



Stool Samples of Acute Diarrhea Inpatients as a Reservoir of ST11 Hypervirulent KPC-2-Producing *Klebsiella pneumoniae*

Beiwen Zheng,^a Hao Xu,^a Tao Lv,^a Lihua Guo,^a Yu Xiao,^a Chen Huang,^a Shuntian Zhang,^a Yunbo Chen,^a Huiming Han,^{b,c} Ping Shen,^a  Yonghong Xiao,^a Lanjuan Li^a

^aCollaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China

^bBasic Medical College, Beihua University, Jilin, China

^cThe Clinical Immunology Research Center, Beihua University, Jilin, China

Beiwen Zheng, Hao Xu, and Tao Lv contributed equally to this work. Author order was determined in order of decreasing seniority.

ABSTRACT The emergence and spread of carbapenem-resistant hypervirulent *Klebsiella pneumoniae* sequence type 11 (ST11-CR-HvKP) in China are a great concern in the public health community. However, the underlying mechanism that enables its wide dissemination in China remains unclear. Here, we investigated the prevalence of carriage of carbapenemase-producing *Enterobacteriaceae* (CPE) among inpatients with diarrhea in a teaching hospital over 1 year to identify ST11-CR-HvKP reservoirs and to understand the genetic background and plasmid profiles of these pathogens. As assessed by stool analysis, the CPE colonization rate (12.4%) among the inpatients with diarrhea was high (12.4%). Antibiotic exposure, surgical history, and CPE positivity were correlated. Genomic investigation of 65 carbapenem-resistant *K. pneumoniae* isolates indicated a shared bacterial population in various wards. According to maximum likelihood phylogenetic tree analysis, these isolates were partitioned into three major clades. Analysis of the *wzi* locus revealed three different K types (KL105, KL47, and K64) among the ST11 isolates, indicating the genetic diversity of these isolates. Genetic and sequence mapping revealed the complexity of virulence and resistance plasmid sets harbored by the isolates. These observations indicate that the dissemination of resistant bacteria is more complex than initially anticipated and possibly involves multiple *K. pneumoniae* ST11 lineages and a variety of virulence plasmids. Collectively, we show for the first time that stool may be a source of ST11-CR-HvKP isolates. Furthermore, the findings reveal the silent dissemination of ST11-CR-HvKP bacteria in Zhejiang Province, China. Future investigations are warranted to determine the association between rectal colonization by ST11-CR-HvKP and clinical infections.

IMPORTANCE China has been experiencing a rapid increase in the number of nosocomial infections caused by carbapenem-resistant *Klebsiella pneumoniae* ST11 (ST11-CRKP) for decades. The emergence of hypervirulent ST11-CRKP (ST11-CR-HvKP) strains is expected to become a serious public health issue in China, considering that carbapenem resistance and virulence have converged in an epidemic clone. *K. pneumoniae* strains that colonize the human intestinal tract may become a reservoir of virulence and carbapenemase-encoding genes. Here, we first characterized the genotypes and antimicrobial phenotypes of ST11-CR-HvKP strains isolated from diarrheal stool samples of inpatients in Zhejiang Province, China. Active surveillance approaches based on the findings of the present study should be implemented, particularly in intensive care units, to combat the spread of ST11-CR-HvKP and to improve treatment.

KEYWORDS gut, hypervirulent, KPC-2, reservoir, genomic characterization

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Address correspondence to Yonghong Xiao, xiaoyonghong@zju.edu.cn.

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Carbapenem-resistant *Klebsiella pneumoniae* (CRKP), which is associated with high mortality rates of up to 50%, has been identified by the World Health Organization as a critical-priority organism (1, 2). Hypervirulent *K. pneumoniae* (HvKP) causes life-threatening infections, and the *rmpA* and *rmpA2* genes are associated with its pathogenicity (3). Recently, a fatal outbreak of sequence type 11 (ST11) carbapenem-resistant, hypervirulent *K. pneumoniae* (ST11-CR-HvKP) in China generated great concern from the public health community (4). The identification of this ST11-CR-HvKP strain demonstrated that carbapenem resistance and virulence had converged in an epidemic clone that could become a serious public health issue (5). Subsequently, cases of ST11-CR-HvKP infection have been documented in diverse regions in China (6–8). These reports suggest that this clone is widespread in China; however, the underlying mechanism that enables its wide dissemination remains unclear.

Intestinal tract colonization by carbapenemase-producing *Enterobacteriaceae* (CPE) may lead to a subsequent nosocomial infection in at-risk patients (9–11). We hypothesized that a relatively silent CPE gastrointestinal reservoir could exist in inpatients with diarrhea and could potentially be involved in the transmission of ST11-CR-HvKP. Accordingly, the aims of the present study were to investigate the prevalence of CPE carriage among inpatients with diarrhea in a teaching hospital in China over 1 year, to identify ST11-CR-HvKP reservoirs, and to explore the genomic complexity of the highly transmissible ST11-CR-HvKP clone in Zhejiang Province.

RESULTS

During the study period, we sampled 811 nonduplicate stool samples from 443 inpatients with diarrhea and screened them for the presence of CPE strains. Ultimately, 87 CPE isolates from 55 patients were included in the study. This identification frequency indicated a high CPE colonization rate (i.e., 12.4% [55/443]) among these inpatients (see Fig. S1 in the supplemental material). Of these 87 isolates, *K. pneumoniae* was the most prevalent species ($n = 65$), followed by *Proteus mirabilis* ($n = 6$) and *Escherichia coli* ($n = 6$) (Table S1). PCR and sequencing revealed that 77 isolates carried *bla*_{KPC-2}, 5 isolates carried *bla*_{NDM-5}, 2 isolates carried *bla*_{NDM-1}, 2 isolates carried *bla*_{IMP-4}, and 1 isolate carried *bla*_{IMP-26}. Furthermore, 44 isolates harbored extended-spectrum β -lactamase (ESBL) genes (Table S2). All CPE isolates were multidrug resistant (i.e., showed phenotypic resistance to three or more drug classes) (Fig. S2). Most of these CPE isolates were resistant to imipenem (99%) and susceptible to colistin (92%) and tigecycline (91%).

Patients in medical intensive care units (ICUs) typically experience a prolonged hospital stay, which results in exposure to potential risk factors. Not surprisingly, therefore, most of the colonization cases were observed among patients from medical ICUs (Table S1). Colonization was detected in 13 wards: 49% (43/87) of cases were from the ICU, 9% were from the hepatobiliary and pancreatic surgery ward, 8% were from the emergency ICU, and the remaining 33% were from 10 other units. Forty-two (76%) of the inpatients were male, and the median inpatient age was 64 years (range, 16 to 97 years). Interestingly, correlations were detected between antibiotic exposure (including linezolid, a β -lactam- β -lactamase inhibitor combination, and carbapenems), surgical history, and being CPE positive (Table 1).

To better define the population structure of the 65 CRKP isolates, we further investigated their comprehensive molecular characteristics. Remarkably, multilocus sequence typing (MLST) analysis revealed that the *K. pneumoniae* isolates belonged to four STs, indicating low diversity (Fig. 1 and Table S1). ST11 was the predominant ST (58/65; 89%), followed by ST37 ($n = 5$), ST15 ($n = 1$), and ST107 ($n = 1$). A notable feature was that patient P1 (single individual) carried two STs, ST37, which was isolated from three different samples, and ST11, which was isolated from eight samples, over the entire study period. Furthermore, 10 patients in the study carried multiple isolates. Several major pulsotypes were found via pulsed-field gel electrophoresis (PFGE) analysis, which is consistent with the MLST results; however, genetic diversity was observed in the profiles (Fig. S3). Roary matrix-based gene sequence analysis generated a large

TABLE 1 Characteristics of the study participants and logistic regression analysis of the carbapenemase-producing *Enterobacteriaceae* strains isolated from stool samples from these inpatients

Variable	Value for group		<i>P</i> ^a	<i>P</i> ^b	OR (95% CI)
	CPE positive	CPE negative			
No. of patients (%)	55 (12)	388 (88)			
No. of male patients (%)	42 (76)	255 (66)	0.116	0.717	1.143 (0.556–2.350)
Median age (yr) ^c (range)	64 (16–97)	55 (13–99)	0.047 ^c	0.677	1.146 (0.603–2.179)
No. of patients (%) with:					
Surgery	16 (29)	54 (14)	0.004	0.047	2.078 (1.008–4.282)
Abdominal pain	13 (24)	130 (34)	0.14	0.578	0.818 (0.402–1.665)
Fever	43 (78)	244 (63)	0.026	0.595	1.257 (0.541–2.919)
No. of hospital-acquired infections (%)	32 (58)	199 (51)	0.124	0.958	0.97 (0.319–2.951)
No. receiving antibiotic therapy (%)					
Cephalosporins	15 (27)	73 (19)	0.141	0.349	1.4 (0.692–2.833)
Carbapenems	42 (76)	221 (57)	0.006	0.156	1.739 (0.81–3.732)
Cephamycin	0 (0)	15 (4)	0.234		
Aminoglycosides	5 (9)	38 (10)	0.869		
Macrolides	3 (6)	18 (5)	0.735		
Glycopeptides	20 (36)	108 (28)	0.192	0.674	1.151 (0.598–2.218)
Oral vancomycin	8 (15)	32 (8)	0.127	0.705	1.196 (0.473–3.021)
Fosfomycin	1 (2)	23 (6)	0.339		
Linezolid	23 (42)	56 (14)	<0.001	0.002	2.871 (1.461–5.642)
Quinolone	16 (29)	160 (41)	0.085	0.174	0.616 (0.306–1.238)
Sulfamethoxazole	4 (7)	68 (18)	0.054	0.457	0.637 (0.194–2.089)
β-Lactam–β-lactamase inhibitor	45 (82)	249 (64)	0.01	0.039	2.276 (1.043–4.969)
Tigecycline	15 (27)	63 (16)	0.044	0.67	0.849 (0.401–1.798)

^aUnivariate analysis; categorical variables were compared using a χ^2 test.

^bMultivariate analysis of variables ($P \leq 0.20$) in the univariate analysis was performed using stepwise backward logistic regression. Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated.

^c*P* values were calculated based on a comparison between the ages of ≥ 60 and < 60 years using a χ^2 test.

pangenome of 10,355 gene clusters across 65 full genomes (Fig. S4). A maximum likelihood phylogenetic tree demonstrated that the 65 strains were partitioned into three major clades (Fig. 1). Seven non-ST11 isolates were clustered into two separate clades, and the 58 ST11 isolates were grouped into three clusters, despite their high similarity. An analysis of the *wzi* locus revealed three different K types (KL105, KL47, and K64) among the ST11 isolates, indicating genetic diversity among these isolates. Of note, ST11 isolates were detected in patients from 12 wards. A review of the cases revealed that these individuals had no contact with one another, lowering the probability of nosocomial transmission.

A broad array of resistance genes associated with various antimicrobials was identified in the *K. pneumoniae* genomes (Fig. 2). Isolates encoding carbapenemases (44/65; 68%) also harbored predicted ESBLs. Among these ESBLs, CTX-M-65 was the most predominant cluster (34/65; 52%), followed by CTX-M-14 (5/65; 8%). Virulence gene analysis revealed that the *K. pneumoniae* isolates carried genes for 45 known virulence factors (Fig. 3). As anticipated, the prevalence of biofilm-encoding type 3 fimbria cluster (*mrk*)- and yersiniabactin (*ybt*)-associated genes was high.

Using *in silico* analysis, we identified 14 *rmpA*- and 19 *rmpA2*-positive ST11-CRKP isolates. Interestingly, S1 nuclease PFGE (S1-PFGE) and Southern blot analyses clearly showed that the ST11-CR-HvKP isolates carried at least four types of *rmpA*- and *rmpA2*-positive plasmids, ranging in size from approximately 146 kb to approximately 218 kb (Fig. 4). The *rmpA* and *rmpA2* genes coexisted on the same plasmid in 14 isolates. We used Pacific Biosciences (PacBio) sequencing to generate five complete *rmpA*- and *rmpA2*-carrying IncHI1B plasmid sequences, which aligned well with the virulence plasmid pLVPK (GenBank accession no. [AY378100](https://www.ncbi.nlm.nih.gov/nuccore/AY378100)), a 219-kb plasmid that harbors a set of virulence genes, including *iroBCDN*, *iucABCD*, *rmpA*, and *rmpA2* (Fig. 5).

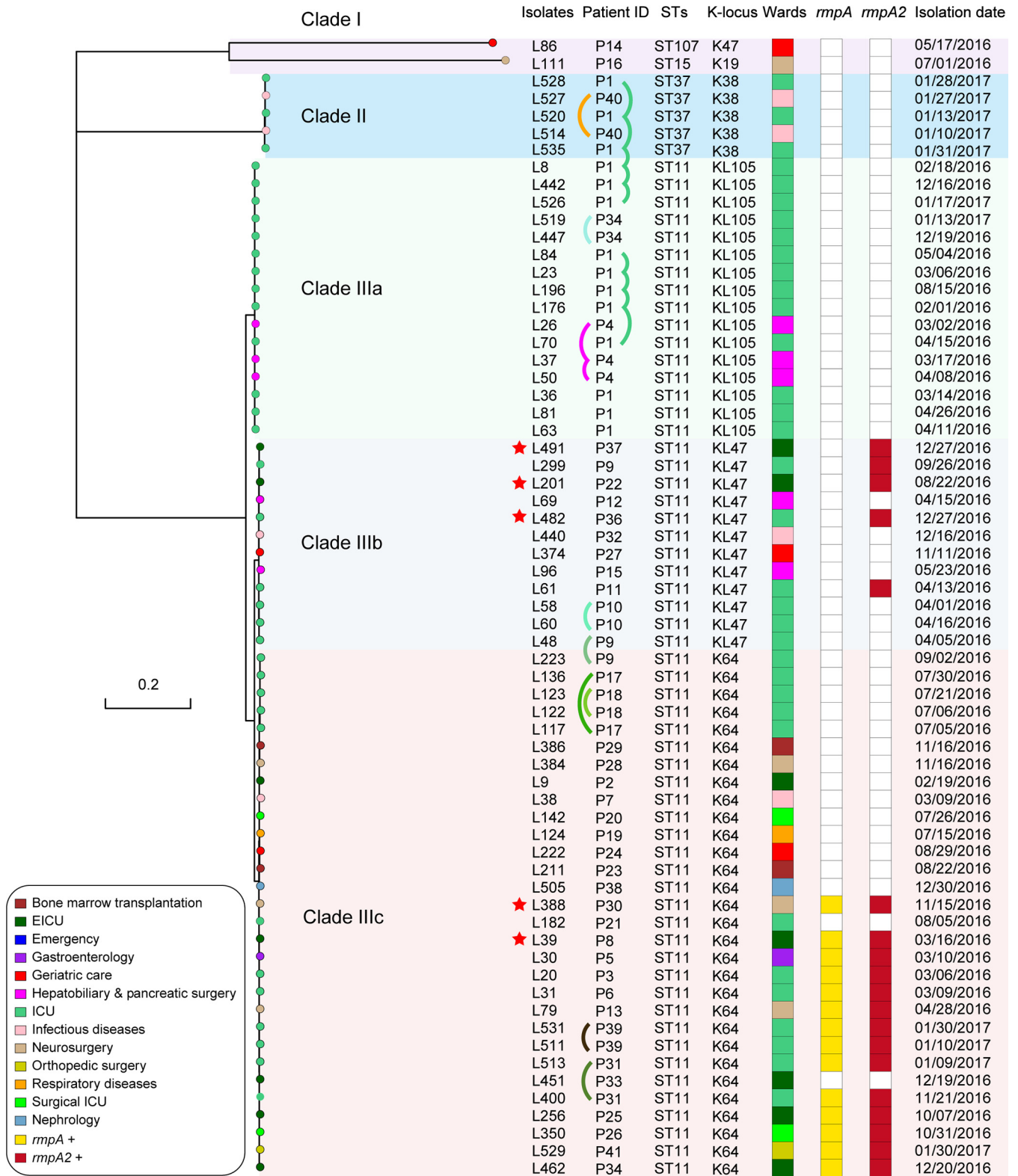


FIG 1 Core-genome phylogeny of the CRKP isolates, associated MLST data, capsule locus genotype, ward of isolation, virulence genes present, and isolation date. The maximum likelihood phylogeny tree is based on single-nucleotide polymorphisms in the core genomes of 65 CRKP isolates. The scale bar indicates nucleotide divergence. The origins of the isolates are shown in different colors. Genome clusters are shaded using different colors. The presence of the *rmpA* and *rmpA2* genes is indicated. The curved lines indicate isolates from the same individual. The red asterisks indicate the isolates that were analyzed by PacBio sequencing. EICU, emergency intensive care unit.

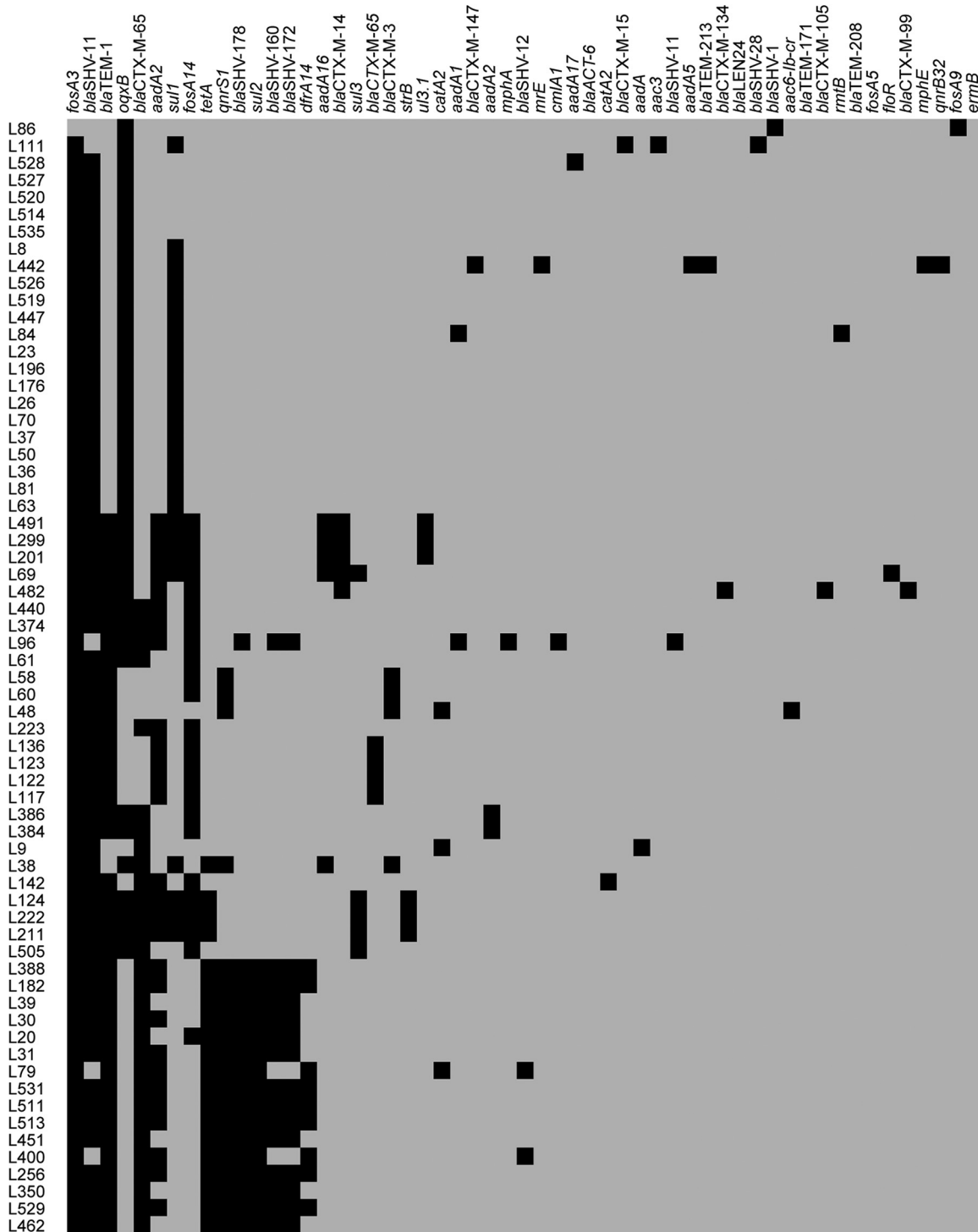


FIG 2 Antimicrobial resistance genes identified in the genomes of CRKP isolates by analyzing the WGS data. The resistance genes are shown at the top, with their presence indicated in black.

DISCUSSION

It is well recognized that intestinal tract colonization by Gram-negative bacteria may lead to a subsequent nosocomial infection in high-risk patients (12). In a recent large cohort study, Frencken et al. (13) demonstrated that rectal carriage of a Gram-negative pathogen is strongly associated with an increased risk of ICU-acquired bacteremia, suggesting that routine surveillance for gut colonization by Gram-negative bacteria

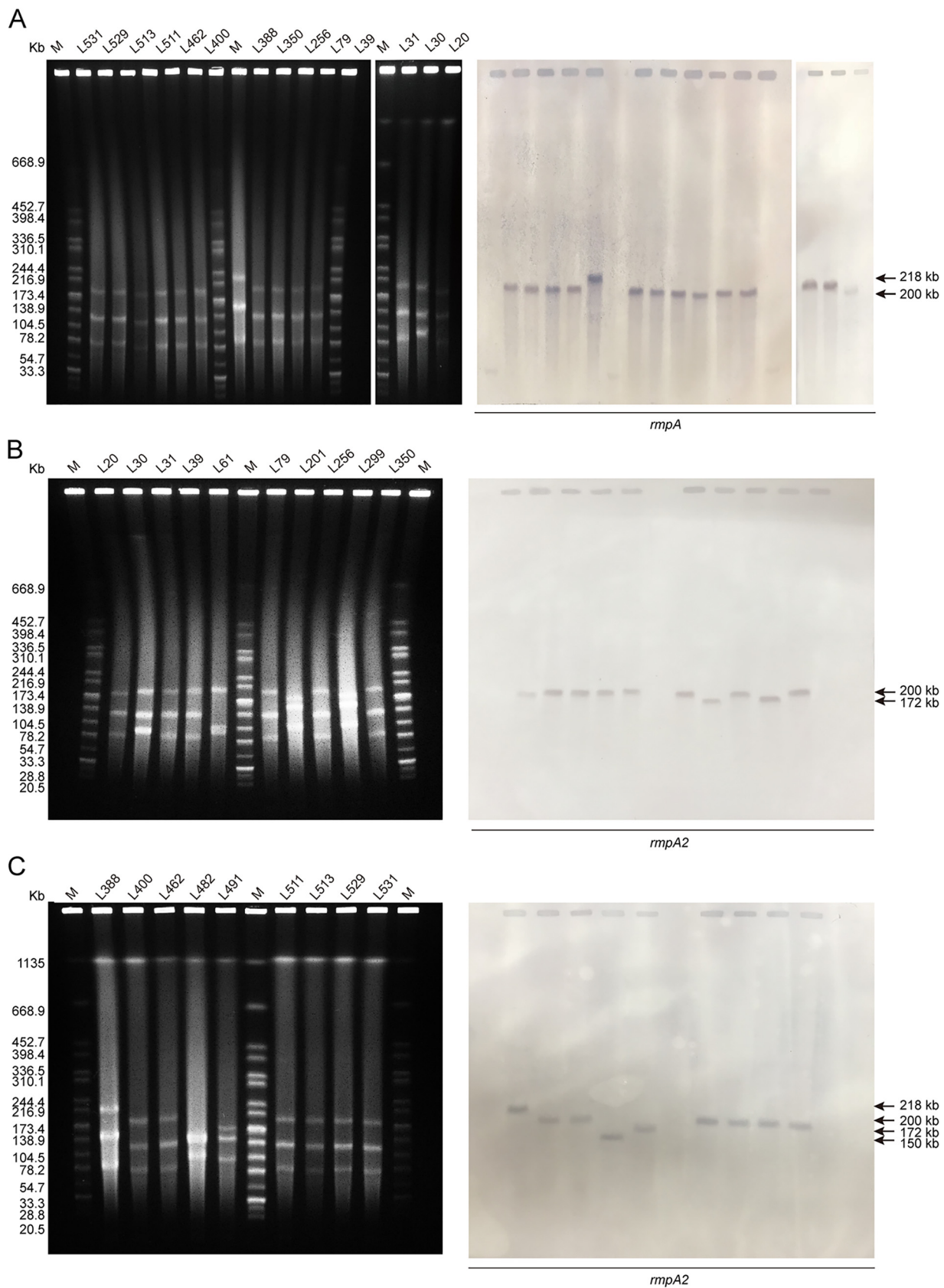


FIG 4 Analysis of plasmids harbored by the ST11-CR-HvKP isolates. The plasmids were analyzed by S1-PFGE and Southern blotting using specific *rmpA* and *rmpA2* probes. (A) Analysis of *rmpA*-carrying isolates. (B and C) Analysis of *rmpA2*-carrying isolates. The arrows indicate the locations of virulence plasmids. S1-PFGE revealed that most of the *K. pneumoniae* strains analyzed harbored three plasmids. M, marker.

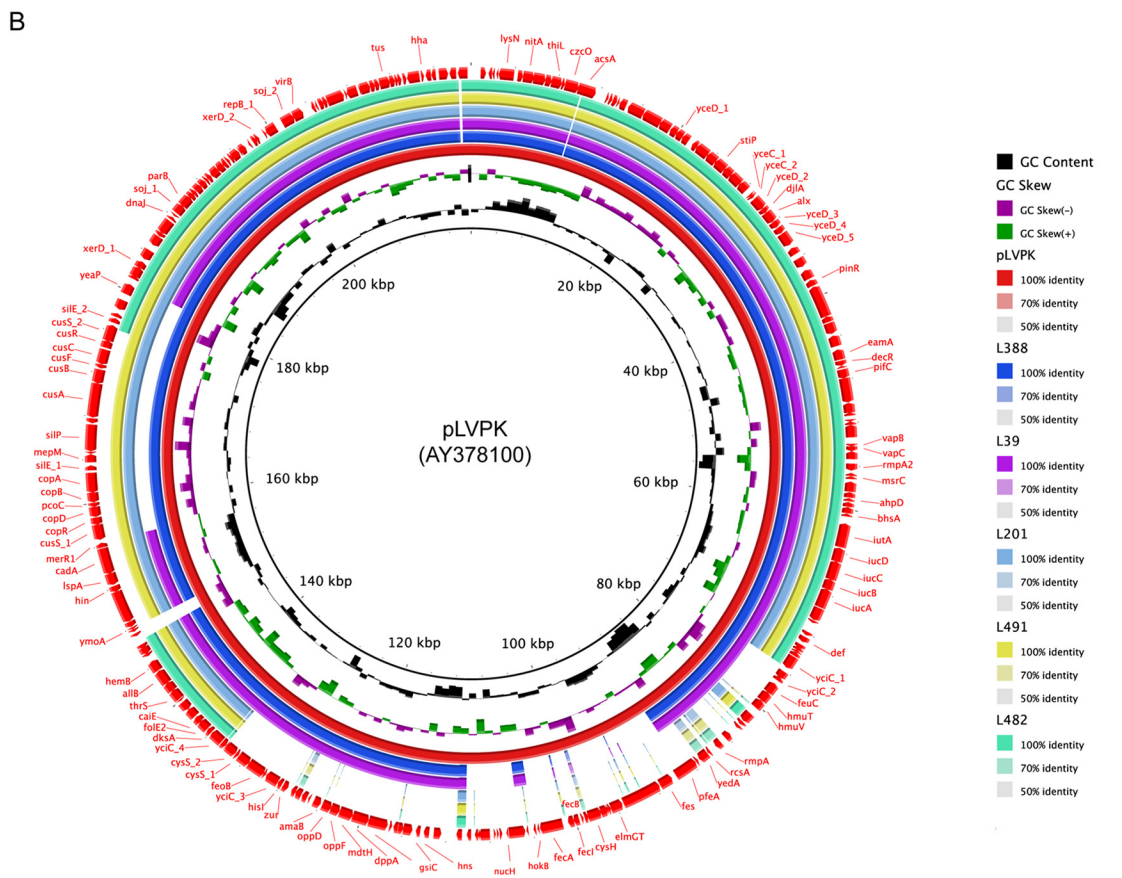
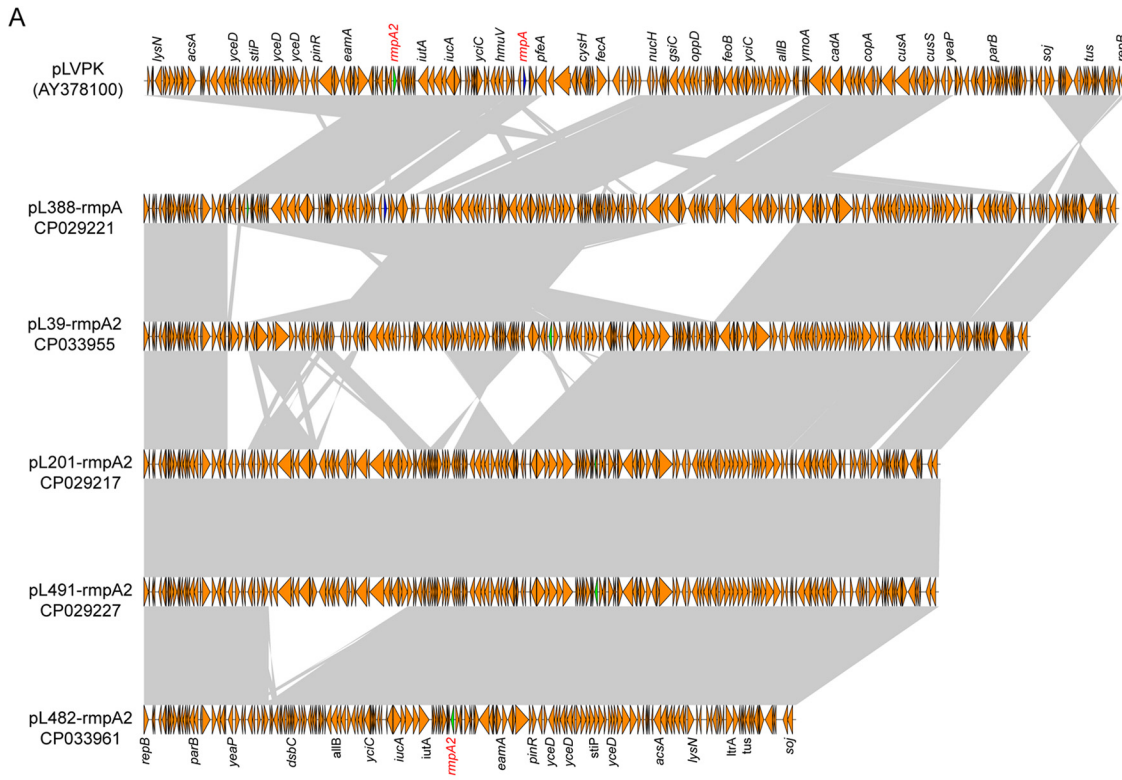


FIG 5 Alignment of the *rmpA*- and *rmpA2*-harboring plasmids recovered in the present study. The circular map was generated using the BLAST Ring Image Generator. (A) Colinear genome alignment of pLVPK (GenBank accession no. AY378100) with five *rmpA*- and *rmpA2*-harboring plasmids. (B) Genomic map of the *rmpA*- and *rmpA2*-harboring plasmids.

may have clinical implications. Therefore, we designed the present study as a prospective survey to evaluate gut colonization by CPE and the associated risk factors among inpatients with diarrhea. We also performed an in-depth characterization of the gut flora CPE isolates.

ST11 is the most prevalent ST among CRKP isolates in China and accounts for up to 59.8% of CRKP isolates (14). We previously reported that 89.9% (124/138) of CRKP strains isolated from a university-affiliated hospital in 2014 carry the *bla*_{KPC-2} gene (15). Furthermore, *K. pneumoniae* ST11 undergoes rapid clonal expansion, resulting in regional outbreaks (16), which poses a challenge to patients, clinicians, and public health. The first ST11-CRKP strain in China was isolated at the First Affiliated Hospital of Zhejiang University (FAHZU) in 2004 (17). In the past few decades, China has witnessed the emergence and subsequent rapid increase in the number of ST11-CRKP nosocomial infections (14). HvKP is rarely resistant to antibiotics. The occurrence of ST11-CR-HvKP threatens the effective management of infections caused by this clinically untreatable pathogen, which could become a serious public health problem and even cause a global CR-HvKP epidemic (5). However, the role of the gut microbiota in the rapid spread of ST11-CR-HvKP has not yet been elucidated. Experimental evidence from the present study, as well as from retrieved clinical data, indicated a disturbing rate of carriage of ST11-CRKP (8.6%) among hospitalized patients with acute diarrhea, particularly in ICU wards. Furthermore, the findings of the present study suggest that stool samples could be a major reservoir of ST11-CR-HvKP. Active surveillance of nondiarrheal patients is required to compare their rates of CPE colonization with those of diarrheal patients and to investigate whether the implementation of contact precautions or other interventions would improve outcomes.

Not surprisingly, in the present study, the majority of colonization cases were observed among patients in the medical ICU. Recently, a systematic review of the clinical epidemiology of CRE identified 13 risk factors associated with CRE acquisition (18). These are medical devices, carbapenem use, invasive procedures, ICU admission, cephalosporin use, glycopeptide use, CRE exposure, underlying diseases, quinolone use, β -lactam use, mechanical ventilation, demographic patient characteristics, and exposure to hospital care. The findings of the present study are consistent with those observations, although we focused on gut colonization by CPE and not on CPE-associated infections. To the best of our knowledge, an association between linezolid exposure and CPE colonization in patients has not been reported to date. The lack of such an association might be explained by the wide use of linezolid, which eliminates Gram-positive bacteria and disrupts the gut microflora, thus promoting gut colonization by *Enterobacteriaceae*, especially by CPE strains.

A previous investigation speculated that the emergence of ST11-CR-HvKP occurred due to a single genetic event in which a pLVKP-like virulence plasmid was acquired by an ST11-CRKP strain that presumably carried a common plasmid such as pKPC-CR-HvKP4 (4). The observed complexity in the existence of virulence plasmids and resistance plasmids in the ST11-CRKP isolates indicates that this process was more complicated than was previously anticipated, as it may have involved multiple ST11 *K. pneumoniae* lineages and a variety of virulence plasmids.

This study has several limitations. First, our study is limited by its single-institution design and 1-year study period; therefore, the findings of this study should be interpreted with caution and might not be generalizable to other hospitals. Second, although we identified 19 fecal ST11-CR-HvKP colonization isolates, none of the colonized patients had ST11-CR-HvKP infections. Third, the findings probably reflect the status of diarrheal patients, i.e., individuals with a disrupted gut microbiota. A previous investigation described the high prevalence and mortality rate of ST11-CR-HvKP meningitis in FAHZU (8), and K64 was also the most common serotype detected in our study. Future investigations addressing the transition from ST11-CR-HvKP carriage to infection in high-risk patients are crucial for the management of such infections.

In conclusion, our genomic epidemiological investigation demonstrated that stool samples of hospitalized patients served as a reservoir of CPE isolates, particularly

ST11-CR-HvKP. Because of the serious clinical outcomes of CRKP infections, more information is needed to understand the potential risk of ST11-CR-HvKP spreading by inpatients and to develop measures for the surveillance or control of these risks.

MATERIALS AND METHODS

Study design. A prospective, observational cohort study involving inpatients with diarrhea was performed between February 2016 and February 2017 (see Fig. S1 in the supplemental material) at the First Affiliated Hospital of Zhejiang University (FAHZU), China. FAHZU is the largest tertiary teaching hospital in Zhejiang Province, with 2,500 beds. *Enterobacteriaceae* isolates cultured from the first collected clinical samples of the respective inpatients were collected. Duplicate isolates (the same species isolated from material from the same patient within 2 weeks of the first positive culture) were excluded. All CPE isolates underwent antimicrobial susceptibility testing, molecular typing, and whole-genome sequencing (WGS). Clinical data for all participants were extracted from the clinical and medical record system using centralized queries. Information on demographic data, characteristics at admission, surgical intervention, hospital-acquired infections, and antibiotic exposure during the hospital stay were collected for CPE carriers and noncarriers.

Bacterial isolation and identification. Fecal samples (1.0 g) were diluted in 5 ml of sterile Luria-Bertani broth and cultured overnight at 37°C. The cultures grown overnight were plated on MacConkey agar supplemented with 1 mg/liter meropenem for 18 to 24 h at 37°C to isolate potential carbapenem-resistant *Enterobacteriaceae* (CRE) strains. Colonies with different morphologies were repeatedly streaked on MacConkey agar with meropenem to obtain pure isolates. Bacterial species were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry and 16S rRNA gene sequencing using universal prokaryotic primers. CRE isolates were confirmed by susceptibility testing in the presence of imipenem, ertapenem, and meropenem via a broth microdilution method (19). Genomic DNA was extracted from cultures grown overnight using the Qiagen (Hilden, Germany) blood/tissue kit. CPE isolates were identified by detecting carbapenemase-encoding genes (*bla_{NDM}*, *bla_{KPC}*, *bla_{IMP}*, and *bla_{VIM}*). The genes were amplified by PCR, with 1 μ l of genomic DNA from each isolate as a template, and the nature of the amplicons was then confirmed by sequencing (20).

Characterization of HvKP. The presence of the virulence-associated genes *rmpA* (regulator of mucoid phenotype A), *rmpA2* (activator for capsule biosynthesis), *iucA*, and *iutA* (21, 22) was examined in HvKP strains by PCR. The PCR products were first visualized by 1% agarose gel electrophoresis, and the nature of the amplicons was then confirmed by direct Sanger sequencing. Furthermore, the hypermucoviscous phenotype was analyzed by using the string test, as described elsewhere (23). The cutoff criterion for positivity in the string test was a viscous string length of >5 mm.

Risk factor analysis. To investigate the risk factors associated with fecal carriage of CPE, carriers were compared with noncarriers in terms of exposure to different variables. Categorical variables were expressed as percentages and compared using the chi-squared test or two-tailed Fisher exact test, as appropriate. Independent predictors for CPE were examined by logistic regression analysis. Variables with a *P* value of ≤ 0.2 (univariate analysis) were included in a logistic regression model to identify the variables with either a negative or a positive impact on the CPE colonization of inpatients with acute diarrhea. The strength of associations was determined by calculating the odds ratios (ORs) and 95% confidence intervals (CIs). Variables with a two-tailed *P* value of < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 24.0.

Antimicrobial susceptibility testing. MIC values of 14 antimicrobial agents (amikacin, aztreonam, cefotaxime, cefpirome, ceftazidime, ciprofloxacin, colistin, fosfomycin, gentamicin, imipenem, meropenem, piperacillin-tazobactam, tobramycin, and tigecycline) were determined by an agar dilution method, except for colistin and tigecycline, for which the broth microdilution method was used. The results were interpreted using Clinical and Laboratory Standards Institute guideline breakpoints (19). Standard reference strains of *Escherichia coli*, ATCC 25922, and *K. pneumoniae*, ATCC 700603, were used for quality control.

Molecular typing of KPC-2-producing *K. pneumoniae* isolates. The genetic relatedness of *K. pneumoniae* isolates was assessed by pulsed-field gel electrophoresis (PFGE). Briefly, DNA fragments were separated by 1% agarose gel electrophoresis (SeaKem gold agarose; Lonza, USA) at 14°C and 6 V/cm and with an alternating pulse at 120°, with a 2- to 40-s pulse-time gradient, for 22 h in 0.5 \times Tris-boric acid-EDTA buffer using a Chef apparatus (Bio-Rad, USA). *Salmonella enterica* serotype Braenderup H9812 was used as a size marker (24). The PFGE pattern dendrogram was constructed using BioNumerics version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium) with UPGMA (unweighted pair group method using average linkages) clustering. Isolates with a similarity cutoff of $\geq 80\%$ were considered to be a pulsotype.

Illumina sequencing and sequence assembly. To characterize the genetic features and resistome of KPC-2-producing *K. pneumoniae* isolates, WGS was performed. Genomic DNA was extracted from cultures grown overnight using the Genra Puregene Yeast/Bact kit (Qiagen). The extracted DNA was evaluated by 1% (wt/vol) agarose gel electrophoresis, and its concentration and purity were determined using a NanoDrop 2000 UV-visible (UV-Vis) spectrophotometer (Thermo Scientific, Waltham, MA, USA) and a Qubit version 2.0 fluorometer (Thermo Scientific). DNA was stored at -20°C until further processing. The sequencing library was prepared by using a Nextera XT kit (Illumina, San Diego, CA, USA) and sequenced using the Illumina HiSeq X 10-PE150 platform (Illumina). A-tailed fragments were ligated with paired-end adaptors and PCR amplified with a 500-bp insert. A mate pair library with an insert size of 5 kb was used for library construction by Beijing Novogene Bioinformatics Technology Co., Ltd. PCR adaptor reads and low-quality reads from the paired-end

and mate pair library were filtered during a quality control step using the Novogene pipeline. Paired reads were then assembled into scaffolds using Velvet version 1.2.10 (25).

Long-read Pacific Biosciences sequencing and assembly. To elucidate the genetic environment of *K. pneumoniae* isolates carrying the *rmpA* and *rmpA2* genes, five *rmpA*- and *rmpA2*-positive isolates were selected based on the size of the *rmpA*- and *rmpA2*-carrying plasmids and analyzed by PacBio sequencing. DNA extracted from isolates L39, L201, L388, L482, and L491 was sequenced by using long-read single-molecule real-time (SMRT) sequencing technology and the PacBio (Menlo Park, CA, USA) RS II platform. Briefly, the extracted high-quality double-stranded DNA (10 μ g) was sequenced using P6-C4 chemistry, PacBio RS II instrumentation, and a complexed 20-kb SMRTbell library. Unicycler was used for a hybrid assembly of three bacterial genomes from a combination of Illumina short reads and PacBio long reads (26). To generate the best assembly, Unicycler was operated in three different modes (conservative, normal, and bold) that alter the cutoff for a minimum acceptable bridge quality.

Bioinformatics analysis and phylogenomic computations. To determine the clonal lineages, the sequence types (STs) of KPC-2-producing *K. pneumoniae* isolates were determined by multilocus sequence typing (MLST) of WGS data, in accordance with protocols described on the Institut Pasteur website (<http://bigsdbs.pasteur.fr/klebsiella/>). ResFinder version 2.1 (<http://cge.cbs.dtu.dk/services/resfinder>) was used to identify antimicrobial resistance genes. Plasmid Finder version 1.3 was used to identify the plasmid incompatibility type (27). The virulence loci in the assembled genome sequences were identified using the BIGSdb *Klebsiella* genome database. A heat map of virulence loci was generated using Genesis software version 1.7.7. The core genes in the genomes of KPC-2-producing *K. pneumoniae* strains were identified using Prokka (28) and Roary (29). Maximum likelihood-based phylogenetic reconstruction was performed with RAxML version 8.2.10 using the generalized time-reversible (GTR) + Γ nucleotide substitution model (30). One hundred bootstrap replicates were evaluated to determine branch support. A maximum likelihood phylogenetic tree based on the core single-nucleotide polymorphism alignments was generated using FastTree (31).

Characterization of the *rmpA*- and *rmpA2*-carrying plasmids. S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) and Southern blot analysis were performed to estimate the size of the *rmpA*- and *rmpA2*-carrying plasmids (32). Plasmid sequences were assembled from WGS data using plasmidSPAdes (33) and annotated using the RAST tool (34). The sequences of representative plasmids were compared against plasmid sequences deposited in the National Center for Biotechnology Information database using BLAST and plotted by using the BLAST Ring Image Generator (35).

Data availability. The complete whole-genome sequences of 65 CRKP isolates have been deposited in the GenBank database under BioProject accession no. PRJNA390758.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 1.9 MB.

FIG S2, TIF file, 1.6 MB.

FIG S3, TIF file, 1.4 MB.

FIG S4, TIF file, 2 MB.

TABLE S1, DOCX file, 0.02 MB.

TABLE S2, DOCX file, 0.01 MB.

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