells to circumvent phagocytosis by immune cells, and survival of CRKP within host cells might serve as a reservoir to protect from antibiotic treatments and enable long-term coexistence with the host (Yang et al., 2018). In this study, all the three inpatients underwent mechanical ventilation, carried CRKP isolates in 2 weeks after hospital admission and had suffered from bloodstream infection caused by CRKP strains during hospitalization. The persistent carriage of CRKP in gastrointestinal tract could last for 1–6 months in our study, inferring that long-term hospital stay and prolonged antibiotic usage could acquire CRKP strains from the settings and enrich them in the host gastrointestinal tract, and CRKP might survive intracellularly to persist in the gastrointestinal colonization. Interestingly, after the half-year rehabilitation therapy in another hospital, CRKP was no longer detected from the stool samples of the Patient 3.

ST11 has been one of the most prevalent MLST in the clinical CRKP strains among different parts of the world. Previous study reported an outbreak of ST11 CR-HvKP in the ICU of our hospital, whose emergence was the result of acquisition of a ∼170 Kb virulence plasmid pLVPK carrying *rmpA2*, *iroBCD*, and *iucABCD* genes by classic ST11 CRKP strains (Gu et al., 2018). A ∼210 Kb pLVPK-like virulence plasmid carrying *rmpA2*, *iroBCD*, and *iucABCD* genes was identified in two ST11 KPC-producing CRKP isolates in the present study, which expressed lower virulence compared to that of the ST11 CR-HvKP previously described but higher virulence than that of the CRKP isolates without virulence plasmids. Notably, a comparison of the two homologous isolates ZHW170 and ZHW183 revealed that ZWH183 carried an additional virulence plasmid (∼105 Kb, co-carrying the *rmpA2* and *iucABCD* genes), which may be a glimpse of dynamic exchange of mobile elements in the gastrointestinal tract. Moreover, as was previously reported, CR-HvKP was found to account for only 3% of infections caused by the ST11 CRKP strains across China (Gu et al., 2018). Consistently, most of CRKP isolates in this study are identified with low virulence. *K. pneumoniae* has a wider ecological distribution, significantly more varied DNA composition, greater antimicrobial resistance gene diversity and a higher plasmid load than other Gram negative opportunists, which means more opportunity to survive within and transfer between multiple environmental and animal-associated hosts; to capture plasmids from environmental microbial communities; to maintain antimicrobial resistance gene-carrying plasmids for long periods; and to transfer plasmids to other clinically important Gram negative bacteria (Wyres and Holt, 2018). Our finding showed that ST11 *rmpA2*-positive CRKP strains harbored three plasmids, of which two carried antimicrobial resistance genes and the third one was a virulence plasmid; *rmpA2*-negative CRKP strains only had two multidrug resistance encoding plasmids without virulence plasmid. It is suggested that the human gastrointestinal tract has a great capacity to colonize and enrich multiple clones of CRKP strains with varied plasmids as well as diverse antimicrobial resistance genes and virulence genes, which presumably due to frequent episodes of antibiotic treatment.

Colonization is believed as an important step in progression to extraintestinal infection which provides the rationale for establishing intervention measures to prevent subsequent infection by identifying the colonized patients. It is urgent matter to take the surveillance of rectal CRE carriage into routine test after admission.

AUTHOR CONTRIBUTIONS

QS did strain characterization and participated in manuscript writing. DG did the whole-genome sequencing and participated in manuscript writing. QW, YH, and JH participated in collecting the clinical data and strain characterization. LS did the *Galleria mellonella* infection experiments. RZ participated in the research design, data interpretation, and manuscript writing. G-XC designed and supervised the study, interpreted the data and wrote the manuscript.

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Antimicrobial Activity of *Lactobacillus* Species Against Carbapenem-Resistant *Enterobacteriaceae*

Chi-Chung Chen[1,2], Chih-Cheng Lai[3], Hui-Ling Huang[4], Wen-Yu Huang[4], Han-Siong Toh[5], Tzu-Chieh Weng[5], Yin-Ching Chuang[1,6], Ying-Chen Lu[2] and Hung-Jen Tang[4,6]**

[1] Department of Medical Research, Chi Mei Medical Center, Tainan, Taiwan, [2] Department of Food Science, National Chiayi University, Chiayi, Taiwan, [3] Department of Intensive Care Medicine, Chi Mei Hospital, Tainan, Taiwan, [4] Department of Health and Nutrition, Chia Nan University of Pharmacy and Science, Tainan, Taiwan, [5] Department of Intensive Care Medicine, Chi Mei Medical Center, Tainan, Taiwan, [6] Department of Medicine, Chi Mei Medical Center, Tainan, Taiwan

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Benjamin Andrew Evans,
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University of Modena and Reggio Emilia, Italy

***Correspondence:**
Ying-Chen Lu
biolyc2016@gmail.com
Hung-Jen Tang
8409d1@gmail.com

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Objective: This study aims to identify suitable lactobacilli that have anti-carbapenem-resistant *Enterobacteriaceae* (CRE) activity with *in vitro* tolerance to pepsin and bile salts.

Methods: Fifty-seven *Lactobacillus* spp. strains encompassing nine species were collected for investigation. Their viabilities in the presence of pepsin and bile salts were tested using tolerance tests. Their anti-CRE effects were assessed by agar well diffusion and broth microdilution assay, as well as time-kill test.

Results: Of the 57 *Lactobacillus* isolates collected, 31 had a less than 2-log reduction in their viability in both pepsin and bile salt tolerance tests. Of these 31 isolates, 5 (LUC0180, LUC0219, LYC0289, LYC0413, and LYC1031) displayed the greatest anti-CRE activity with a CRE zone of inhibition greater than 15 mm in agar well diffusion assays. The minimal inhibitory percentages of supernatants from these five strains against CREs ranged from 10 to 30%. With the exception of LUC0180, which had a minimal bactericidal percentage $\geq$ 40%, the bactericidal percentage of all the strains ranged from 20 to 40%. The inhibitory effect of the cell-free culture supernatants from these *Lactobacillus* strains did not change after heating but was abolished as the pH changed to 7.0. After a 24-h incubation, five of the *Lactobacillus* strains at a concentration of 10^8 CFU/ml totally inhibited the growth of carbapenem-resistant *Escherichia coli* (CRE316) and *Klebsiella pneumoniae* (CRE632). After a 48-h incubation, the growth of CRE316 was completely inhibited under each concentration of lactobacilli based on time-kill test. Furthermore, when the concentration of lactobacilli was at 10^8 CFU/ml, the decline in pH was faster than at other concentrations.

Conclusion: Some *Lactobacillus* strains exhibit anti-CRE activity, which suggests potential applications for controlling or preventing CRE colonization or infection.

Keywords: *Lactobacillus*, carbapenem-resistant, *Enterobacteriaceae*, *in vitro* – antibacterial, activity

INTRODUCTION

Although *Enterobacteriaceae* are normal flora of the human intestinal system, they are also common pathogens causing human infections in the setting of both community-acquired and healthcare-associated infections (Hsueh et al., 2010; Toh et al., 2012; Lai et al., 2014; Jean et al., 2016). In this era of widespread antibiotic resistance, *Enterobacteriaceae* are no exception. Recently, the emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) has become a more critical issue due to the limited therapeutic options available for these pathogens and the significant morbidity and mortality associated with CRE infections (Tang et al., 2016a; Rodriguez-Bano et al., 2018). Therefore, there is an urgent need for new treatments for these critical CRE-associated conditions.

Lactobacillus is one of a number of probiotics considered to be biological therapeutics and host immune-modulating biologicals that are generally recognized as safe (GRAS). Recent studies demonstrated several antimicrobial mechanisms of *Lactobacillus* such as nutrient competition, production of inhibitory compounds, immune-stimulation and competition for binding sites. In addition, *Lactobacillus* can produce lactic acid, acetic acid, formic acid and other acids to reduce intestinal pH, which may be the most important mechanism. These bacteria can also secrete certain antimicrobial molecules, such as ethanol, fatty acid, hydrogen peroxide and bacteriocins to exert the antimicrobial activity (Georgieva et al., 2015; Inglin et al., 2015). Through these mechanisms, *Lactobacillus* has demonstrated its ability to inhibit several bacterial pathogens, including *Clostridium difficile* (McFarland, 2015), *Escherichia coli* (Kumar et al., 2016), *Shigella* spp. (Mirnejad et al., 2013), *Streptococcus mutans* (Ahn et al., 2018), *Pseudomonas aeruginosa* (Jamalifar et al., 2011), and *Staphylococcus aureus* (Kang et al., 2017). However, no previous studies have assessed the antimicrobial activity of *Lactobacillus* against CRE. Thus, we conducted this study to identify suitable lactobacilli that have anti-CRE activity with *in vitro* tolerance to pepsin and bile salts.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Fifty-seven *Lactobacillus* spp. strains encompassing nine species were obtained from Department of Food Science at the National Chiayi University in Chiayi, Taiwan. Species confirmation was performed by 16S rDNA sequencing. A fragment of the 16S rDNA was amplified by PCR. After amplification, the amplicons were separated by gel electrophoresis and sequenced. Sequences were compared with the NCBI GenBank database using the BLAST search tool to find the closest matches. The basic growth media for LAB were Man-Rogosa-Sharpe (MRS; Oxoid Inc., Ogdensburg, NY, United States).

Twenty clinical strains, including 10 different pulse field gel electrophoresis (PFGE) genotyped carbapenem-resistant *Escherichia coli* (CREC) and *Klebsiella pneumoniae* (CRKP) strains were isolated from Chi Mei Medical Center (Lai et al., 2016; Tang et al., 2016b). Species confirmations were performed using the VITEK 2 automated system (bioMérieux, Marcy l'Etoile, France) with standard biochemical methods. Mueller Hinton (MH) broth (Difco Laboratories, Detroit, MI, United States) were used for bacterial pathogens. The isolates were stored at −80°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, United Kingdom) before use.

Carbapenem susceptibility testing was performed using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute [CLSI], 2015). In brief, a 0.5 McFarland turbidity standard inoculum from overnight cultures was followed by incubation of Mueller-Hinton agar plates at 35°C. The antibiotic disks were placed on the agar surface. After 16–18 h of incubation at 35°C, results were interpreted as either sensitive, intermediate, or resistant according to the inhibitory zone diameters around the disks using CLSI breakpoints. Carbapenem resistance was defined as resistance to imipenem, meropenem, doripenem, or ertapenem.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) for the *Escherichia coli* and *K. pneumoniae* isolates were performed as described previously (Lai et al., 2016) with a CHEF DR II apparatus (Bio-Rad Laboratories, Hercules, CA, United States). Briefly, bacterial chromosomal DNAs were digested using XbaI (New England Biolabs, Beverly, MA, United States). Electrophoresis was carried out for 22 h at 14 μC, with pulse times ranging from 2 to 40 s at 6 V/cm, using a Bio-Rad CHEF MAPPER apparatus (Bio-Rad Laboratories, Richmond, CA, United States). A dendrogram based on the unweighted pair group was generated using methods previously described. The PFGE patterns were visually examined and interpreted according to the

TABLE 1 | Name and number of each species among the 57 total *Lactobacillus* isolates.

Lactobacillus brevis (*N* = 2)	LYC1152, LYC1113
Lactobacillus casei (*N* = 4)	LUC0095, LUC0123, LUC0197, LYC1229,
Lactobacillus fermentum (*N* = 6)	LUC0113, LUC0168, LUC0174, LUC0182, LUC0191, LYC1120
Lactobacillus furfuricola (*N* = 1)	LYC1039
Lactobacillus futsaii (*N* = 2)	LYC1037, LYC1038
Lactobacillus paracasei (*N* = 16)	LUC0018, LUC0040, LUC0044, LUC0048, LUC0097, LUC0180,
	LYC1119, LYC1142, LYC1149, LYC1151, LYC1154, LYC1156,
	LYC1162, LYC1164, LYC1235, LYC1237
Lactobacillus plantarum (*N* = 17)	LUC0125, LUC0128, LUC0219, LYC0289, LYC1031, LYC1088,
	LYC1112, LYC1115, LYC1117, LYC1138, LYC1141, LYC1143,
	LYC1144, LYC1146, LYC1159, LYC1303, LYC1322
Lactobacillus rhamnosus (*N* = 8)	LUC0103, LUC0103, LUC0115, LUC0127, LUC0192, LYC0413,
	LYC1065, LYC1118
Lactobacillus sakei (*N* = 1)	LYC1287

TABLE 2 | Distribution of different antimicrobial agent MICs (mg/l) for 57 *Lactobacillus* isolates.

	≤0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128
AMP	2	1		3	6	33	11	1					
CHL					1	5	46	5					
CLI	20	19	8	7	1	2							
ERY	33	22				1			1				
FA					2	2	1	1	7	13	2	15	14
GM				1	2	9	25	15	3	1	1		
KAN											14	26	17
LNZ					1	4	47	5					
STR						1	1	6	29	13	7		
TEC								1	1	3	2	5	45
SXT					2	4	1		1		1	10	38
VAN												1	56

(1) SXT, only the MIC of trimethoprim is shown. (2) AMP: ampicillin; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; FA, fusidic acid; GM, gentamicin; KAN, kanamycin; LNZ, linezolid; STR, streptomycin; TEC, teicoplanin; SXT, trimethoprim/sulfamethoxazole (1/19); VAN, vancomycin.

TABLE 3 | Pepsin tolerance test results for 57 *Lactobacillus* isolates at pH 2.5 with 3.5 mg/ml pepsin.

Lactobacillus strain	Cell viability (log CFU/ml)		Reduction in cell viability (log)
	pH 7.0	pH 2.5, 3.5 mg/ml pepsin	
LUC0018	7.04 ± 0.08	4.54 ± 0.09	2.50
LUC0040	7.14 ± 0.07	7.01 ± 0.08	0.13
LUC0044	6.81 ± 0.00	4.59 ± 0.16	2.22
LUC0048	6.93 ± 0.04	4.50 ± 0.28	2.43
LUC0095	6.89 ± 0.02	0.00 ± 0.00	6.89
LUC0097	7.08 ± 0.03	4.60 ± 0.08	2.48
LUC0103	6.54 ± 0.00	0.00 ± 0.00	6.54
LUC0113	6.15 ± 0.00	0.00 ± 0.00	6.15
LUC0114	7.11 ± 0.00	0.00 ± 0.00	7.11
LUC0115	6.90 ± 0.08	0.00 ± 0.00	6.90
LUC0123	6.50 ± 0.14	0.00 ± 0.00	6.50
LUC0125	6.83 ± 0.18	0.00 ± 0.00	6.83
LUC0127	6.69 ± 0.21	5.89 ± 0.06	0.80
LUC0128	6.89 ± 0.06	0.00 ± 0.00	6.89
LUC0168	6.83 ± 0.25	6.68 ± 0.19	0.15
LUC0174	6.92 ± 0.15	6.81 ± 0.16	0.11
LUC0180	7.05 ± 0.10	6.60 ± 0.08	0.45
LUC0182	7.08 ± 0.08	6.90 ± 0.04	0.18
LUC0191	6.86 ± 0.06	6.36 ± 0.26	0.50
LUC0192	6.63 ± 0.04	0.00 ± 0.00	6.63
LUC0197	6.80 ± 0.02	0.00 ± 0.00	6.80
LUC0219	6.48 ± 0.25	6.23 ± 0.05	0.25
LYC0289	6.39 ± 0.12	4.85 ± 0.00	1.54
LYC0413	6.48 ± 0.00	6.11 ± 0.02	0.37
LYC1031	6.72 ± 0.03	5.09 ± 0.12	1.63
LYC1037	6.50 ± 0.14	0.00 ± 0.00	6.50
LYC1038	6.44 ± 0.06	0.00 ± 0.00	6.44
LYC1039	6.63 ± 0.04	0.00 ± 0.00	6.63
LYC1065	7.10 ± 0.05	6.89 ± 0.06	0.21
LYC1088	6.65 ± 0.00	6.50 ± 0.14	0.15
LYC1112	6.81 ± 0.10	6.59 ± 0.16	0.22
LYC1113	7.24 ± 0.01	4.93 ± 0.04	2.31
LYC1115	6.85 ± 0.21	6.54 ± 0.09	0.31
LYC1117	6.57 ± 0.12	6.24 ± 0.09	0.33
LYC1118	6.93 ± 0.04	6.77 ± 0.10	0.16
LYC1119	6.83 ± 0.02	6.54 ± 0.09	0.29
LYC1120	7.11 ± 0.02	6.68 ± 0.03	0.43
LYC1138	7.04 ± 0.03	6.95 ± 0.00	0.09
LYC1141	6.99 ± 0.05	6.70 ± 0.00	0.29
LYC1142	6.85 ± 0.11	6.81 ± 0.10	0.04
LYC1143	7.09 ± 0.06	6.94 ± 0.05	0.15
LYC1144	6.98 ± 0.06	0.00 ± 0.00	6.98
LYC1146	7.00 ± 0.03	6.03 ± 0.11	0.97
LYC1149	6.90 ± 0.12	6.44 ± 0.06	0.46
LYC1151	6.78 ± 0.05	6.20 ± 0.03	0.58
LYC1152	7.03 ± 0.04	5.15 ± 0.00	1.88
LYC1154	7.19 ± 0.04	6.89 ± 0.06	0.30
LYC1156	6.90 ± 0.08	3.60 ± 0.08	3.30
LYC1159	6.96 ± 0.08	6.88 ± 0.10	0.08
LYC1162	7.22 ± 0.02	0.00 ± 0.00	7.22
LYC1164	7.18 ± 0.04	6.81 ± 0.05	0.37
LYC1229	7.02 ± 0.06	6.96 ± 0.05	0.06
LYC1235	6.95 ± 0.10	0.00 ± 0.00	6.95
LYC1237	6.97 ± 0.02	0.00 ± 0.00	6.97
LYC1287	6.85 ± 0.11	0.00 ± 0.00	6.85
LYC1303	6.83 ± 0.02	0.00 ± 0.00	6.83
LYC1322	7.07 ± 0.01	5.72 ± 0.03	1.35

criteria of Tenover et al. (1995). The similarities of the PFGE profiles of each strain were compared using a Dice coefficient at 1.0% of tolerance and 1.0% of optimization. Isolates that

TABLE 4 | Bile salt tolerance test results for 57 *Lactobacillus* isolates at pH 7.3 with 0.3% Oxgall.

Lactobacillus strain	Cell viability (log CFU/ml)		Reduction in cell viability (log)
	pH 7.3	pH 7.3, 0.3% Oxgall	
LUC0018	9.67 ± 0.10	8.62 ± 0.11	1.05
LUC0040	9.78 ± 0.18	8.60 ± 0.08	1.18
LUC0044	9.99 ± 0.02	8.48 ± 0.00	1.51
LUC0048	9.93 ± 0.07	8.79 ± 0.07	1.14
LUC0095	8.99 ± 0.08	8.08 ± 0.00	0.91
LUC0097	9.88 ± 0.10	8.67 ± 0.10	1.21
LUC0103	9.03 ± 0.14	8.41 ± 0.40	0.62
LUC0113	9.80 ± 0.02	8.97 ± 0.10	0.83
LUC0114	9.65 ± 0.00	9.06 ± 0.05	0.59
LUC0115	9.81 ± 0.00	9.00 ± 0.11	0.81
LUC0123	9.07 ± 0.20	8.41 ± 0.34	0.66
LUC0125	9.85 ± 0.00	8.38 ± 0.38	1.47
LUC0127	9.25 ± 0.07	8.44 ± 0.06	0.81
LUC0128	9.65 ± 0.00	8.65 ± 0.00	1.00
LUC0168	9.20 ± 0.14	8.83 ± 0.13	0.37
LUC0174	9.40 ± 0.00	9.14 ± 0.06	0.26
LUC0180	9.30 ± 0.00	8.70 ± 0.06	0.60
LUC0182	9.81 ± 0.16	9.13 ± 0.09	0.68
LUC0191	9.34 ± 0.45	8.88 ± 0.10	0.46
LUC0192	10.02 ± 0.00	8.85 ± 0.11	1.17
LUC0197	9.19 ± 0.16	8.30 ± 0.14	0.89
LUC0219	9.33 ± 0.20	8.99 ± 0.12	0.34
LYC0289	9.16 ± 0.02	8.11 ± 0.05	1.05
LYC0413	9.02 ± 0.00	8.59 ± 0.27	0.43
LYC1031	9.98 ± 0.00	8.88 ± 0.10	1.10
LYC1037	9.81 ± 0.00	9.11 ± 0.05	0.70
LYC1038	8.74 ± 0.06	6.99 ± 0.05	1.75
LYC1039	9.10 ± 0.06	8.55 ± 0.21	0.55
LYC1065	9.18 ± 0.00	8.87 ± 0.04	0.30
LYC1088	9.74 ± 0.00	9.09 ± 0.06	0.65
LYC1112	9.40 ± 0.00	8.88 ± 0.10	0.52
LYC1113	9.88 ± 0.00	9.36 ± 0.41	0.52
LYC1115	9.13 ± 0.01	8.13 ± 0.02	1.00
LYC1117	9.30 ± 0.25	8.41 ± 0.41	0.89
LYC1118	9.15 ± 0.21	8.94 ± 0.18	0.21
LYC1119	9.10 ± 0.17	8.44 ± 0.37	0.66
LYC1120	9.81 ± 0.00	8.88 ± 0.10	0.93
LYC1138	9.14 ± 0.06	8.37 ± 0.32	0.77
LYC1141	9.02 ± 0.20	8.68 ± 0.03	0.34
LYC1142	9.06 ± 0.12	8.26 ± 0.31	0.80
LYC1143	9.26 ± 0.19	8.76 ± 0.03	0.50
LYC1144	9.78 ± 0.00	9.06 ± 0.08	0.72
LYC1146	9.54 ± 0.00	8.47 ± 0.10	1.07
LYC1149	9.13 ± 0.00	8.08 ± 0.08	1.05
LYC1151	9.08 ± 0.09	8.65 ± 0.07	0.43
LYC1152	9.48 ± 0.00	9.02 ± 0.03	0.46
LYC1154	9.39 ± 0.30	8.78 ± 0.00	0.61
LYC1156	9.24 ± 0.23	8.36 ± 0.26	0.88
LYC1159	9.85 ± 0.00	8.04 ± 0.03	1.81
LYC1162	9.65 ± 0.00	8.48 ± 0.25	1.17
LYC1164	9.12 ± 0.06	7.02 ± 0.06	2.10
LYC1229	9.42 ± 0.17	8.45 ± 0.21	0.97
LYC1235	9.22 ± 0.24	8.71 ± 0.15	0.51
LYC1237	9.10 ± 0.02	8.94 ± 0.05	0.16
LYC1287	9.23 ± 0.05	8.05 ± 0.11	1.18
LYC1303	9.78 ± 0.00	8.74 ± 0.00	1.04
LYC1322	9.54 ± 0.00	8.12 ± 0.03	1.42

had < 80% similarity on the PFGE profiles were considered different types (**Figure 1**).

Antimicrobial Susceptibility Test

The procedure for broth microdilution was adapted from the CLSI protocol for antimicrobial susceptibility testing (Clinical and Laboratory Standards Institute [CLSI], 2009). Antibiotic solutions were prepared in LAB susceptibility media (90% LSM and 10% MRS broth, adjusted to pH 6.7) (Klare et al., 2005). The following antibiotics were tested: ampicillin, chloramphenicol, clindamycin, erythromycin, fusidic acid, gentamycin, kanamycin, linezolid, streptomycin, teicoplanin, trimethoprim/sulfamethoxazole (1/19), and vancomycin (Sigma-Aldrich, St. Louis, MO, United States). The antibiotic susceptibility tests and their interpretations were carried out according to the CLSI guidelines (British Society for Antimicrobial Chemotherapy [BSAC], 2014; Clinical and Laboratory Standards Institute [CLSI], 2016).

Pepsin Tolerance

A pepsin solution was prepared by suspending 3 mg/ml pepsin (Sigma-Aldrich, St. Louis, MO, United States) in a 0.85% sterile saline solution adjusted to pH 2.5. To test bacterial viability in the presence of pepsin, the 3 mg/ml pepsin solution, or saline at a pH of 7.0 as control, was inoculated with 10^6 CFU/ml of *Lactobacillus* spp. and then incubated at 37°C for 4 h. The viable cell population was determined using the spread plate method. Each experiment was conducted in triplicate (Tokatli et al., 2015).

Bile Salt Tolerance

The bile salt tolerance assay was performed as previously described (Jacobsen et al., 1999). In brief, each strain was adjusted to 1 × 106 CFU/ml in 0.3% Oxgall (Sigma-Aldrich, St. Louis, MO, United States) in PBS pH 7.3, or in PBS pH 7.3 alone as a control. Survival was tested at 37°C after 24 h by the spread plate method. Each experiment was conducted in triplicate.

Cell-Free Supernatant (CFS) Preparation

Lactobacilli cell-free supernatants were cultured in MRS broth at 37°C for 24 h. The cultures were then centrifuged at 4000 rpm

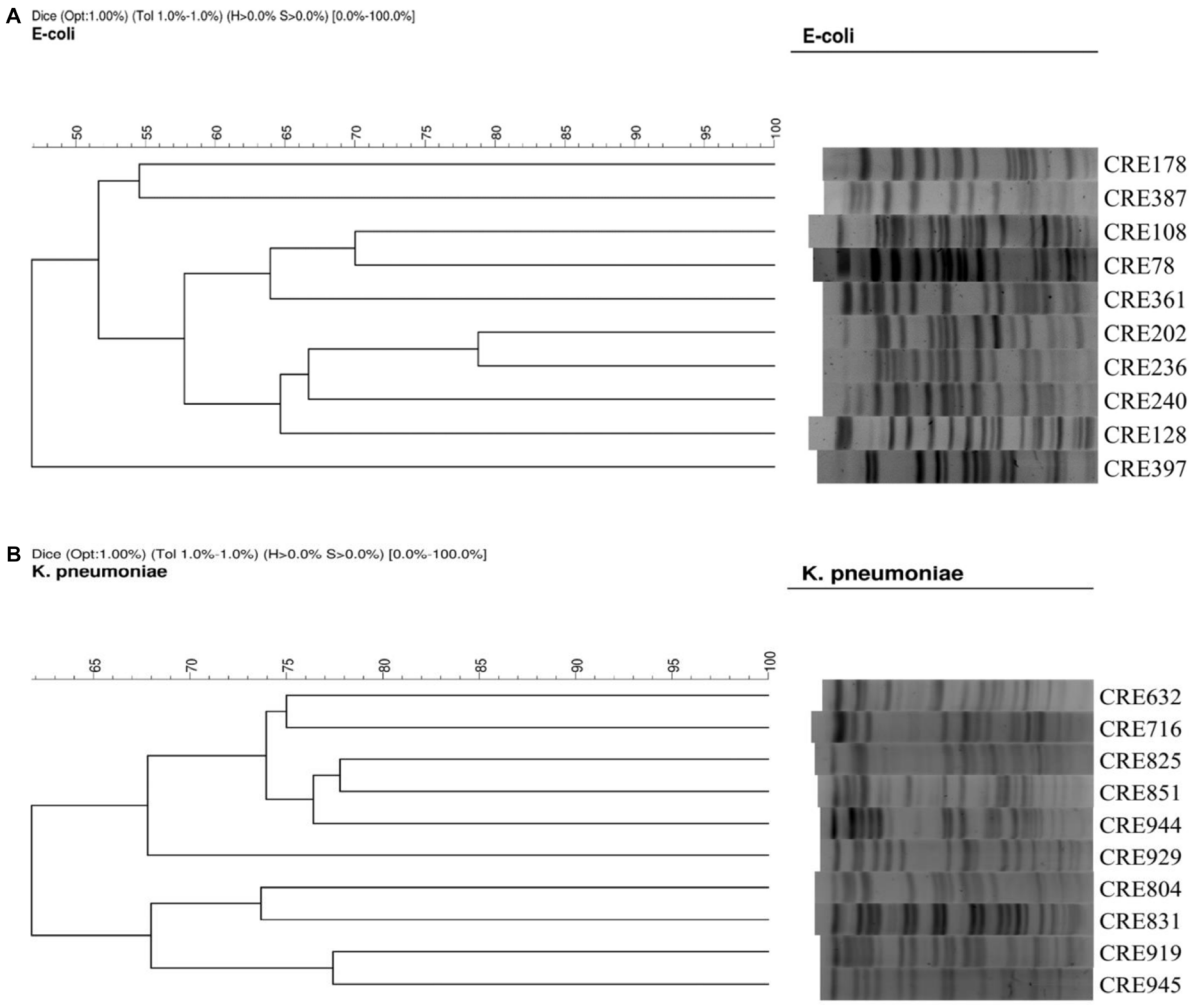

FIGURE 1 | (A) PFGE of 10 isolates of carbapenem-resistant *E. coli.* **(B)** PFGE of 10 isolates of carbapenem-resistant *K. pneumoniae.*

at 4°C for 30 min. The supernatants were sterilized by filtration through a 0.22 μm filter (Millipore, Billerica, MA, United States).

Well Diffusion Assay

The agar well diffusion method (Tagg and McGiven, 1971) was modified to detect antimicrobial activities of supernatants isolated from *Lactobacillus* strains. First, MH agar plates were swabbed on the surface with CRE bacterial cultures. Then, 6 mm diameter wells were prepared and cell-free supernatants from isolated lactobacilli were loaded in the wells (100 μl/well). Following a 24-h incubation at 37°C, inhibition zones were recorded.

Broth Microdilution Assay

Broth microdilution assay was carried out as previously described (Arena et al., 2016) with some modifications. Overnight cultures of pathogenic bacteria were inoculated into fresh MHB media and seeded into 96-well plates (BD Discovery Labware, Bedford, MA, United States). The CFS of each *Lactobacillus* culture was separated into three aliquots: The first aliquot received no treatment, the second aliquot was heated at 80°C for 10 min, and the third aliquot was neutralized to pH 7.0 with 1N NaOH. A 200 μl volume of test solution, consisting of 100 μl of the pathogenic bacterial culture (final inoculum was approximately 10^6 CFU/ml) and 100 μl of one of the CFS aliquots, was mixed into the wells. The CFSs were diluted with MRS broth and used at different percentages (i.e., 10, 20, 30, 40, 50%) in the final 200 μl volume. The minimum inhibitory percentage (MIP), defined as the lowest percentage of supernatant that can inhibit the growth of pathogen, was monitored by measuring optical density (OD600 nm). The minimum bactericidal percentage (MBP) was defined as the lowest percentage of CFS that can kill all the pathogenic bacteria, as detected by subculturing treated samples onto MH agar. All tests were done in triplication.

Time-Kill Test in Co-cultures

Carbapenem-resistant *Enterobacteriaceae* and *Lactobacillus* strains were individually cultured in their respective broth

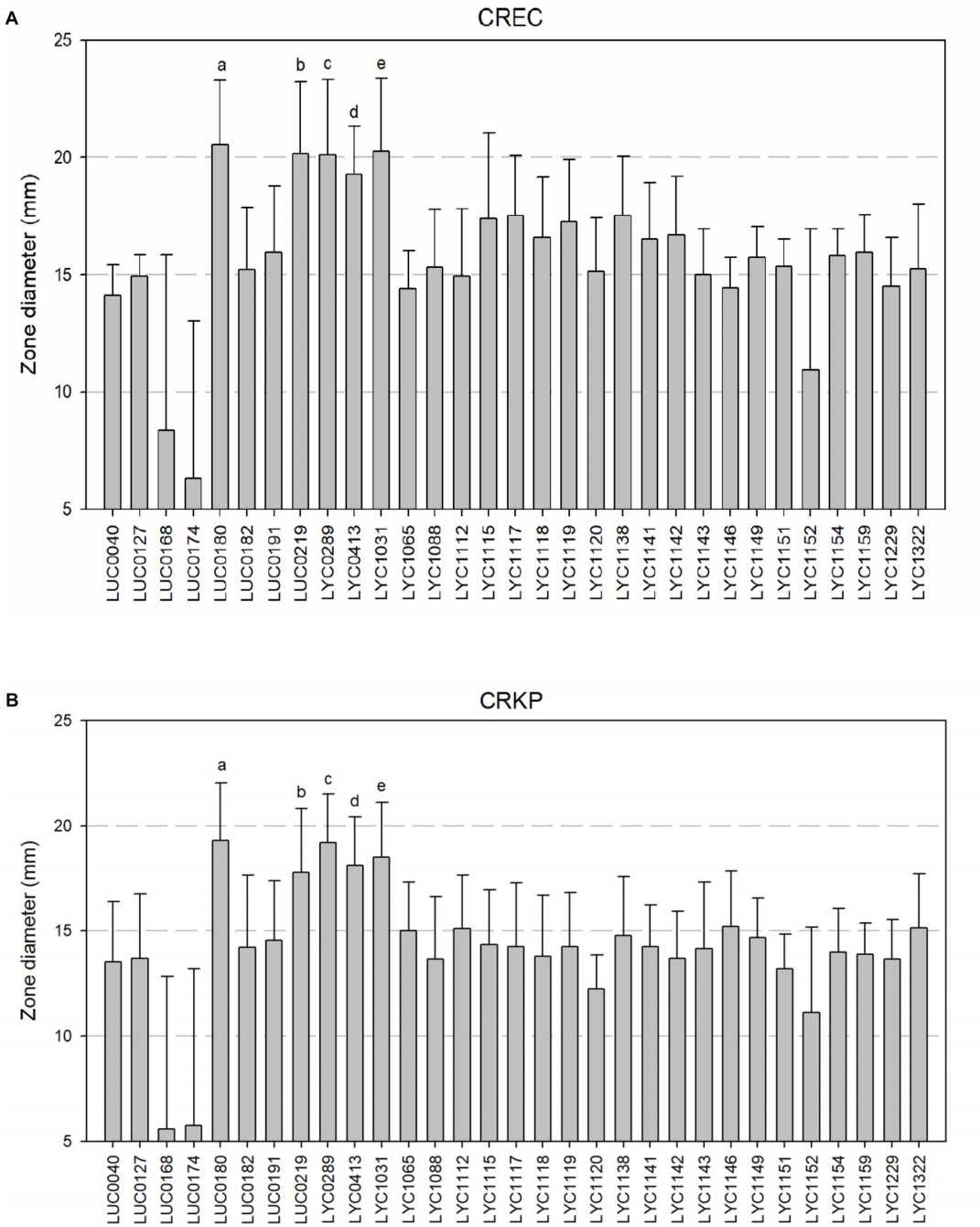

FIGURE 2 | (A) Zone of inhibition of 31 *Lactobacillus* isolates against carbapenem-resistant *E. coli* by well diffusion assays. (a, b, c, d, e five isolates showed the significantly better activity than all of the other strains (all $p < 0.05$). However, there was no significant differences among them). **(B)** Zone of inhibition of 31 *Lactobacillus* isolates against carbapenem-resistant *K. pneumoniae* by well diffusion assays. [a, b, c, d, e five isolates showed the significantly better activity than all of the other strains (all $p < 0.05$). However, there was no significant differences among them].

medium at 37°C for 24 h. The cultures were centrifuged at 6000 rpm, 22°C for 10 min to collect the cell pellet. Then, pathogenic bacteria were inoculated at 1×10^6 CFU/ml and lactobacilli at 1×10^5, 1×10^6, 1×10^7, or 1×10^8 CFU/ml into mono-cultures or 1×10^6 CFU/ml pathogenic bacteria co-culture with 1×10^5, 1×10^6, 1×10^7, or 1×10^8 CFU/ml lactobacilli in tubes containing 10 ml of MRS-MH broth (1:1) (Drago et al., 1997). Mono-cultures and co-cultures were incubated at 37°C for 48 h. Samples were collected at 0, 2, 4, 8, 24, and 48 h for the determination of viable cell count and pH measurements. A 1 ml aliquot of each sample was used to prepare serial dilutions that were poured onto the appropriate

TABLE 5 | The resulting pH and MIPs/MBPs of five *Lactobacillus* strain supernatants (%) against carbapenem-resistant *E. coli* (CREC) and *K. pneumoniae* (CRKP).

CREC	pH	CRE78	CRE108	CRE128	CRE178	CRE202	CRE236	CRE240	CRE361	CRE387	CRE397
LUC0180	4.01	20/40	20/40	20/40	20/40	20/40	20/30	20/40	20/40	30/>50	30/>50
LUC0219	3.86	10/30	10/40	10/40	10/40	10/30	10/30	10/40	10/40	20/40	20/40
LYC0289	3.76	10/30	10/40	10/30	10/40	10/30	10/30	10/30	10/30	10/30	10/40
LYC0413	3.82	10/20	10/30	10/20	10/30	10/20	10/20	10/30	10/30	10/30	10/30
LYC1031	3.84	10/30	10/40	10/30	10/40	10/30	10/30	10/30	10/30	10/30	10/30
CRKP	**pH**	**CRE632**	**CRE716**	**CRE804**	**CRE825**	**CRE831**	**CRE851**	**CRE919**	**CRE929**	**CRE944**	**CRE945**
LUC0180	4.01	20/40	20/40	20/30	20/30	20/30	20/30	20/30	20/40	20/30	20/30
LUC0219	3.86	10/40	10/30	10/30	10/30	10/30	10/30	10/20	10/30	10/20	10/30
LYC0289	3.76	10/30	10/30	10/30	10/20	10/20	10/30	10/20	10/30	10/20	10/20
LYC0413	3.82	10/20	20/20	10/20	20/20	10/20	20/20	10/20	20/20	10/20	20/30
LYC1031	3.84	10/40	10/30	10/30	10/20	10/30	10/30	10/20	10/30	10/20	10/20

agar plates; MRS agar was used for *Lactobacillus* spp., while MacConkey Agar was used for *Enterobacteriaceae*. Plates were incubated at 37°C for 24 h and colonies were counted. The assay detection limit was 100 CFU/ml (Shah et al., 2016). All tests were done in triplication.

Statistical Analysis

The paired *t*-test was used for statistical analysis. The level of significance for all analysis was $p < 0.05$.

RESULTS

Microbiological Characteristics of *Lactobacillus* Isolates

A total of 57 *Lactobacillus* isolates, including *L. plantarum* ($n = 17$), *L. paracasei* ($n = 16$), *L. rhamnosus* ($n = 8$), *L. fermentum* ($n = 6$), *L. casei* ($n = 4$), *L. brevis* ($n = 2$), *L. futsaii* ($n = 2$), *L. furfuricola* ($n = 1$), and *L. sakei* ($n = 1$) were collected for this study (**Table 1**). Based on the findings from MIC testing of these *Lactobacillus* isolates, we determined that these isolates were highly susceptible to chloramphenicol, clindamycin, erythromycin, linezolid and streptomycin with susceptibility rates ranging from 91.2 to 100%. The resistance rates of these isolates against fusidic acid, kanamycin, trimethoprim/sulfamethoxazole, teicoplanin, and vancomycin ranged from 89.5 to 100% (**Table 2**).

In vitro Viability of *Lactobacillus* Isolates

Table 3 displays the results of the pepsin tolerance tests. The viability of 32 of the isolates was reduced by <2 log. Another 19 isolates were totally inhibited in these simulated conditions. **Table 4** shows the results of the bile salt tolerance tests. With the exception of LYC1164, which had a 2.1-log reduction in growth, all of the isolates had a less than a 2-log reduction in cell viability after incubation in this simulated intestinal condition for 24 h. Based on the findings of these *in vitro* viability tests, we chose 31 *Lactobacillus* isolates with less than 2-log reductions in viability in both pepsin and bile salt tolerance tests. Because of their ability to survive in these simulated gastric and intestinal environments, we selected these strains for the assessment their antibacterial activity.

The Results of the Agar Well Diffusion Method

The 21 isolates displayed the greatest activity against carbapenem-resistant *E. coli* with zones of inhibition greater than 15 mm (**Figure 2A**). The greatest activity against carbapenem-resistant *K. pneumoniae* isolates, with zones of inhibition greater than or equal to 15 mm, were observed in the nine isolates (**Figure 2B**). Overall, we chose five *Lactobacillus* strains (LUC0180, LUC0219, LYC0289, LYC0413, and LYC1031), which had the significantly better antibacterial activities than the most of the other strains ($p < 0.05$) as determined by the agar well diffusion and time-kill assay for further tests of antibacterial activity.

The Results of MIP/MBP Tests

Table 5 shows the MIP and MBP for the five most potent *Lactobacillus* supernatants against CRE. The MIP of these five strains ranged from 10 to 30%. Except for LUC0180, which had an MBP of ≥40%, all of the strains had MBPs ranging from 20 – 40%. The inhibitory effects of these *Lactobacillus* strains did not change after heating up to 80°C for 10 min. However, the inhibitory effect disappeared once the pH increased to 7.0 (data not show). Additionally, the pH in the presence of LUC0180 seemed to be higher than the other four isolates.

The Results of Time-Kill Test

Figure 3 shows the results from time-killing test and assessment of the association between pH and antibacterial effects. After a 24-h incubation, there was no significant change between mono-cultures of lactobacilli and co-culture of lactobacilli and carbapenem-resistant *E. coli* (CRE316) in term of the concentration of lactobacilli (all $p > 0.05$) (**Figure 3A**). In contrast, the growth of CRE 316 was significantly inhibited after co-culture with different concentration lactobacilli after 24 h (all $p < 0.05$) and even 48 h ($p < 0.0001$) when comparing with mono-culture of CRE (**Figure 3B**). Furthermore, the decreases in pH were observed in the mono-cultures of lactobacilli,

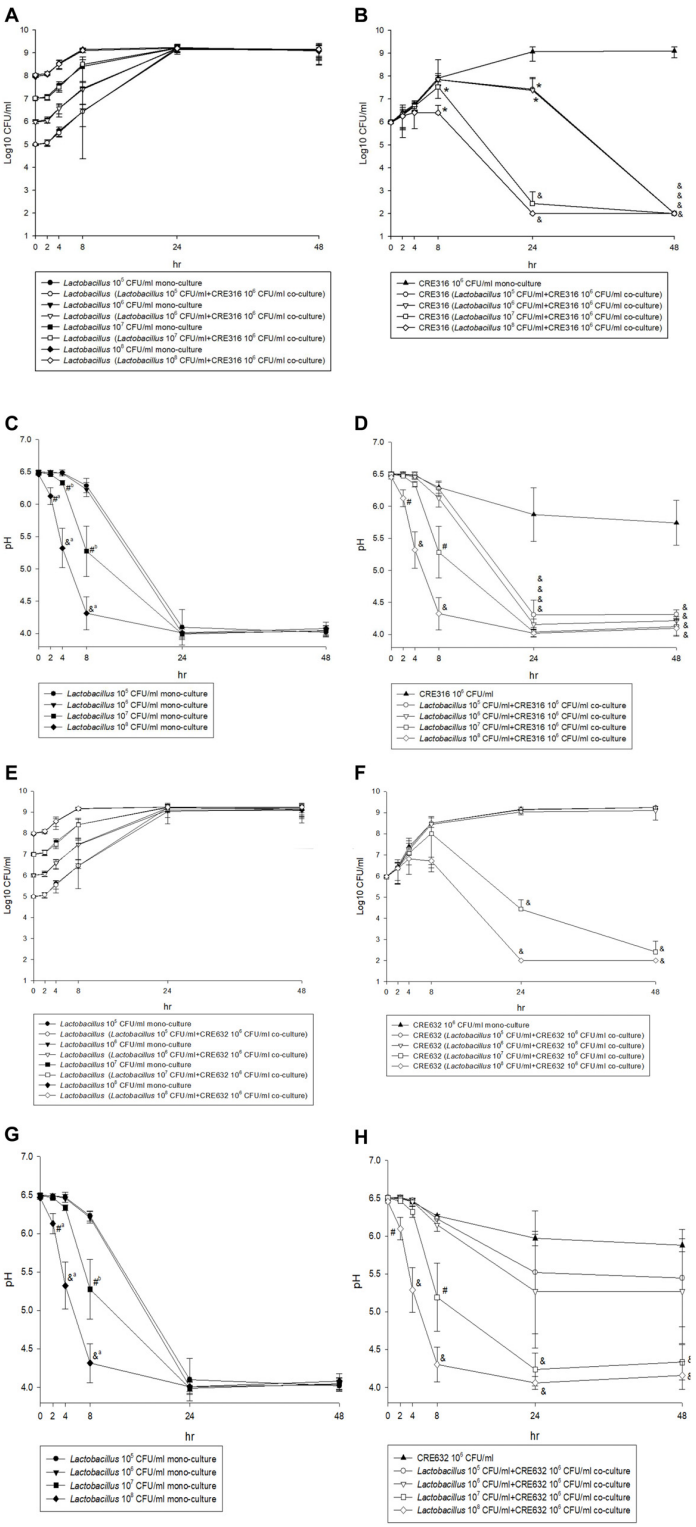

FIGURE 3 | (A,E) The CFU change of five different concentration (10^5 to 10^8 CFU/ml) *Lactobacillus* isolates after mono-culture or co-culture with CRE316/ 632 for 48 h. **(B,F)** The CFU change of CRE316/ 632 after mono-culture or co-culture with five different concentration (10^5 to 10^8 CFU/ml) *Lactobacillus* isolates for 48 h and comparison between mono-culture and co-culture. **(C,G)** The pH changes of five different concentration (10^5 to 10^8 CFU/ml) *Lactobacillus* isolates mono-culture for 48 h. **(D,H)** The pH changes of CRE316/ 632 after mono-culture or co-culture with five different concentration (10^5 to 10^8 CFU/ml) *Lactobacillus* isolates and comparison between mono-culture and co-culture. ([a] Means the comparisons between 10^8 and $10^{5,6,7}$ CFU/ml lactobacillus isolates. [b] Means the comparisons between 10^7 and $10^{5,6}$ CFU/ml *Lactobacillus* isolates. $*P$-value < 0.05, #P-value < 0.001, &P-value < 0.0001).

especially at the concentration of 10^8 and 10^7 CFU/ml (both $p < 0.05$) (**Figure 3C**). The decrease in pH remains significant while in the co-cultures with CRE316 compared with CRE316 monoculture (all $p < 0.05$, **Figure 3D**). The similar findings were also noted for the co-culture with carbapenem-resistant *K. pneumonia* (CR632) (**Figures 3E–H**).

DISCUSSION

In this study, five *Lactobacillus* strains, including three *L. plantarum* strains (LUC0219, LYC0289, LYC1031) and one each of *L. paracaseri* (LUC0180) and *L. rhamnosus* (LYC0413), exhibited good antibacterial activity against CREs. These inhibitory effects were demonstrated in through various tests including the agar well diffusion method, broth microdilution assay, and time-kill test. *Lactobacillus* spp. are non-pathogenic Gram-positive rods that are also recognized as normal human flora. Recently, several studies have reported that *Lactobacillus* strains can exhibit antibacterial activity through several mechanisms such as the production of antimicrobial substances or metabolites (hydrogen peroxide, lactic acid, bacteriocin), competition for nutrients, inhibition of bacterial adhesion to the mucosa, and enhancement of the immune response (Gill et al., 2001; Reid and Burton, 2002; Servin, 2004; Saulnier et al., 2009). Some *in vitro* studies have also shown that *Lactobacillus* strains can exhibit antimicrobial activity against *C. difficile*, *E. coli*, *Shigella* spp., *S. mutans, P. aeruginosa,* and *S. aureus* (Jamalifar et al., 2011; Mirnejad et al., 2013; Kumar et al., 2016; Kang et al., 2017; Ahn et al., 2018). To the best of our knowledge, this is the first study to document evidence of *Lactobacillus* antimicrobial activity against CRE. Although *in vitro* activity cannot be directly translated to *in vivo* effect, our findings suggest a promising role for *Lactobacillus* strains in the prevention and treatment of CRE colonization or infection. However, further animal studies are warranted to clarify this issue.

In the time-kill studies, the co-cultures with CREs did not influence the growth of lactobacilli. Additionally, effective inhibitory activity was generally observed at a pH of less than 4.2. These results were comparable with what we observed in our MIP/MBP experiments. By contrast, the antibacterial activity of *Lactobacillus* strains was dependent on an acidic environment and the inhibitory effect disappeared once the pH became greater than 6.5 during the MIP/MBP tests. Importantly, antimicrobial activity was not influenced by heating. This indicates that the inhibitory effect is mostly due to the acidic conditions, and not the production of bacteriocin-like substances. Similar findings were noted in the time-kill test. Previous reports have shown that the production of organic acids by probiotic organisms and the resulting decrease in culture pH is considered to be the principal antimicrobial effect (Zhang et al., 2011; Tejero-Sarinena et al., 2012). In addition to lowering the culture pH, Alakomi et al. (2000) reported that organic acids could also function as outer membrane permeabilizers of some gram-negative bacteria and enhance the activity of other antimicrobial metabolites. These findings suggest that acidic pH and/or the presence of organic acids may be essential for the antibacterial activity observed in the present study.

In this study, only 31 of the 57 *Lactobacillus* strains that were initially collected for assessment were found to be able to survive in the simulated gastric and intestinal environment. For clinical application, these characteristics are extremely important. To be functional in the lower enteric tract, the tolerance of lactobacilli to pepsin, low pH and bile salts is essential.

This study had several limitations. First, in addition to the effect of acid environment, we did not investigate the detail mechanism or molecular effectors of these function traits. Second, we used separated tests instead of a unique trait by using *in vitro* GIT system to evaluate the GIT resistance in this study. Further study is warranted to clarify these issues.

CONCLUSION

Several *Lactobacillus* strains exhibit antibacterial activity against CRE. We suggest that this effect may have potential applications through the use of *Lactobacillus* strains as starter cultures in fermented foods or as food preservatives for controlling or preventing CRE infections. Additional studies are required to determine the effects of complex nutrients on the synthesis of the antibacterial substance, as well as to elucidate the mechanisms and genetic basis of the bactericidal activity. Further animal models are also necessary to document the *in vivo* survival of these acid, pepsin and bile salt tolerant lactobacilli in the lower enteric tract. If the same inhibitory effect can be documented *in vivo*, further studies including vancomycin-resistant *Enterococcus* should be performed in the future.

AUTHOR CONTRIBUTIONS

H-JT and Y-CL are the guarantors of this manuscript. C-CC, C-CL, H-LH, W-YH, H-ST, T-CW, and Y-CC contributed to the conception and design of the study. C-CC and H-JT analyzed and interpreted the data. C-CC, C-CL, and H-JT drafted the manuscript. All authors reviewed the manuscript.

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High Prevalence of Metallo-β-Lactamase-Producing *Enterobacter cloacae* From Three Tertiary Hospitals in China

Yimei Cai[1†], Cha Chen[2,3†], Mei Zhao[4†], Xuegao Yu[1†], Kai Lan[2,3], Kang Liao[1], Penghao Guo[1], Weizheng Zhang[2,3], Xingyan Ma[1], Yuting He[1], Jianming Zeng[2,3], Liang Chen[5], Wei Jia[4], Yi-Wei Tang[6,7*] and Bin Huang[1*]*

[1] Department of Laboratory Medicine, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, [2] Department of Laboratory Medicine, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China, [3] Department of Laboratory Medicine, Guangdong Provincial Hospital of Chinese Medicine, Guangzhou, China, [4] Department of Laboratory Medicine, Ningxia Hospital of Ningxia Medical University, Yinchuan, China, [5] Public Health Research Institute Tuberculosis Center, New Jersey Medical School, Rutgers University, Newark, NJ, United States, [6] Department of Laboratory Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, United States, [7] Department of Pathology and Laboratory Medicine, Weill Medical College of Cornell University, New York, NY, United States

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***Correspondence:**
Wei Jia
13519299090@126.com
Yi-Wei Tang
tangy@mskcc.org
Bin Huang
huangb3@mail.sysu.edu.cn

[†]*These authors have contributed equally to this work*

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Enterobacter cloacae has recently emerged as one of the most common carbapenem-resistant *Enterobacteriaceae*. The emergence and spread of metallo-β-lactamase-producing *E. cloacae* have posed an immediate threat globally. Here, we investigated the molecular characteristics of 84 carbapenem-resistant *Enterobacter cloacae* (CREL) collected from three tertiary hospitals in China between 2012 and 2016. Species identification and antimicrobial susceptibility testing were performed using a VITEK-2 system. Carbapenems, polymyxins B, and tigecycline were tested by broth microdilution method. The carbapenem in activation method (CIM) and cefoxitin three-dimensional test were used to detect carbapenemase and AmpC β-lactamase, respectively. Isolates were screened for β-lactam resistance genes by PCR, and expression of *ompC* and *ompF* was determined by qRT-PCR. Genetic relatedness was performed by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), while selected isolates were subjected to whole-genome sequencing. Among the 84 CREL isolates, 50 (59.5%) were detected as carbapenemase producers. NDM-1 was the dominant carbapenemase (80.0%), followed by IMP-26 (8.0%) and IMP-4 (6.0%). Notably, we identified the first NDM-1 and IMP-1 co-producing *E. cloacae*, carrying plasmids of several incompatibility (Inc) groups, including IncHI2, IncHI2A, and IncN. Most isolates showed decreased expression of *ompC* and/or *ompF*, and contained a broad distribution of ESBLs and AmpC β-lactamases. These findings suggested that different molecular mechanisms, including carbapenemase, ESBL and/or AmpC plus loss of porins, have contributed to carbapenem resistance. The bla_{NDM-1}-harboring plasmids contained highly conserved gene environment around bla_{NDM-1} (bla_{NDM-1}-ble_{MBL}-*trpF-dsbD-cutA1-groES-groEL*), which could be associated with the potential dissemination of bla_{NDM-1}. IMP-type MBL was located within a variety of integrons and usually contained various gene cassettes encoding multidrug resistance. These isolates produced 54 different pulsotypes, and were

classified into 42 STs by MLST. Nineteen bla_{NDM-1}-positive *E. cloacae* isolates obtained from Ningxia had the same pulsotype (PFGE type 1), belonging to ST78 within clonal complex 74 (CC74). The plasmid-based replicon typing indicated that IncX3 plasmids mediated the dissemination of bla_{NDM-1} among these homologous strains. This is the first report on the outbreak of NDM-1-producing *E. cloacae* ST78 with contribution of IncX3 plasmids in Northwestern China. There's an immediate need to intensify surveillance attentively to prevent and control the further spread of NDM-1 in China.

Keywords: carbapenem-resistant, *Enterobacter cloacae*, outbreak investigation, NDM-1, IncX3 plasmids, ST78

INTRODUCTION

Enterobacter cloacae, an opportunistic pathogen ranking the third among all *Enterobacteriaceae* in healthcare-associated infections, may cause various nosocomial infections involving urinary tract, lower respiratory tract, skin and soft tissues, biliary tract, intravenous catheters, and central nervous system (Davin-Regli and Pagès, 2015). *E. cloacae* is intrinsically resistant to ampicillin, amoxicillin/clavulanic, cephamycin, and the 1st and 2nd generation cephalosporins owing to chromosomally encoded AmpC β-lactamase (Jacoby, 2009). Recently, severe comorbid conditions, extensive invasive procedures, and heavy exposure to antibiotics have involved in the global emergence of carbapenem-resistant *E. cloacae* (CREL). Carbapenems have increasingly been used as a common drug for the treatment of nosocomial infections. According to CHINET, one of the largest antimicrobial resistance surveillance networks in China, carbapenem resistance rates among *E. cloacae* were <1.0% in 2007. Whereas, in the year of 2017, the resistance rates to imipenem, meropenem, and ertapenem have rapidly increased to 6.9, 7.0, and 8.2%, respectively. Infections caused by CREL usually result in higher mortality rates, longer hospitalization and higher costs of treatment, which has posed an immediate threat to public health (Kelly et al., 2017).

Resistance to carbapenems is associated with several mechanisms. Carbapenemase, which is largely responsible for carbapenem resistance in *Enterobacteriaceae*, has been classified into three functional groups: class A (mostly KPC, GES), class B metallo-β-lactamase (MBL, mostly IMP, VIM and NDM) as well as class D OXA-type β-lactamase (mostly OXA-48-like) (Tzouvelekis et al., 2012). Genes encoding MBLs are most commonly identified in *E. cloacae* and often carried on mobile genetic elements such as plasmids and transposons. Integrons located on such mobile elements play a crucial role in the horizontal transfer of MBL genes between bacteria (Villa et al., 2014; Lee et al., 2017a). In China, carbapenemase production is largely attributed to MBLs such as VIM-1 (Yang et al., 2014), IMP-4 (Wang et al., 2017), IMP-8 (Yan et al., 2002), and NDM-1 (Dai et al., 2013), among which NDM-1 was even more worrisome for its sharp increase. Since the first case of carbapenem-resistant *E. cloacae* harboring NDM-1 was detected in Chongqing (Wang et al., 2017), NDM-1-producing *E. cloacae* have emerged in various regions across the country (Jia et al., 2018; Jin et al., 2018). Moreover, moderate- to high-level carbapenem resistance in most isolates was closely related to an additional mechanism of resistance, e.g., decreased porin permeability (Majewski et al., 2016). Alteration or loss of non-specific porins, coupled with ESBL and/or AmpC overexpression is considered as one of the main mechanisms of resistance (Wozniak et al., 2012).

However, there has been little epidemiological data on carbapenem-resistant *E. cloacae* (CREL) in certain regions in China, such as Southern (e.g., Guangdong) and Northwestern (e.g., Ningxia) China. To gain insights into the evolution of CREL isolates in these two regions, we conducted a molecular epidemiological study to describe the resistance mechanism to carbapenems, clonal relatedness, and the genetic environment of carbapenemases (NDM-1 or IMP) -encoding plasmids among CREL isolates.

MATERIALS AND METHODS

Bacterial Strains and Characterization

From 2012 to 2016, a total of 84 non-duplicate strains from patients infected by CREL were collected from three tertiary hospitals including hospital A (First Affiliated Hospital of Sun Yat-sen University, Guangdong, China, 25 strains), hospital B (Guangdong Provincial Hospital of Chinese Medicine, Guangdong, China, 18 strains), and hospital C (Ningxia Hospital of Ningxia Medical University, Ningxia, China, 41 strains). Species identification and initial antibiotic susceptibility were analyzed by a VITEK-2 system (bioMerieux, France). Mueller-Hinton broth (Oxoid, UK) supplemented with calcium and magnesium (25.0 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+}) was used to test five antibiotics, including imipenem (ApexBio, USA), meropenem (ApexBio, USA), ertapenem (Menlunbio, China), polymyxin B (Sigma, USA), and tigecycline (ApexBio, USA). Minimum inhibitory concentrations (MICs) were performed according to the criteria of the Clinical and Laboratory Standards Institute (CLSI) (M100-S27). Isolates with an MIC of 4 μg/mL were considered resistant to polymyxin B. A susceptibility breakpoint of <2 μg/mL for tigecycline was interpreted in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Carbapenemase production was assessed by the carbapenem in activation method (CIM) (Aguirre-Quiñonero et al., 2017), while AmpC β-lactamase production was examined by three-dimensional test. Strains used in quality control were *Escherichia coli* ATCC 25922 and *Enterobacter cloacae* ATCC 700323.

Genotype Analysis

Crude DNA extracts prepared by boiling method were used as template in polymerase chain reactions (PCR). Various β-lactam resistance genes were examined, including carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{OXA-48}), ESBL-related genes (bla_{TEM}, bla_{CTX-M}, and bla_{SHV}) and AmpC-β-lactamase genes (bla_{EBC}, bla_{MOX}, bla_{CIT}, and bla_{DHA}). Moreover, *mcr-1* colistin resistance gene and *tetX* tigecycline resistance gene were also determined by using primers as described previously (Pérez-Pérez and Hanson, 2002; Pagani et al., 2003; Biendo et al., 2008; Poirel et al., 2011; Liu et al., 2016). Positive PCR products were sequenced and analyzed by nucleotide homology comparison against GenBank database by BLAST (www.ncbi.nlm.nih.gov/blast/). Details about oligonucleotides and thermal conditions are presented in **Supplementary Table 1**.

Outer Membrane Protein Gene Expressions

Expression of genes encoding outer membrane proteins (OmpF and OmpC porins) was determined using real-time reverse transcription PCR. The total RNA was extracted using the Trizol method and reversely transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TAKARA, China). Real-time quantitative PCR was performed using SYBR® Green I assay with analysis of dissociation curve (2× SYBR Green q-PCR Master Mix, Biomake, USA) on Applied Biosystems ViiA™ 7 Dx (Life Technologies, USA). Each experiment was performed in triplicate. The expression of porin-encoding genes, *ompF* and *ompC*, relative to *rpoB* was determined using specific primers and conditions described previously (Majewski et al., 2016). Relative expression was derived from the 2-Δ(ΔCT) formula, in which ΔCT represented the difference of cycle threshold (CT) between target genes and *rpoB*, while Δ(ΔCT) represented the difference of ΔCT between CREL strains and a standard strain, *Enterobacter cloacae* ATCC 700323.

Genetic Homologeity Analysis

All isolates were analyzed by PFGE to determine their genetic relatedness. In brief, the bacterial suspension mixed with equal volumes of low-melting-temperature agarose was cleaved with 1% sodium dodecyl sulfate and proteinase K (Sigma, USA) at 54°C overnight, and then digested with restriction endonuclease *Xba* I (Takara, Dalian, China) at 37°C for 8 h. DNA separation was performed in 0.5× TBE buffer using a pulsed-field electrophoresis system (CHEF MAPPER; Bio-Rad Laboratories, California, USA) under the following conditions: temperature 14°C; voltage 6.0 V/cm; switch angle, 120°; and switch ramp of 2.16–54.17 s for 18 h. Comparison of pulsotypes was performed with Bionumerics software version 6.6. Isolates were allocated into genetic similarity clusters using an 80% cut-off value. MLST was performed as described previously (https://pubmlst.org/ecloacae/). New alleles and sequence types were submitted to the MLST website and approved (https://pubmlst.org/ecloacae/). EnteroBase together with GrapeTree was used to analyze population evolutionary relationship (https://enterobase.readthedocs.io/en/latest/index.html).

Whole-Genome Sequencing

Six representative strains producing single NDM-1, six strains producing single IMP (according to clonal relatedness), one strain co-producing NDM-1 and IMP-1, and eight strains negative for all β-lactamase genes were selected for whole-genome sequencing. Genomic DNA was extracted using a MiniBEST Bacteria Genomic DNA Extraction Kit (TaKaRa, Dalian, China). DNA library was prepared using a QIAseq FX DNA Library Kit (Qiagen Inc., Valencia, CA) following the manufacturer's recommendations. The quantity and quality of the libraries were assessed with a Qubit dsDNA HS Assay Kit (Life technologies, USA) and LabChip GX Analyzer (Perkin Elmer; Waltham, MA). All barcoded libraries were pooled together in equimolar amounts and sequenced on NextSeq 500 platform (illumina Inc., San Diego, CA). Sequencing raw reads were processed for library adapter removal and filtering using FASTQ preprocessor Fastp v0.12.5 (Chen et al., 2018), followed by *de novo* assembly with SPAdes v3.13.0 (Bankevich et al., 2012) and annotating using Prokka annotation pipeline (Seemann, 2014). Antimicrobial resistance genes and plasmid-typing identification were mined using ABRicate program (https://github.com/tseemann/abricate, v0.8.2), and the contig containing carbapenemase genes, such as bla_{NDM} and bla_{IMP}, were extracted with an in-house Python script, followed by BLAST against NCBI sequence database. The sequence containing bla_{IMP} of the variable regions of integrons were analyzed followed by the Integron Database (http://integrall.bio.ua.pt/). Genetic organization analysis was visualized using EasyFig v2.2.3 (Sullivan et al., 2011).

Statistical Analysis

All the statistical analyses were performed by SPSS 18.0 (IBM Corp., Armonk, USA). The chi-square test was applied to evaluate difference in antibiotic resistance between carbapenemase-positive and carbapenemase-negative subpopulations. Relative changes in gene expression were indicated with median and extremum. All the relevant data were analyzed by non-parametric rank sum test, and was considered statistically significant if $P < 0.05$.

Ethical Considerations

This study was approved by Institutional Review Board of Second Affiliated Hospital of Soochow University. The study was retrospective and patients were not identified during data collection. Informed consent was not needed for this study.

RESULT

Characteristics of Clinical CREL Isolates

Eighty-four clinical CREL isolates were found to be resistant to at least one carbapenem. These isolates were recovered from different sources: body fluids ($n = 40$), sputum ($n = 19$), urine ($n = 8$), wound secretion ($n = 5$), blood ($n = 5$), pus ($n = 2$), catheter ($n = 2$), cannula ($n = 1$), semen ($n = 1$), and tissue ($n = 1$). These CREL isolates were obtained from patients admitted to the hepatobiliary surgery ($n = 25$, 29.8%), intensive care unit ($n = 17$, 20.2%), general surgery ($n = 11$, 13.1%), neurosurgery

TABLE 1 | *In vitro* activities of antimicrobial agents against 84 CREL isolates.

Antibiotic	Hospital A ($n = 25$)			Hospital B ($n = 18$)			Hospital C ($n = 41$)			Total ($n = 84$)		
	S%	I%	R%	S%	I%	R%	S%	I%	R%	S%	I%	R%
IPM	36.0	20.0	44.0	5.5	66.7	27.8	0.0	0.0	100.0	11.9	20.2	67.9
MEM	36.0	20.0	44.0	83.3	5.6	11.1	0.0	0.0	100.0	28.6	7.1	64.3
ETP	0.0	0.0	100.0	50.0	16.7	33.3	0.0	0.0	100.0	10.7	3.6	85.7
CAO	0.0	4.0	96.0	61.1	11.1	27.8	0.0	0.0	100.0	13.1	3.6	83.3
CAZ	24.0	8.0	68.0	72.2	0.0	27.8	0.0	0.0	100.0	22.6	2.4	75.0
TZP	28.0	0.0	72.0	88.9	0.0	11.1	14.6	2.4	82.9	34.5	1.2	64.3
FEP	24.0	8.0	68.0	83.3	0.0	16.7	0.0	0.0	100.0	25.0	2.4	72.6
ATM	16.0	0.0	84.0	55.6	27.8	16.6	2.4	0.0	97.6	17.8	6.0	76.2
CIP	48.0	4.0	48.0	72.2	0.0	27.8	31.7	7.3	61.0	45.2	4.8	50.0
LEV	56.0	0.0	44.0	72.2	0.0	27.8	29.3	7.3	63.4	46.4	3.6	50.0
GEN	76.0	12.0	12.0	72.2	0.0	27.8	61.0	0.0	39.0	67.8	3.6	28.6
TOB	52.0	24.0	24.0	72.2	0.0	27.8	4.9	73.1	22.0	33.3	42.9	23.8
ATM	96.0	0.0	4.0	88.9	0.0	11.1	95.1	4.9	0.0	94.0	2.4	3.6
SXT	72.0	0.0	28.0	72.2	0.0	27.8	4.9	0.0	95.1	39.3	0.0	60.7
NIT	44.0	44.0	12.0	72.2	16.7	11.1	46.4	34.1	19.5	51.2	33.3	15.5
PB*	92.0	0.0	8.0	33.3	0.0	66.7	97.6	0.0	2.4	82.1	0.0	17.9
TGC	88.0	8.0	4.0	94.6	5.6	0.0	82.9	0.0	17.1	86.9	3.6	9.5

*S, susceptible; I, intermediate; R, resistant. IPM, imipenem; MEM, meropenem; ETP, ertapenem; CAO, cefatriaxone; CAZ, cefepime; TZP, piperacillin/tazobactam; FEP, cefepime; ATM, aztreonam; CIP, ciprofloxacin; LEV, levofloxacin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; SXT, sulfamethoxazolen; NIT, furazolidin; PB, polymyxin B; TGC, tigecycline. Hospital A, First Affiliated Hospital of Sun Yat-sen University, Guangdong, China; Hospital B, Guangdong Provincial Hospital of Chinese Medicine, Guangdong, China; Hospital C, Ningxia Hospital of Ningxia Medical University, Ningxia, China. *Two isolates obtained from Hospital B were suspected of heteroresistance to polymyxin B.*

TABLE 2 | *In vitro* activities of carbapenems to 84 CREL isolates with or without carbapenemase.

Antibiotics	Carbapenemase positive ($n = 50$)				Carbapenemase negative ($n = 34$)				*P
	R%	MIC (μg/mL)			R%	MIC (μg/mL)			
		MIC range	MIC50	MIC90		MIC range	MIC50	MIC90	
IPM	100	8–256	128	256	20.6	1–64	2	4	$P < 0.01$
MEM	100	16–256	128	256	11.8	0.5–8	1	4	$P < 0.01$
ETP	100	32–256	128	256	64.7	0.5–64	2	4	$P < 0.01$

*IPM, imipenem; MEM, meropenem; ETP, ertapenem. R, resistant. *P-value for comparisons of resistance rates between carbapenemase-positive and carbapenemase-negative groups.*

($n = 7$, 8.3%), pediatrics ($n = 4$, 4.8%), burns surgery ($n = 5$, 6.0%), respiratory department ($n = 3$, 3.6%), gastroenterological surgery ($n = 2$, 2.4%), cardiology department ($n = 2$, 2.4%), department of orthopedics ($n = 2$, 2.4%), nephrology department ($n = 1$, 1.2%), and other surgery wards ($n = 5$, 6.0%). Among 84 CREL isolates, 85.7% (72/84) of the isolates were classified as MDR as they were resistant to three or more classes of antibiotics. Seventy-two (85.7%) isolates were resistant to ertapenem, followed by 57 (67.9%) and 54 (64.3%) resistant to imipenem and meropenem, respectively. The strains showed high resistance rates to cefatriaxone (83.3%), ceftazidime (75.0%), cefepime (72.6%), and aztreonam (76.2%), while 43 (51.2%), 51 (60.7%), and 27 (32.1%) strains were resistant to fluoroquinolones, sulfanilamide, and aminoglycosides, respectively. In contrast, 94.0 and 86.9% were susceptible to amikacin and tigecycline. Thirteen *E. cloacae* isolates were resistant to polymyxin B and two isolates were suspected of heteroresistance. Two *E. cloacae* isolates (ECL-ZY07 and ECL-ZY14) with resistance to polymyxin B and tigecycline showed promising *in-vitro* activity against amikacin. Carbapenemase producers presented a dramatically higher resistance rate than negative ones for carbapenemase ($P < 0.01$). Descriptive statistics on antimicrobial susceptibility tests were presented in **Tables 1**, **2**.

Phenotype and Genotype Analysis

Fifty-four isolates were considered positive in the CIM test according to Tijet et al. (2016), but only 50 (59.5%) were demonstrated as carbapenemase producers by PCR analysis. All KPC, NDM, VIM, or IMP producing isolates were unequivocally detected with the CIM test. Four isolates non- producing carbapenemase (one with $bla_{\mathrm{TEM-1}}$, $bla_{\mathrm{SHV-12}}$, $bla_{\mathrm{CTX-M}}$, and $bla_{\mathrm{DHA-1}}$, three hyperproducing AmpC) were positive by the test. The sensitivity and specificity of the detection assay compared with molecular methods were 100 and 89.5%. The

bla_{NDM-1} gene was found the most prevalent carbapenemase gene (40/50), followed by bla_{IMP-26} (4/50), bla_{IMP-4} (3/50), bla_{IMP-1} (1/50), bla_{VIM-4} (1/50), and bla_{KPC-2} (1/50). We observed one *E. cloacae* isolate co-harboring bla_{NDM-1} and bla_{IMP-1}, which resulted in strongly high MICs of all carbapenems (MICs = 256 μg/L). Remarkably, 97.6% (40/41) of the isolates obtained from Ningxia were MBL producers, but only 20.9% (9/43) produced MBL in Guangdong. No carbapenemase gene was detected in hospital B (Guangdong Provincial Hospital of Chinese Medicine, Guangdong, China). Moreover, 83.3% of the isolates continuously produced high AmpC β-lactamase, and 10.7% were pAmpC producers (eight with bla_{DHA-1}, and one with bla_{LAP-2}). All carried chromosomal-mediated ACT-type β-lactamase. ESBL producers were found in 36 (42.9%) isolates, among which 25 isolates overproduced AmpC. 19.0% (16/84), 15.5% (13/84), 26.2% (22/84), and 23.8% (20/84) of the isolates were detected as positive for bla_{TEM-30}, bla_{TEM-1}, bla_{CTX-M}, and bla_{SHV-12}, respectively. Meanwhile, 31.0% (26/84) of the isolates co-expressed carbapenemase and ESBL, and 7.1% (6/84) produced carbapenemase, ESBL and pAmpC simultaneously. None of the isolates were PCR-positive for bla_{OXA-48}, *mcr-1 or tetX*.

Expression of *ompC* and *ompF* Genes

Relative expression of *ompF* gene fell within a range of 0.01–1.58, with a median value of 0.12, while expression of *ompC* gene ranged from 0.01 to 36.36, with a median value of 2.80. Thirty-two isolates lost or had lower expression of both major porins, while 52 isolates had decreased expression of one porin, especially for OmpF porin (50/52). Isolates with a combination of carbapenemase and porin loss showed extensively high carbapenem MICs. All 34 non-carbapenemase-producing isolates had lost at least one porin, among which 22 isolates had reduced expression of both major porins. Additionally, 7 isolates produced both ESBL and AmpC, and 24 isolates produced only one type of enzyme. The remaining 3 isolates were negative for ESBLs (TEM, SHV, or CTX-M) and AmpC. Porin loss together with AmpC overexpression typically resulted in insensitivity to one or two carbapenems. Compared with isolates non-susceptible to three carbapenems and isolates only non-susceptible to one or two carbapenems, the latter had significantly lower expression of *ompC* ($P < 0.01$), but there was no difference in *ompF* expression among two groups ($P = 0.398$). Further, lower expression of *ompC* was significantly associated with increasing MIC of imipenem ($P < 0.01$). All details about antibiotic susceptibilities and porin expression of CREL isolates were given in **Table 3**.

PFGE Analysis

Pulsed-field gel electrophoresis patterns of the 84 CREL isolates were identified into 54 pulsotypes (clusters 1–54), and of these, PFGE types 2, and 3 could be classified into 3 subtypes, while types 4, 5, and 7 were classified into 2 subtypes (**Figure 1**). PFGE revealed genetic diversity, whereas PFGE types 1, 2, 3, 4, 5, 6, and 7 were observed in 19, 6, 3, 2, 2, 2, and 2 different patients, respectively. Notably, all the 19 strains with a single dominant pulsotype (PFGE type 1) were isolated from patients in hepatobiliary surgery (17/19), intensive care unit (1/19), and vascular surgery (1/19) during a detection peak period (from Jan. 2016 to Sep. 2016) in hospital C. As shown in **Figure 2**, the strain ECL-NX38 which was isolated from a 78-year-old patient in hepatobiliary surgery on January 4, 2016 could be the first strain of these outbreak cases. Besides, three strains of type 3 were isolated from patients in two different wards in hospital C during 1-year period. All the six strains of PFGE type 2 and two strains of type 6 were distributed sporadically in different wards in hospital C. All details about clinical characteristics and PFGE patterns of 41 CREL isolates from hospital C was shown in **Table 4**. However, all pulsotypes were single isolates in hospital B. Two strains of PFGE type 4 were isolated from patients in ICU in hospital A within 1 year; two strains of type 5 were isolated from patients in two different wards within 2 months; while two strains of type 7 were isolated from patients in hepatobiliary surgery within 1 month (data not shown).

MLST Analysis

MLST distinguished 42 STs including 22 novel STs (ST1211-ST1229, ST1231, ST1232, and ST1234). The most prevalent ST was ST78 ($n = 27$, 32.1%), followed by ST51 ($n = 7$, 8.3%), ST1226 ($n = 5$, 6.0%), ST114 ($n = 4$, 4.8%), ST93 ($n = 2$, 2.4%), ST346 ($n = 2$, 2.4%), and ST1044 ($n = 2$, 2.4%). The remaining STs contained one isolate for each. MLST showed subtype diversity, which was relatively consistent with PFGE. As expected, PFGE was more discriminatory than MLST. Most isolates belonging to different subtypes of the same pulsotype had an identical ST. The only exceptions were one single-locus variant pairs (SLVs) (ST114 and ST1229) and five SLVs (ST51 and ST778), among which isolates of one ST were clustered into one of the pulsotypes of the other ST. Of the 42 STs, 33 (78.6%) were singletons, 6 (14.3%) had single SLVs and 3 (7.1%) clustered into a bigger group within CC114. The ST114-centered group encompassed 6 isolates of 3 STs including ST114 ($n = 4$), ST1229 ($n = 1$), and ST1231 ($n = 1$), and isolates were all obtained from hospital A. Twenty-eight isolates from hospital A and C belonged to ST78 ($n = 27$) and ST1221 ($n = 1$) within CC74, while eight isolates represented ST51 ($n = 7$) and ST50 ($n = 1$) within CC234. Other single SLVs consisted of ST1044 and ST1228, indicating direct clonal evolution between the SLVs. All of the STs were analyzed by Grapetree in order to reveal their relationships (**Figure 3**). The ST78 clones of eight PFGE pulsotypes exhibited various β-lactamase profiles. The most prevailing type harboring NDM-1 belonged to ST78 and carried plasmids of two or three Inc types, including IncFIB, IncX3, and IncX4 (**Supplementary Table 2**). Plasmid-based replicon typing revealed that the IncX3 type epidemic plasmid carrying bla_{NDM-1} caused an outbreak of *E. cloacae*. These dominated clones carried several other β-lactamase genes, including bla_{ACT-5} and bla_{OXA-1}. However, ST78 *E.cloacae* isolates were not always associated with NDM-1. Three ST78 strains from hospital A produced different ESBLs but not NDM-1, and one ST78 *E.cloacae* from hospital C produced IMP-4. Each of these comprised various Inc types of plasmids. Six ST51 isolates with highly similar pulsotypes produced NDM-1-type MBL, and four isolates belonged to ST1226 diversified into three pulsotypes, were frequently related to IMP-26. Due to the

TABLE 3 | Drug-resistance characteristics of 84 CREL isolates.

Isolate	MIC (μg/L)			Carbapenemase	ESBL	AmpC	OMPF	OMPC
	IPM	MEM	ETP					
ECL-ZY01	64	64	64	NDM-1	–	+	0.19↓	1.56↑
ECL-ZY02	64	64	64	NDM-1	SHV-12	+	0.07↓	0.13↓
ECL-ZY03	1	1	4	–	CTX-M; TEM-30	+	0.10↓	2.07↑
ECL-ZY04	1	1	2	–	CTX-M; TEM-30	–	0.13↓	1.34↑
ECL-ZY05	1	1	4	–	–	+	0.02↓	0.02↓
ECL-ZY06	8	8	64	–	CTX-M; TEM-1; SHV-12	–	0.01↓	1.36↑
ECL-ZY07	1	1	4	–	CTX-M; TEM-30	+	0.28↓	0.58↓
ECL-ZY08	256	256	256	NDM-1	CTX-M; TEM-1; SHV-12	+	0.26↓	0.66↓
ECL-ZY09	128	128	128	NDM-1	CTX-M; TEM-30; SHV-12	+	0.31↓	3.31↑
ECL-ZY10	2	2	4	–	–	+	0.01↓	0.01↓
ECL-ZY11	32	32	128	IMP-8	–	+	0.01↓	0.02↓
ECL-ZY12	1	1	4	–	–	+	0.29↓	3.69↑
ECL-ZY13	256	256	256	NDM-1	TEM-1; SHV-12	+	0.54↓	0.63↓
ECL-ZY14	2	2	4	–	SHV-12	+	0.01↓	0.82↓
ECL-ZY15	1	1	2	–	–	+	0.28↓	0.74↓
ECL-ZY16	2	2	8	–	CTX-M	+	0.01↓	0.21↓
ECL-ZY17	2	2	2	–	CTX-M; TEM-1	+	0.19↓	2.76↑
ECL-ZY18	128	128	128	IMP-4	CTX-M; TEM-1	+	0.01↓	0.09↓
ECL-ZY19	1	1	4	–	–	+	0.06↓	6.48↑
ECL-ZY20	1	1	2	–	–	+	0.22↓	7.52↑
ECL-ZY21	1	1	2	–	–	+	0.19↓	5.41↑
ECL-ZY22	256	256	256	KPC-2	CTX-M; TEM-1; SHV-12	+	0.01↓	0.01↓
ECL-ZY23	8	16	64	VIM-4		+	0.01↓	2.70↑
ECL-ZY24	256	256	256	NDM-1	TEM-1; SHV-12	+	0.20↓	0.47↓
ECL-ZY25	2	2	4	–	CTX-M	+	0.74↓	0.01↓
ECL-NX01	128	128	128	NDM-1	–	+	0.61↓	10.92↑
ECL-NX02	64	64	32	NDM-1	CTX-M; TEM-1	+	0.38↓	36.36↑
ECL-NX03	128	128	128	NDM-1	–	+	0.23↓	11.28↑
ECL-NX04	128	128	128	NDM-1	–	+	0.32↓	13.81↑
ECL-NX05	32	32	32	IMP-26	TEM-30; SHV-12	+	0.65↓	6.99↑
ECL-NX06	256	256	256	NDM-1	CTX-M; TEM-30	–	0.12↓	7.07↑
ECL-NX07	32	32	32	IMP-26	TEM-30; SHV-12	+	0.01↓	6.06↑
ECL-NX08	256	256	256	NDM-1	CTX-M; TEM-30	–	0.09↓	0.01↓
ECL-NX09	128	128	128	NDM-1	–	+	0.08↓	12.44↑
ECL-NX10	128	128	128	NDM-1	–	+	0.20↓	8.37↑
ECL-NX11	128	128	128	NDM-1	–	+	0.22↓	10.12↑
ECL-NX12	128	128	128	NDM-1	–	+	0.14↓	7.62↑
ECL-NX13	128	128	128	NDM-1	–	+	0.15↓	9.35↑
ECL-NX14	128	128	128	NDM-1	–	+	0.16↓	10.11↑
ECL-NX15	128	128	256	NDM-1	–	+	0.15↓	9.91↑
ECL-NX16	128	128	128	NDM-1	–	+	0.22↓	11.3↑
ECL-NX17	256	256	256	NDM-1	CTX-M; TEM-30	–	0.11↓	8.41↑
ECL-NX18	64	8	64	–	CTX-M; TEM-1; SHV-12	+	0.06↓	0.49↓
ECL-NX19	128	128	128	IMP-26	TEM-1; SHV-12	+	0.15↓	6.92↑
ECL-NX20	256	256	256	NDM-1	SHV-12	+	0.12↓	5.83↑
ECL-NX21	128	128	128	NDM-1	–	+	0.14↓	10.05↑
ECL-NX22	128	128	128	NDM-1	–	+	0.14↓	9.20↑
ECL-NX23	128	128	128	NDM-1	–	+	0.09↓	12.28↑
ECL-NX24	32	32	32	IMP-4	SHV-12	+	1.58↓	13.48↑

(Continued)

TABLE 3 | Continued

Isolate	MIC (μg/L)			Carbapenemase	ESBL	AmpC	OMPF	OMPC
	IPM	MEM	ETP					
ECL-NX25	256	256	256	NDM-1	CTX-M; TEM-30	–	0.03↓	10.59↑
ECL-NX26	128	128	128	NDM-1		+	0.12↓	15.44↑
ECL-NX27	32	32	64	IMP-4	CTX-M; SHV-12	+	0.06↓	6.73↑
ECL-NX28	256	256	256	NDM-1	CTX-M; TEM-30	–	0.09↓	0.01↓
ECL-NX29	256	256	256	NDM-1	CTX-M; TEM-30	–	0.09↓	0.05↓
ECL-NX30	128	128	128	NDM-1	–	+	0.15↓	8.98↑
ECL-NX31	128	128	128	NDM-1	–	+	0.19↓	6.43↑
ECL-NX32	128	128	128	NDM-1	–	+	0.16↓	8.14↑
ECL-NX33	128	128	128	NDM-1	–	+	0.08↓	7.85↑
ECL-NX34	128	128	128	NDM-1	TEM-30	+	0.07↓	4.04↑
ECL-NX35	256	256	256	NDM-1 IMP-1	TEM-30; SHV-12; LAP-2	+	0.01↓	6.31↑
ECL-NX36	128	128	128	NDM-1	–	+	0.17↓	3.06↑
ECL-NX37	128	128	128	IMP-26	TEM-30; SHV-12	+	0.16↓	3.06↑
ECL-NX38	128	128	128	NDM-1	–	+	0.07↓	2.79↑
ECL-NX39	64	64	64	NDM-1	CTX-M; TEM-30; SHV-12	–	0.23↓	5.66↑
ECL-NX40	64	64	64	NDM-1	CTX-M; TEM-1; SHV-12	–	0.09↓	2.81↑
ECL-NX41	128	128	128	NDM-1	TEM-1; SHV-12	+	0.12↓	1.29↑
ECL-SZY01	2	0.5	0.5	–	–	+	0.01↓	0.01↓
ECL-SZY02	2	0.5	0.5	–	–	–	0.01↓	0.01↓
ECL-SZY03	4	0.5	0.5	–	–	+	0.01↓	0.01↓
ECL-SZY04	4	4	4	–	–	+	0.01↓	0.01↓
ECL-SZY05	2	0.5	0.5	–	–	+	0.01↓	0.02↓
ECL-SZY06	2	0.5	0.5	–	–	+	1.35↑	0.01↓
ECL-SZY07	2	0.5	0.5	–	–	+	0.06↓	0.01↓
ECL-SZY08	4	1	1	–	–	+	0.01↓	0.01↓
ECL-SZY09	2	0.5	1	–	–	+	0.01↓	0.01↓
ECL-SZY10	2	0.5	0.5	–	–	+	0.01↓	0.20↓
ECL-SZY11	2	0.5	0.5	–	–	+	0.27↓	0.82↓
ECL-SZY12	2	0.5	0.5	–	–	+	0.01↓	0.01↓
ECL-SZY13	1	1	4	–	–	–	0.90↓	0.01↓
ECL-SZY14	4	1	1	–	–	+	1.20↑	0.01↓
ECL-SZY15	2	2	4	–	–	+	0.03↓	0.01↓
ECL-SZY16	4	4	2	–	TEM-1	–	0.01↓	4.5↑
ECL-SZY17	2	1	4	–	–	–	0.01↓	3.91↑
ECL-SZY18	2	1	4	–	–	+	0.01↓	0.08↓

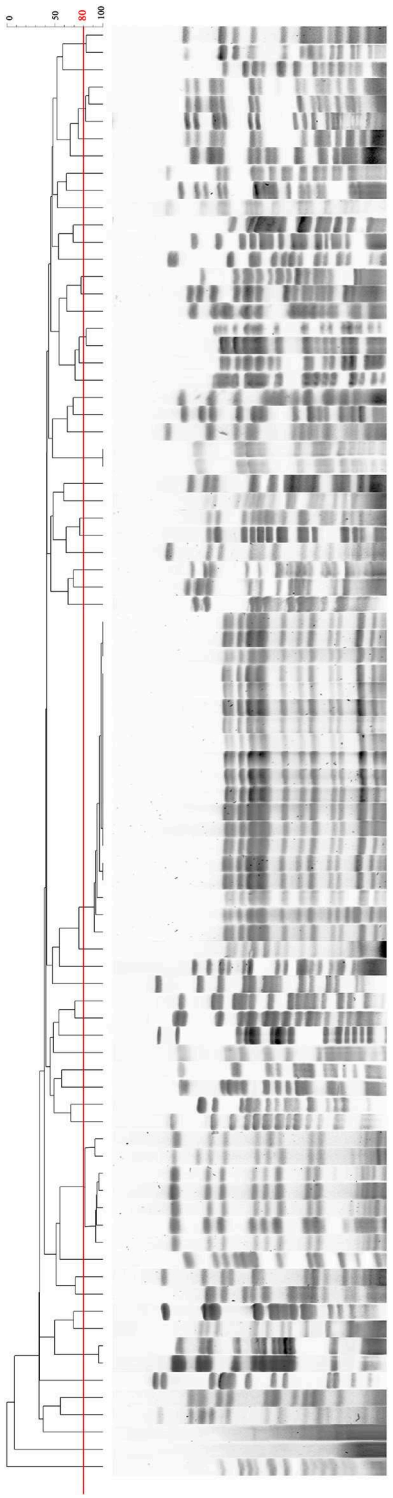
IPM, imipenem; MEM, meropenem; ETP, ertapenem. Arrows indicated the relative variation of expression in ompC and ompF genes. AmpC β-lactamase was examined by cefoxitin in three-dimensional test. The plus and minus were used to describe the positive or negative results of β-lactamases. Arrows represents up- and down- regulation of OM expression.

deficiency of clinic case and limited data, all these observations were still to be further validated.

Genetic Environments of NDM-1 or IMP Carrying Contigs

As shown in **Figure 4**, seven NDM-1-harboring plasmids had a common genetic structure, which was highly similar to some previous plasmids, such as pNDM-ECN49 (GenBank Accession No. KP765744). Compared with another epidemic plasmid pNDM-BJ01 in *A. lwoffii* (GenBank Accession No. JQ001791), however, a complete or partial IS*5* supplanted the IS*Aba125* upstream of the bla_{NDM-1} gene. The bla_{NDM-1}-like-carrying plasmids could be divided into two different types. Type A plasmids mainly differed by the insertion of a Tn*3*-like transposon downstream of *groES*. The Tn*3*-like transposon consisted of *tnpA*, *tnpR*, and two ORFs encoding hypothetical proteins, flanked by a pair of 38 bp inverted repeats (IR). The inverted repeat motifs of target site 6 bp at the boundaries of the Tn*3*-like element indicated insertion by transposition. However, IS*91*-like element, which probably transpose via a rolling-circle replication mechanism, was located downstream of *groES* among two plasmids of Type B.

All of the bla_{IMP} genes were situated within class 1 integrons. The bla_{IMP-8} gene was identified in In655 containing



Strain No.	Beta-lactamases	PFGE patterns	STs	Hospital
ECL-ZY08	NDM-1, CTX-M, TEM-1, SHV-12	4a	1229	A
ECL-ZY09	NDM-1, CTX-M, TEM-30, SHV-12	4b	114	A
ECL-NX15	NDM-1	8	78	C
ECL-NX05	IMP-26, TEM-30, SHV-12, DHA-1	3a	1226	C
ECL-NX37	IMP-26, TEM-30, SHV-12	3b	1226	C
ECL-NX41	NDM-1, TEM-1, SHV-12, DHA-1	3c	1226	C
ECL-NX19	IMP-26, TEM-1, SHV-12	9	1226	C
ECL-NX07	IMP-26, TEM-30, SHV-12, DHA-1	10	1226	C
ECL-SZY11		11	1216	B
ECL-ZY14	SHV-12	12	114	A
ECL-NX27	IMP-4, CTX-M, SHV-12, DHA-1	13	78	C
ECL-SZY03	DHA-1	14	1224	B
ECL-SZY10		15	1217	B
ECL-SZY13		16	200	B
ECL-ZY21		17	1044	A
ECL-ZY23	VIM-4	18	422	A
ECL-ZY19		19	544	A
ECL-ZY03	CTX-M, TEM-30	5a	78	A
ECL-ZY06	CTX-M, TEM-1, SHV-12	5b	78	A
ECL-ZY01	NDM-1	20	78	A
ECL-ZY04	CTX-M, TEM-30	21	78	A
ECL-NX24	IMP-4, SHV-12	22	127	C
ECL-ZY11	IMP-8	23	27	A
ECL-NX02	NDM-1, CTX-M, TEM-1, DHA-1	24	88	C
ECL-NX39	NDM-1, CTX-M, TEM-30, SHV-12	6	93	C
ECL-NX40	NDM-1, CTX-M, TEM-1, SHV-12	6	93	C
ECL-NX20	NDM-1, SHV-12	25	50	C
ECL-ZY10		26	125	A
ECL-NX16	NDM-1	27	78	C
ECL-SZY18		28	1223	B
ECL-ZY02	NDM-1, SHV-12	29	1227	A
ECL-ZY07	CTX-M, TEM-30	30	346	A
ECL-ZY15		31	346	A
ECL-SZY01		32	365	B
ECL-NX11	NDM-1	1	78	C
ECL-NX36	NDM-1	1	78	C
ECL-NX32	NDM-1	1	78	C
ECL-NX01	NDM-1	1	78	C
ECL-NX03	NDM-1	1	78	C
ECL-NX04	NDM-1	1	78	C
ECL-NX09	NDM-1	1	78	C
ECL-NX10	NDM-1	1	78	C
ECL-NX12	NDM-1	1	78	C
ECL-NX14	NDM-1	1	78	C
ECL-NX21	NDM-1	1	78	C
ECL-NX23	NDM-1	1	78	C
ECL-NX26	NDM-1	1	78	C
ECL-NX30	NDM-1	1	78	C
ECL-NX33	NDM-1	1	78	C
ECL-NX38	NDM-1	1	78	C
ECL-NX13	NDM-1	1	78	C
ECL-NX31	NDM-1	1	78	C
ECL-NX22	NDM-1	1	78	C
ECL-ZY25	CTX-M	33	1221	A
ECL-ZY16	CTX-M	34	1231	A
ECL-SZY05	DHA-1	35	1232	B
ECL-NX34	NDM-1, TEM-30	36	78	C
ECL-SZY04		37	1211	B
ECL-SZY09		38	1218	B
ECL-NX35	NDM-1, IMP-1, TEM-30, SHV-12, LAP-2	39	97	C
ECL-SZY16	TEM-1	40	1213	B
ECL-SZY17		41	1212	B
ECL-SZY02		42	1225	B
ECL-SZY07		43	1222	B
ECL-NX28	NDM-1, CTX-M, TEM-30	2a	778	C
ECL-NX29	NDM-1, CTX-M, TEM-30	2b	51	C
ECL-NX06	NDM-1, CTX-M, TEM-30	2c	51	C
ECL-NX08	NDM-1, CTX-M, TEM-30	2c	51	C
ECL-NX25	NDM-1, CTX-M, TEM-30	2c	51	C
ECL-ZY24	NDM-1, TEM-1, SHV-12	2c	51	A
ECL-NX17	NDM-1, CTX-M, TEM-30	2c	51	C
ECL-SZY15		44	1214	B
ECL-ZY13	NDM-1, TEM-1, SHV-12	45	56	A
ECL-ZY18	IMP-4, CTX-M, TEM-1	46	1044	A
ECL-SZY08		47	1219	B
ECL-ZY17	CTX-M, TEM-1	48	1234	A
ECL-ZY12		7a	114	A
ECL-ZY20		7b	114	A
ECL-SZY06		49	712	B
ECL-NX18	CTX-M, TEM-1, SHV-12, DHA-1	50	51	C
ECL-ZY22	KPC-2, CTX-M, TEM-1, SHV-12	51	175	A
ECL-SZY14		52	1220	B
ECL-SZY12		53	1215	B
ECL-ZY05		54	561	A

FIGURE 1 | Dendrogram of the PFGE profiles of CREL isolates. The Dice coefficient was used to identify different types with a cutoff of 80.0% similarity. Hospital A, First Affiliated Hospital of Sun Yat-sen University, Guangdong, China; Hospital B, Guangdong Provincial Hospital of Chinese Medicine, Guangdong, China; Hospital C, Ningxia Hospital of Ningxia Medical University, Ningxia, China.

bla$_{IMP-8}$-*aacA4′-3*, which was previously detected in *E. cloacae* (GenBank Accession No. MF344574). Three genetic mutations in coding region of *intI1* (A38978C, G38958C, and T38953C) were observed in the integron mediated IMP-8. The integron harboring *bla*$_{IMP-26}$ contained the gene cassette array: *bla*$_{IMP-26}$-*ereA2*–*qacEΔ1*/*sul1*, and was detected in 3 isolates. The integron had a structurally similar cassette to pIMP26 in *E. cloacae* (GenBank Accession No. MH399264), but the difference in *ereA2* gene insertion resulted in erythromycin resistance. Integrons of *bla*$_{IMP-4}$ gene had a similar genetic structure to pAR0161 (GenBank Accession No. MF344574), and 3′-CS *qacEΔ1 -sul1* were also observed downstream. However, we were unable to sequence the complete integron-related gene cassettes due to the limitations of short-read sequencing. The *bla*$_{IMP-1}$ gene was determined in a shorter contig accompanied with a partial *aacA4* gene downstream. The sequence alignment revealed that one synonymous mutation (T39C) were observed, compared with the widely reported sequence of *bla*$_{IMP-1}$ gene (GenBank Accession No. MG287118). This resultant sequence was almost completely consistent with the partial published sequence in *K. oxytoca* p7121-IMP (GenBank Accession No. KX784502.1).

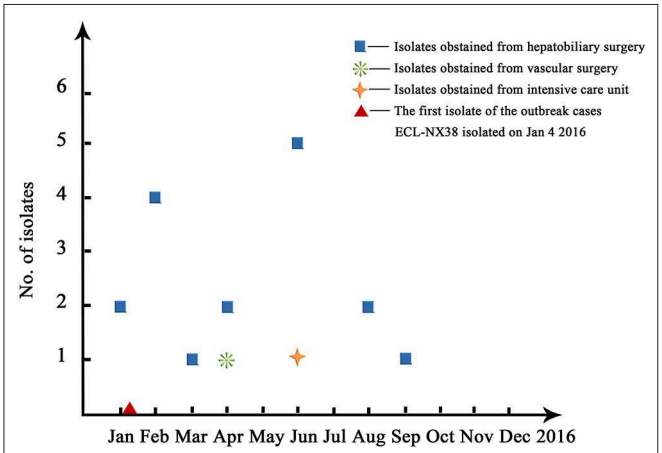


FIGURE 2 | An overall time distribution of predominated ST78 clones in Hospital C in the year of 2016.

DISCUSSION

E. cloacae is frequently implicated in serious healthcare-associated infections, with a high proportion of carbapenemase

TABLE 4 | PFGE patterns and clinical characteristics of 41 CREL isolates obtained from Hospital C.

Pulsotypes	Strain no.	Departments (*n*)	Specimens (*n*)	Years (*n*)				
				2012	2013	2014	2015	2016
		Hepatobiliary surgery (17)						19
1	19	Intensive care unit (1),	bi (17), su (1), pf (1)					
		Vascular Surgery (1)						
2a	1	Intensive care unit (1)	ur (1)		1			
2b	1	Intensive care unit (1)	pf (1)	1				
		Neurosurgery (1)						
2c	4	Cardiology department (1)	sp (2), ur (1), su (1)	1		2	1	
		Respiratory Medicine (1)						
		Intensive care unit (1)						
3a	1	Burns surgery (1)	se (1)					1
3b	1	Burns surgery (1)	se (1)					1
3c	1	Hepatobiliary surgery (1)	bl (1)				1	
6	2	Hepatobiliary surgery (1)	bi (1), sp (1)		1			1
		Intensive care unit (1)						
8	1	Vascular surgery (1)	su (1)					1
9	1	Burns surgery (1)	se (1)					1
10	1	Burns surgery (1)	ps (1)		1			
13	1	Burns surgery (1)	ca (1)					1
22	1	General surgery	sp (1)					1
24	1	Oral surgery (1)	sb (1)		1			
25	1	PICU (1)	ca (1)				1	
27	1	Orthopedics (1)	ps (1)					1
36	1	Hepatobiliary surgery (1)	bi (1)					1
39	1	Cardiology department (1)	pf (1)					1
50	1	Pediatrics	bl (1)					1
Total	41			2	4	2	3	30

bi, bile; su, drainage; sp, sputum; ur, urine; se, secretion; ps, pus; ca, catheter; bl, blood.

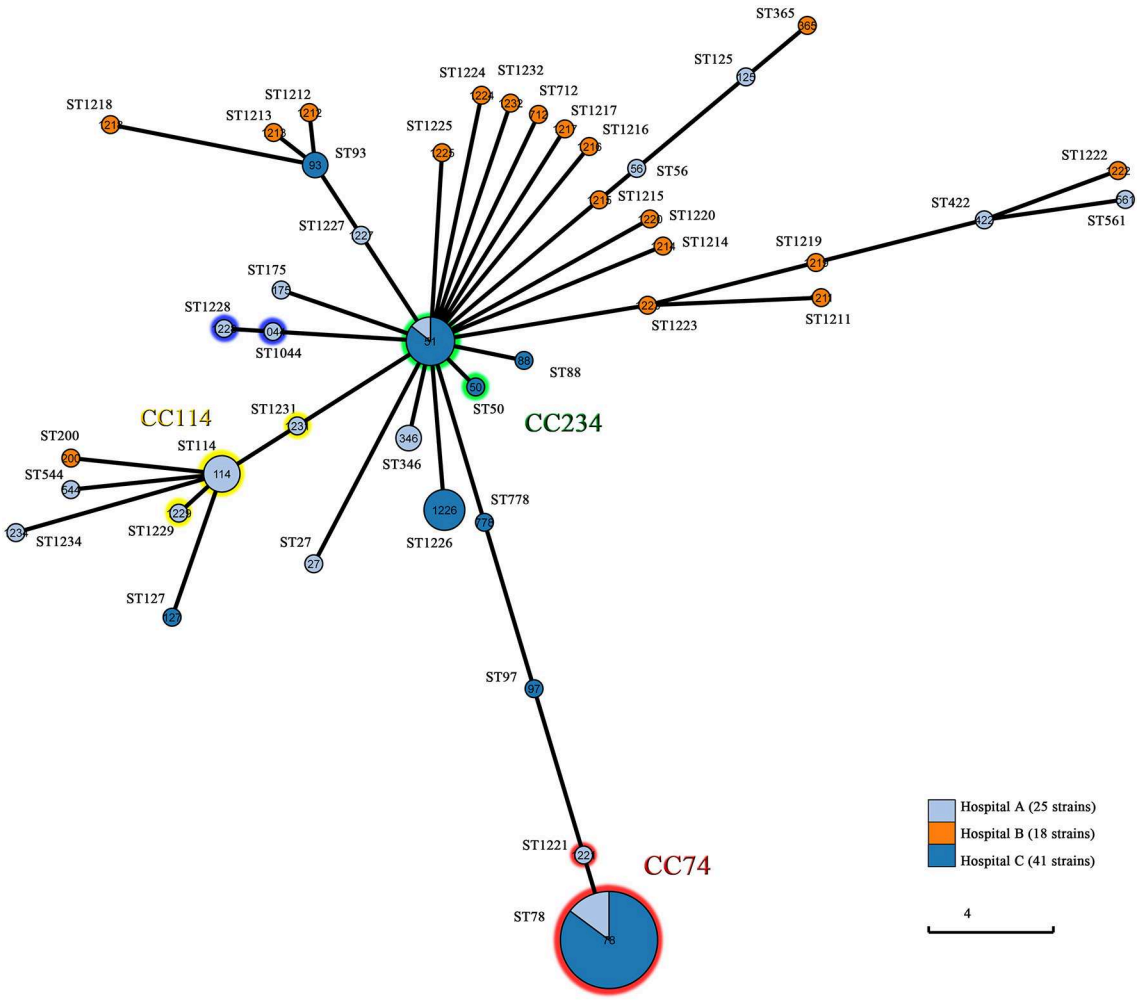


FIGURE 3 | Population structure of the 84 CREL isolates by GrapeTree analysis. Each circle is a Node and each line is a branch. Node size is dependent on the number of strains within that node. Numbers in circles represent ST types. Branch length varies on the distance between nodes. Hospital A, First Affiliated Hospital of Sun Yat-sen University, Guangdong, China; Hospital B, Guangdong Provincial Hospital of Chinese Medicine, Guangdong, China; Hospital C, Ningxia Hospital of Ningxia Medical University, Ningxia, China.

producers among carbapenem-resistant *Enterobacteriaceae* (Davin-Regli and Pagès, 2015). *E. cloacae* has been reported to acquire carbapenemases including KPC-2 (Andrade et al., 2018), NDM-1 (Torres-González et al., 2015), IMP-4 (Lee et al., 2017b), IMP-26 (Dai et al., 2013), IMP-8 (Pang et al., 2016), IMP-1 (Aoki et al., 2018), and VIM-4 (Sonnevend et al., 2017). Our study demonstrated the high prevalence of carbapenemase among carbapenem-resistant *E. cloacae* (CREL) in south and northwest of China (59.5%). MBLs, especially NDM-1 was, the dominant mechanism of resistance to carbapenems in *E. cloacae* in China. Most cases of infections or colonizations of NDM-1-producing bacteria originated in southern Asia, most commonly in India (Walsh and Toleman, 2011). The different prevalence of NDM-1 between countries might be explained by its high genetic transfer rate among unrelated bacterial species. Meanwhile, IMP-type MBL has been identified as the second common carbapenemase (10.7%). Compared to the worldwide distribution of IMP-4 (Leung et al., 2013; Lee et al., 2017b) and IMP-8 (Wang et al., 2015; Pang et al., 2016), IMP-26 and IMP-1 were also detected in our study. Although KPC or VIM-producing *E. cloacae* has been reported to spread rapidly in the last decade (Kanamori et al., 2017; Sonnevend et al., 2017; Daniels et al., 2018), the prevalence remained relatively low in our study. Moreover, the carriage rate of ESBL-related genes among CREL isolates was high (42.9%), though no dominant ESBL type was identified. Our study showed that 83.3% of the isolates continuously produced high AmpC β-lactamase. ACT-type inducible AmpC enzyme remained conservative. DHA-type, however which has become the most prevalent plasmid-mediated AmpC β-lactamase, was less frequently described in the literature with regard to *E. cloacae*. These additional β-lactamases might explain a slight increase of carbapenem resistance among CREL isolates (Souna et al., 2014). Of note, two MBLs, NDM-1 and IMP-1, were firstly identified to be co-expressed in *E. cloacae,* which was isolated

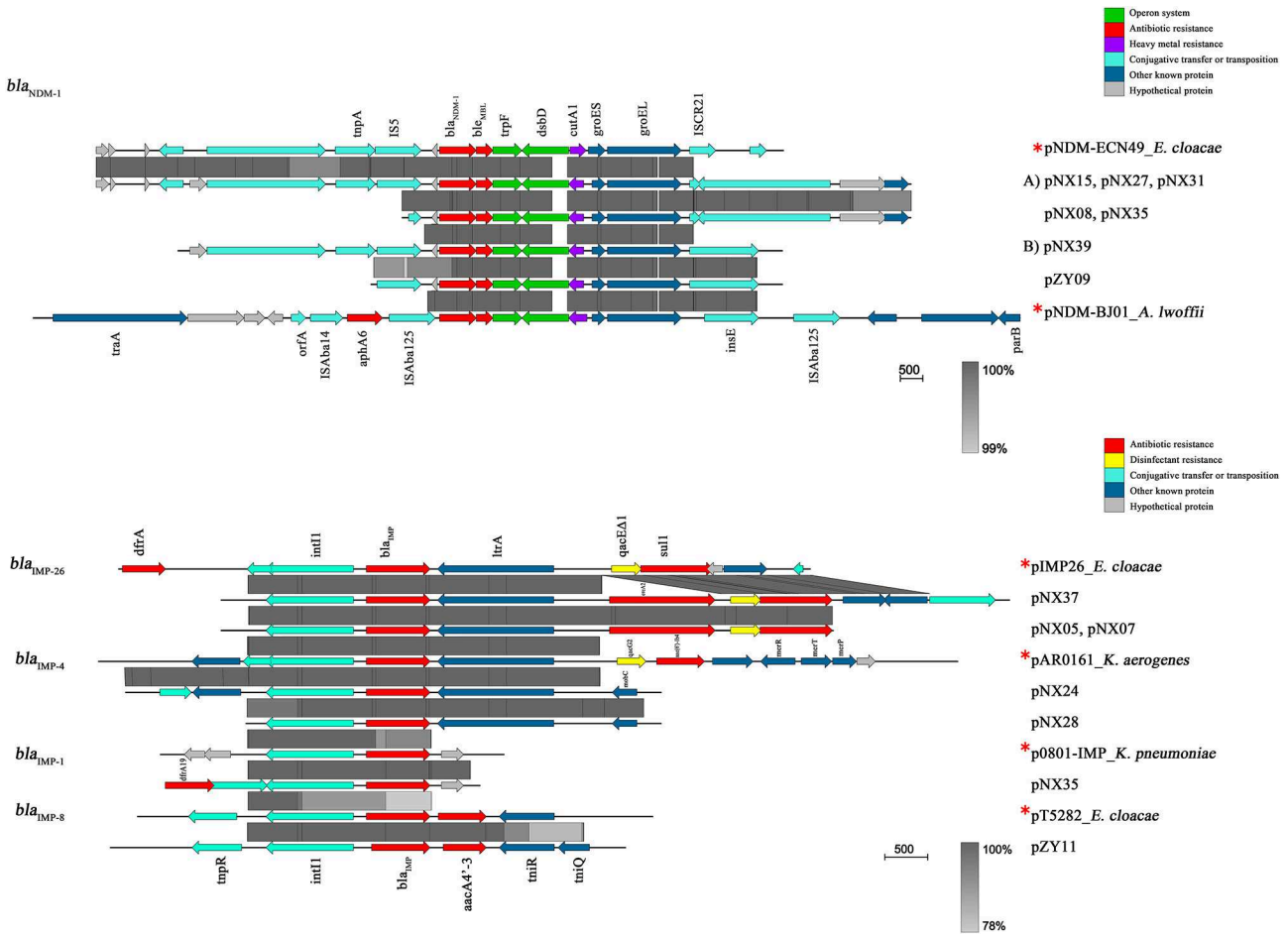


FIGURE 4 | Linear comparison of the representative plasmid sequences carrying NDM-1 or IMP. *Reference plasmids were as following: pNDM-1-ECN49 in *E. cloacae* (GenBank Accession No. KP765744), pNDM-BJ01 in *A. lwoffii* (GenBank Accession No. JQ001791), pIMP26 in *E. cloacae* (GenBank Accession No. MH399264), pAR0161 in *K. aerogenes* (GenBank Accession No. MF344574), p0801-IMP in *K. pneumoniae* (GenBank Accession No. KT345947), and pT5282 in *E. cloacae* (GenBank Accession No. MF344574). Block arrows indicate confirmed or putative open reading frames (ORFs) and their orientations. Arrow size is proportional to the predicted ORF length. Regions of homology are marked by gray shading. The color code is as follows: red, antibiotic resistance genes; purple, heavy metal resistance genes; yellow, disinfectant-resistance genes; green, operon; cyan, genes associated with the transfer; dark blue, other known genes; hypothetical and unknown genes are represented by light-gray arrows.

from a patient after cardiac surgery in Ningxia. This XDR *E. cloacae* isolate co-harbored bla_{SHV-12}, bla_{TEM-30}, bla_{LAP-2}, bla_{ACT-5}, *qnrS1*, *aac(6')-IIc*, *aadA6*, *aph(3')-Ia*, *aph(6)-Id*, *catA2*, *sul1*, and *dfrA10*. Resistance genes existed in multiple incompatibility groups, including IncN, IncHI2A, and IncHI2, which could further aggravate the dissemination under the selective pressure of antibiotics (**Supplementary Table 2**).

Besides, the absence or reduced expression of porins coupled with ESBL and/or AmpC overexpression, could lead to increased carbapenem MICs (Babouee Flury et al., 2016). In our study, most isolates had decreased membrane permeability caused by low expression of *ompC* or *ompF*, or both. Most isolates exhibited down-regulation of *ompF*, but up-regulation of *ompC*. These results were consistent with the previous reports and indicated that the decreased expression of *ompF* might lead to the potential role of OmpC-directed OM protein polarization (Majewski et al., 2016). Combinations of either ESBL or AmpC and porin loss played a pivotal role especially among carbapenemase-negative isolates. These isolates had lost at least one porin, among which 91.2% (31/34) of the ioslates produced different types of β-lactamase. Analysis of *ompC* expression showed a significant decrease among isolates only non-susceptible to imipenem. The decrease or loss of OmpC, a smaller pore size membrane porin compared to that of OmpF, might be correlated with the slightly increase in imipenem MIC (Lavigne et al., 2012). Hence, porin loss combined with ESBL and/or AmpC overexpression was one of the major mechanisms of drug resistance. Carbapenemase, ESBL and/or AmpC hyperproduction plus porin loss usually resulted in extensively high carbapenem MICs.

In this study, we observed significantly differential distribution and molecular characteristics of CREL isolates in two

geological regions. In Ningxia, China, MBLs, especially NDM-1, was identified in the highest proportion, indicating a great potential risk of spread of drug-resistant strains in northwest of China. However, lower prevalence of carbapenemase producers has been described in Guangdong, China during our investigation, and that approximately half of isolates showed intermediate resistance to one or two carbapenems. This phenomenon might be mainly due to additional β-lactamases and impaired permeability. No carbapenemase producer was found in hospital B (Guangdong Provincial Hospital of Chinese Medicine, Guangdong, China). The possible interpretation was that carbapenems might be utilized less frequently in these hospitals. In most cases, patients with infections caused by CREL usually suffer from critical basic diseases, lower immune function, and all kinds of invasive operations, and they may be less inclined to TCM hospitals. Another explanation, probably different genetic events among *E. cloacae* isolates have occurred over time in TCM hospitals and carbapenemase genes was very rare or unique. Besides, about other mechanisms, especially high level of AmpC β-lactamases plus porin loss, and overexpression of efflux pumps, would play a more important part in carbapenem resistance among these *E. cloacae* isolates. Therefore, a variety of mechanisms could induce resistance in varying degrees in the process of drug resistance in *E. cloacae*.

The most susceptible antimicrobials to CREL isolates was amikacin (94.0%), followed by tigecycline (86.9%), and polymyxin B (82.1%). Amikacin, an aminoglycoside group antibiotic, has been restricted in recent years due to its side effects such as nephrotoxicity and ototoxicity (Wargo and Edwards, 2014). Polymyxin B and tigecycline are currently the most active therapeutic option for carbapenem-resistant *Enterobacteriaceae* including CREL (Rafailidis and Falagas, 2014). However, polymyxin B and tigecycline resistance has sporadically occurred in recent years (Kumar, 2016; Karaiskos et al., 2017). We found 13 isolates resistant to polymyxin B and 11 isolates non-susceptible to tigecycline, but *mcr-1* and *tetX* were not detected. Resistance to polymyxins might be associated with alterations in the lipopolysaccharide structure and overexpression of efflux pumps (Lim et al., 2010). Besides, ribosome protection and excessive expression of efflux pumps might contribute to decreased susceptibility to tigecycline (Pournaras et al., 2016). Remarkably, polymyxin B heterogeneous subpopulations were detected in two isolates, indicating that more consideration should be taken to heterogeneously colistin resistant *E. cloacae*.

Our results suggested that NDM-1-harboring plasmids contained a highly conserved region around $bla_{\mathrm{NDM-1}}$ ($bla_{\mathrm{NDM-1}}$-ble_{MBL}-*trpF-dsbD-cutA1-groES-groEL*), which might be involved in the further spread of $bla_{\mathrm{NDM-1}}$. The highly similar genetic structure was also obtained from various NDM-1-encoding plasmids, such as *C. freudii* from China (Yang et al., 2015) and *K. pneumoniae* from Australia (Wailan et al., 2016). More importantly, the region flanking $bla_{\mathrm{NDM-1}}$ was highly homologous to some *Acinetobacter* spp. isolated from China (Bogaerts et al., 2013). Recent studies proposed that the acquisition of $bla_{\mathrm{NDM-1}}$ in *Enterobacteriaceae* might be derived from *Acinetobacter* spp. via horizontal transmission of drug-resistant plasmids (Bogaerts et al., 2013). These findings suggested that the potential dissemination of acquired $bla_{\mathrm{NDM-1}}$ gene among different bacterial species had become an emerging global threat. IMP-type MBL was located within a variety of integrons. Similar to previous studies, IMP-producing *E. cloacae* presented a diverse genomic environment due to the transfer and rearrangement of plasmids and integrons in all probability (Aoki et al., 2018). Different genetic events might occur among *E. cloacae* isolates and other isolates, leading to the diversity of integrons in our study. The presence of various integrons associated with plasmids could facilitate horizontal or clonal transmission among bacteria of different genera and species through conjugation.

PFGE revealed that 84 CREL isolates possessed 54 pulsotypes. Nineteen NDM-1-producing *E. cloacae* isolates obtained from Ningxia had a single dominant pulsotype. These predominated clones have obtained fast transmission in hepatobiliary surgery, vascular surgery, and ICU during a peak period (from Jan. 2016 to Sep. 2016), which might be the evidence of intra-hospital clonal dissemination. The further examining revealed that all outbreak strains were ST78, belonging to clonal complex CC74, which was considered as the most versatile genetic lineages among the world (Izdebski et al., 2015). The uniform genetic background among NDM-1-producing *E. cloacae* with conjugative IncX3 plasmids showed horizontal transmission between epidemic isolates. These results suggested that IncX3-type plasmids might contribute significantly to the outbreak of $bla_{\mathrm{NDM-1}}$ within *E. cloacae* in China. Previous studies have reported that the IncX3-type plasmid was a serious threat, particularly because of the global spread of NDM-1-producing *Enterobacteriaceae* spp (Yang et al., 2015). The outbreak strains producing NDM-1 co-harbored multiple resistance genes, including $bla_{\mathrm{ACT-5}}$, $bla_{\mathrm{OXA-1}}$, *sul1*, *dfrA15b*, *mphA*, *catB3*, *arr3* as well as the *qnrA1* and *aac(6')Ib-cr* genes encoding quinolone resistance. Studies have reported the emergency and spread of *E. cloacae* ST120, ST74, ST418, and ST88 producing NDM-1 in China (Liu et al., 2015; Jia et al., 2018; Jin et al., 2018). This implied that the $bla_{\mathrm{NDM-1}}$ gene might have been transmitted to multiple clones of *E. cloacae* in many parts of China. To the extent of our knowledge, this is the first identification of an outbreak of $bla_{\mathrm{NDM-1}}$ harboring *E. cloacae* ST78 isolates in China. Our study suggested that ST78 has become a successful hospital-related clone with a unique ability to accept plasmids for further dissemination in China. ST78 was one of the leading clonal lineages with increased epidemic potential, which might be associated with the spread of carbapenem resistance (Miyoshi-Akiyama et al., 2013). In summary, we reported that CREL isolates were still distributed popularly in Guangdong, China during our investigation. However, we emphasized the spread of NDM-1-producing *E. cloacae* ST78 with contribution of IncX3 plasmids in Ningxia, China. The outbreak has provided a new model for the dissemination of the $bla_{\mathrm{NDM-1}}$-haboring *E. cloacae* in China. Therefore, epidemiological surveillance of resistance is vital and should be performed routinely, especially in high-risk departments.

MLST distinguished 42 STs with ST78 (32.1%), ST51 (8.3%), ST1226 (6.0%), and ST114 (4.8%), and being the predominant

STs. Previous studies revealed that ST66, ST78, ST108, and ST114 were the most prevalent and widespread *E. cloacae* STs (Izdebski et al., 2015). Our study showed that 36.9% (31/84) of the isolates belonged to the potentially high-risk international clones, with ST78 prevailing. We found that *E. cloacae* ST78 producing NDM-1 with a higher epidemic potential might be more prone to cause severe drug-resistant outbreaks. The ST78 clones demonstrated various β-lactamase profiles. Early studies also indicated a lack of strict correlation between β-lactamase profiles and ST78. The ST78 *E. cloacae*, originally identified in Japan, was a major international clone. The ST78 clones was widely distributed and independently obtained plasmids with β-lactamase genes, making specific pulsotypes more suitable for further spread (Miyoshi-Akiyama et al., 2013). In addition, seven ST51 isolates belonged to CC234, one of dominant clonal groups with high prevalence and wide distribution. Four ST114 isolates were diversified into multiple pulsotypes and caused some sporadic isolates. As the central genotype of CC114, ST114 has a wide global distribution formerly detected in Japan, France, Italy, Spain, Greece and Israel, mainly associated with $bla_{\mathrm{CTX-M-15}}$ (Izdebski et al., 2015). Moreover, we identified a novel ST type, ST1226 detected in 5 isolates with highly similar pulsotypes, and might be related to the carriage of $bla_{\mathrm{IMP-26}}$ in our study. Strikingly, we described in detail one *E. cloacae* ST97, which co-harbored $bla_{\mathrm{NDM-1}}$ and $bla_{\mathrm{IMP-1}}$ as well as multiple resistance genes.

Compared with carbapenem-resistant *K. pneumoniae* (CRKP), our study demonstrated that NDM-1-type MBL was the most commonly identified in *E. cloacae*. Recent surveillance data in China indicated that NDM was predominant in *E. coli* and *E. cloacae*, whereas KPC appeared to be most common in *K. pneumoniae* (Wang et al., 2018). Most previous studies revealed that the substantial burden of KPC-type carbapenemase mainly lied in North America, Latin America and Europe (Munoz-Price et al., 2013). However, NDM-1-mediated carbapenem resistance was widespread in Asia, such as India, Pakistan, and China (Nordmann et al., 2012). In contrast to the close connection between ST258/ST11 and bla_{KPC} in *K. pneumoniae* (Liu et al., 2018), $bla_{\mathrm{NDM-1}}$ apparently has a higher association with IncX3-type plasmids regardless of ST type in *E. cloacae*. Diverse clones of NDM-1-carrying *E. cloacae* have been widely distributed in geography. Evidence showed that clonal expansion was the cause of most transmitted cases. Thus the rapid spread of NDM-1-producing *E. cloacae* has comprised one of the greatest challenges to global health.

This study had several limitations. Our collection may not represent the prevalence and evolution of carbapenem-resistant *E. cloacae* in China. Our study lacks a comprehensive analysis of clinical risk factors. We were unable to determine complete genetic structures owing to the limitation of short-read sequencing. Long-read sequencing techniques would provide more detailed analysis of integron structures and plasmid backbones.

In conclusion, our study revealed that carbapenemase, ESBL and/or AmpC overexpression combined with porin loss were the primary mechanisms responsible for carbapenem resistance. Moreover, we initially reported the nosocomial outbreak caused by NDM-1-producing *E. cloacae* ST78 in Northwest China. Therefore, it emphases a critical concern to monitor and control the further spread of NDM-1 in China.

AUTHOR CONTRIBUTIONS

BH, LC, and Y-WT designed the study. YC, XY, KLa, MZ, XM, and YH carried out the experiments. CC, WJ, JZ, KLi, PG, and WZ analyzed the data. YC wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01610/full#supplementary-material

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