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ORIGINAL RESEARCH

Evidence of Sharing of Carbapenem-Resistant *Klebsiella pneumoniae* Strains Between Intensive Care Unit Patients and the Environment

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Purpose: Carbapenem-resistant *Klebsiella pneumoniae* (CR-KP) has emerged as an important public health threat. Intestinal colonization with CR-KP increases the risk of infection and death, especially in intensive care unit patients. To clarify the source of colonizing bacteria is very important to prevent the spread of CR-KP, so the purpose of this study was to explore the relationship between the ward environment and intestinal colonization of CR-KP.

Methods: In this study, 353 environmental swabs from ICU (Intensive Care Unit) wards and 241 anal swab samples from ICU patients were collected and screened on MacConkey plates containing 2 μg/mL ertapenem, and the origin and genotype of CR-KP were analyzed by PCR and sequencing. The sequence type of the strains was also obtained by multi-locus sequence type (MLST) analysis, and plasmid conjugation test was used to clarify whether CR-KP can promote the transmission of drug resistance genes through plasmid integration and rearrangement.

Results: A total of 20 CR-KP environmental strains and 7 intestinal strains were obtained, most of which were $bla_{\text{OXA-48}}$ resistant genotypes. Four different STs were identified by multi-locus sequence type (MLST) analysis, among which the large logarithm was ST485 type, and PFGE clustering showed that the similarity between them was >85%. In the plasmid transcoupling assay, we report that one of the *Klebsiella pneumoniae* drug-resistant plasmids was successfully transferred to E. coli, indicating that it may promote the spread of drug-resistant genes through plasmid integration and rearrangement.

Conclusion: Our research suggests that the environment may be a potential source of CR-KP and that there is a need for us to adopt more effective disinfection measures.

Keywords: carbapenem-resistant *Klebsiella*, intestinal, colonization, environment, ICU, homology

Introduction

Klebsiella pneumoniae has long been recognized as an agent of disease since it was first described by Carl Friedländer as the cause of pneumonia in 1882, and remains among the world's most common nosocomial gram-negative pathogens.[1] It can cause severe pneumonia, bloodstream infections and urinary tract infections[2] in patients in the ICU (Intensive Care Unit). *Klebsiella pneumoniae* can carry a variety of drug resistance genes and can be resistant to *β*-lactams, tetracyclines[3] and quinolones.[4] For a long time carbapenems were considered to be an effective treatment for multi-drug resistant *Klebsiella pneumoniae*. However, the widespread use of antimicrobial drugs has led to the production of an increasing number of extended spectrum-ß-lactamases (ESBLs) and metallo-ß-lactamases (MBLs),[5] which are thus resistant to

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carbapenems. Carbapenem-resistant *Klebsiella pneumoniae* (CR-KP) has few treatment options and is a serious public health threat.[6]

Intestinal colonization of CR-KP is an independent risk factor for systemic CR-KP infection, and colonization precedes or coincides with infection.[7] Studies have shown that CRE colonization more than triples the risk of CRE infection in ICU patients, with a concomitant increase in the risk of death from infection.[8] In the ICU ward, the mobility of personnel is high, most patients are critically ill and immunocompromised, and there are many invasive treatment procedures, which leads to a greater possibility of bacterial transmission, including through fecal contamination. Asymptomatic colonized patients and healthy medical staff can serve as important reservoirs of infectious agents. And multiple studies have shown that the spread of bacteria can occur between patients, medical staff, medical equipment and clinical settings.[9] Previous studies have focused on CR-KP molecular epidemiology and resistance mechanisms,[10,11] and have reported how biofilms in the environment exert a high degree of resistance to antimicrobial agents.[12] To the best of our knowledge, no studies on environmental-human gut sharing have been performed on CR-KP. However, this information is critical for better understanding the epidemiology of this important pathogen. Therefore, in this study, we screened the ICU environment and the intestinal colonization of CR-KP in patients, and studied its molecular characteristics, aiming to provide some clues about the transmission route of CR-KP.

Materials and Methods

Reagents

Imipenem, meropenem and ertapenem was purchased from Pfifizer. Trypticase Soy Broth (TSB), Mueller Hinton and MacConkey agar were purchased from OXOID. Primer synthesis was performed by Sangon Biotech Shanghai Co. Ltd. Premix Taq and Xba I restriction enzymes were purchased from Takara Biotech Dalian Co. Ltd.

Sample Collection, Bacterial Identification, and Reference Strains

All fecal and anal swab samples were collected from the General Hospital of Ningxia Medical University, a large hospital located in the northwestern city of Yinchuan, China, and were collected non-invasively after informed, written consent was obtained. Reference strain isolates (E. coli ATCC25922, *Klebsiella* pneumoniae ATCC BAA-1705, and *Klebsiella* pneumoniae ATCC BAA-1706) and bacterial identification isolates were also obtained from the same hospital. The study population included all patients in the ICU and RICU (Respiratory Intensive Care Unit). Prior to inclusion, all human participants were informed about the main objectives of the study and asked to sign a consent form. The first anal swab was taken within 2 hours of the patient's admission to the ICU and daily thereafter until CR-KP was detected or the patient left the ICU. If CR-KP was not detected in the stool or anal swab on the first collection but was detected on a subsequent occasion, the participant was considered a CR-KP coloniser and was included in the study. The patient's age and gender are not limited. Before the disinfection operation of indoor items at 2:00 p. m., environmental bacteria were collected with a physiological saline cotton swab, and then the swab was placed in 3 mL of TSB broth containing a piece of meropenem drug sensitive paper (10μg), and cultured overnight. Patients and surface swabs from ICUs were collected between June and December 2020, and then cultured on MacConkey plates containing 2 μg/mL ertapenem. Species identification and initial sensitivity analysis were performed using MALDI-TOF MS and Vitek2 compact from Biomerieux, France.

Identification of *Klebsiella pneumoniae* by PCR and Sequencing

Genomic DNA was obtained by boiling method, and then used as a template to detect 16S rRNA gene segment (1465 bp) by PCR. The sequences of primers:27F:AGAGTTTGATCMTGGCTCAG, and 1492R:TACGGYTACCTTGTT ACGACTT. The PCR programs were as follows: denaturation at 94°C 5 min, followed by 30 cycles of 94°C 30s,55°C 40s, and 72°C 1 min, and extension at 72°C 5 min. The PCR products were sequenced by Sangon Biotech Shanghai Co. Ltd.

Drug Susceptibility Test and Detection of Resistance Genes

CR-KP was determined by measuring the minimum inhibitory concentrations of isolates for imipenem and meropenem by the agar dilution method, and the results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines (M100, 30th). The CR-KP strains were screened by PCR for the presence of the following Drug-resistant genes:bla_{TEM}, bla_{SHV}, bla_{KPC}, bla_{NDM-1}, bla_{IMP}, bla_{VIM} and bla_{OXA-48}. After preparing a 50 μL reaction system, proceed as follows: denaturation at 94 °C for 5 min, followed by 30 cycles of 94°C 30s,55°C~57°C 40s, and 72°C 45s, and extension at 72°C 5 min. The primer sequences, product lengths and annealing temperatures for drug resistance genes are shown in Table 1. Positive amplification products were sequenced and the sequencing results were compared by BLAST.

Genetic Homogeneity Analysis

To determine the genetic homology of CR-KP, pulsed-field gel electrophoresis (PFGE) methods and multilocus sequence typing (MLST) were used. The primers used for MLST and the PCR reaction conditions were referred to the literature.[13] Bacterial DNA embedded in small plastic blocks was treated with proteinase K and cleaved by the restriction enzyme XbaI at 37°C for 2.5h. DNA digested by xbai was electrophoresed at 6V for 18.5h, the pulse angle was 120◦, and the pulse time was 6.8 to 35.4 seconds.[14] PFGE was repeated three times. K. pneumoniae strains with unique pulse types from environment or human were further typed by MLST according to the published consensus MLST scheme (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). The PFGE and MLST results were analyzed by BioNumerics to construct a similarity tree.

Plasmid Conjugation Test

The conjugation test was performed by the broth mating method using CR-KP (environmental source) as the donor strain and E coli. J53 as the recipient strain. First, take 500 μL each of the donor bacteria and acceptor bacteria in the exponential growth phase and add them to 4 mL of LB broth, and let stand overnight in a 35°C incubator. Then, on the MH solid medium of 2μg/mL meropenem and 200 μg/mL NaN_3, the cells were cultured overnight in a 35°C incubator. In this process, the donor and recipient strains were used as negative controls. Finally, combined with the 16S rDNA sequencing results and the agar dilution method to determine the MIC values of imipenem and meropenem, the conjugation strains were identified.[15] The experiment was repeated three times.

Results

Source and Genotype of Carbapenem-Resistant *Klebsiella pneumoniae*

A total of 353 environmental swabs and 241 patients were CR-kp screened at admission to the ICUs from from June to December 2020. 7(2.9%) were CR-kp colonized:5 strains in Ward A of ICU and 2 in RICU. At the same time, we

Table 1 Primer Sequence, Product Length and Annealing Temperature of β-Lactamase Genes

β-Lactamase Gene	Primer Sequence (5′→3′)	Annealing Temperature (°C)	Product Length (bp)
SHV	F: ATGCGTTATATTCGCCTGTG R: TTAGCGTTGCCAGTGCTCGATC	56	861
TEM	F:GAGTATTCAACATTTCCGTGTCGC R: TACCAATGCTTAATCAGTGAGGC	56	858
KPC	F:ATGTCACTGTATCGCCGTCT R:TTTTCAGAGCCTTACTGCCC	56	893
NDM	F:ATGGAATTGCCCAATATTATGC R:TCAGCGCAGCTTGTCGG	56	813
VIM	F:TTATGGAGCAGCAACGATGT R:CAAAAGTCCCGCTCCAACGA	55	920
IMP	F:TGAGCAAGTTATCTGTATTC R:TTAGTTGCTTGGTTTTGATG	55	740
0XA-48	F:GCGTGGTTAAGGATGAACAC R:CATCAAGTTCAACCCAACCG	57	438

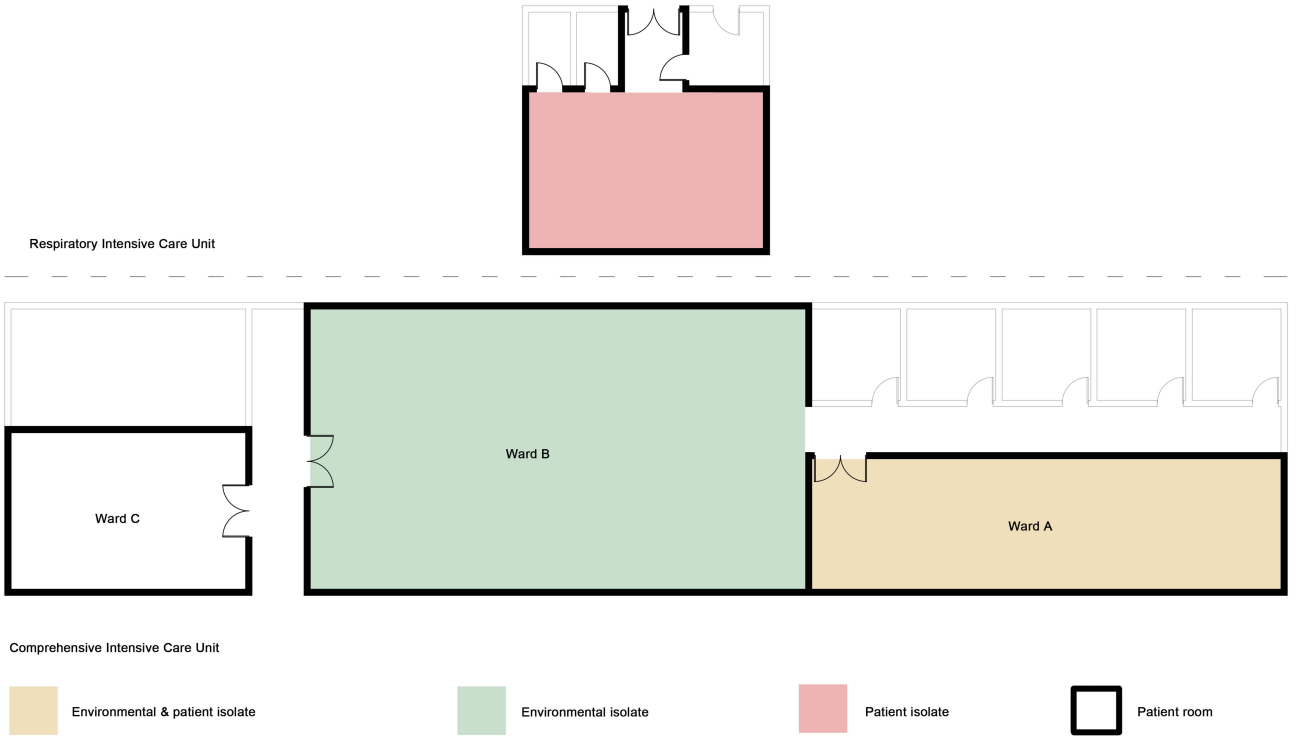


Figure 1 Distribution of carbapenem-resistant *Klebsiella pneumoniae* in intensive care units. Both environmental and patient strains were isolated (yellow), red means only patient strains were isolated and green means only environmental strains were isolated. The respiratory intensive care unit and the comprehensive intensive care unit are not in the same building, and the tested wards are highlighted with a black border.

screened 20 strains of CR-KP from the environment. The distribution of these environmental bacteria in the ward is shown in Figure 1, and most of them (18/20) originated from the ward A. Of the environmental CR-KP isolates, 7 were collected from stethoscopes, 4 from bed rail,4 from the computer and 2 from the nurse station. There was only one strain from the ventilator and rack. In terms of time, these environmental bacteria were isolated from September and October, which is close to the isolation time of CR-KP colonized in the patient's intestine. In addition, 2 intestinal colonizing CR-KP strains were isolated from the respiratory intensive care unit in July. As shown in Table 2, all strains carried β-lactamases gene SHV, and 25 strains carried TEM. Carbapenem resistance genes were mainly $bla_{\text{OXA-48}}$ (21/27), followed by metalloenzymes (bla_{IMP}, 4/27). Two of the 27 strains were found to be New Delhi metallo-lactamase-1 ($bla_{\text{NDM-1}}$). KPC and VIM genes were not detected.

Molecular Typing

The molecular profiles of all isolates were determined by pulsed field gel electrophoresis (PFGE), and the sequence profiles of the strains were obtained by multi-site sequence typing (MLST) analysis, both of which were used to characterize the molecular types at the same time. The dendrogram of Figure 2 was generated based on PFGE analysis without weighting the ST included in the graph. Based on a cutoff of 80% genetic similarity, these isolates were grouped into A (isolate No: 229), B (isolate No: 97), C (isolate No: 225, 203, 237, 279, 243, 207, 132, 161, 165, 141, 147, 236, 240, 333, 340, 343, 339, 344, 345, 335, 336, 337, 338, 223), and D (isolate No: 102) clusters (Figure 2). A total of 4 distinct STs were identified among 27 CR-KP. As depicted in Figure 2, ST485 was the most prevalent ST (85.19%). There was one strain each of ST101, ST1737 and ST1905. One strain could not find the corresponding ST type. The patient strains that are clonal to the strains in the environment are 4 out of 7.

Table 2 Screening Results and β-Lactamase Gene of Carbapenem-Resistant *Klebsiella pneumoniae*

Source	Isolate ID	Date	Location			β-Lactamase Gene
			Ward	Bed Number	Sampling Point	
E	132	Sept.28th	ICU/A	2	Ventilator	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	141	Sept.28th	ICU/A	2	Bed rail	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	147	Sept.28th	ICU/A	/	Nurse station	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	161	Sept.28th	ICU/A	2	Stethoscope	bla_{SHV}, bla_{IMP}
E	165	Sept.28th	ICU/A	2	Bed rail	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	225	Oct.10th	ICU/A	6	Stethoscope	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	236	Oct.10th	ICU/B	11	Computer	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	240	Oct.10th	ICU/A	6	Bed rail	bla_{SHV}, bla_{TEM}, bla_{IMP}
E	243	Oct.10th	ICU/A	2	Computer	bla_{SHV}, bla_{TEM}, bla_{IMP}
E	279	Oct.17th	ICU/A	/	Nurse station	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	333	Oct.17th	ICU/A	1	Bed rail	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	335	Oct.17th	ICU/A	/	Rack	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	336	Oct.17th	ICU/A	1	Computer	bla_{SHV}, bla_{TEM}, bla_{IMP}
E	337	Oct.17th	ICU/A	2	Stethoscope	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	338	Oct.17th	ICU/A	2	Bedside table	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	339	Oct.17th	ICU/A	4	Stethoscope	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	340	Oct.17th	ICU/A	5	Stethoscope	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	343	Oct.17th	ICU/A	4	Computer	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	344	Oct.17th	ICU/A	1	Stethoscope	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
E	345	Oct.17th	ICU/B	9	Stethoscope	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
P	97	Jul.6th	RICU	/	/	bla_{NDM-1}, bla_{SHV}, bla_{TEM}
P	102	Jul.6th	RICU	/	/	bla_{NDM-1}, bla_{SHV}, bla_{TEM}
P	203	Sept.27th	ICU/A	2	/	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
P	207	Oct.10th	ICU/A	2	/	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
P	223	Oct.12th	ICU/A	1	/	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
P	229	Oct.22nd	ICU/A	3	/	bla_{OXA-48}, bla_{SHV}, bla_{TEM}
P	237	Nov.17th	ICU/A	2	/	bla_{OXA-48}, bla_{SHV}

Abbreviations: E, environment; P, patient.

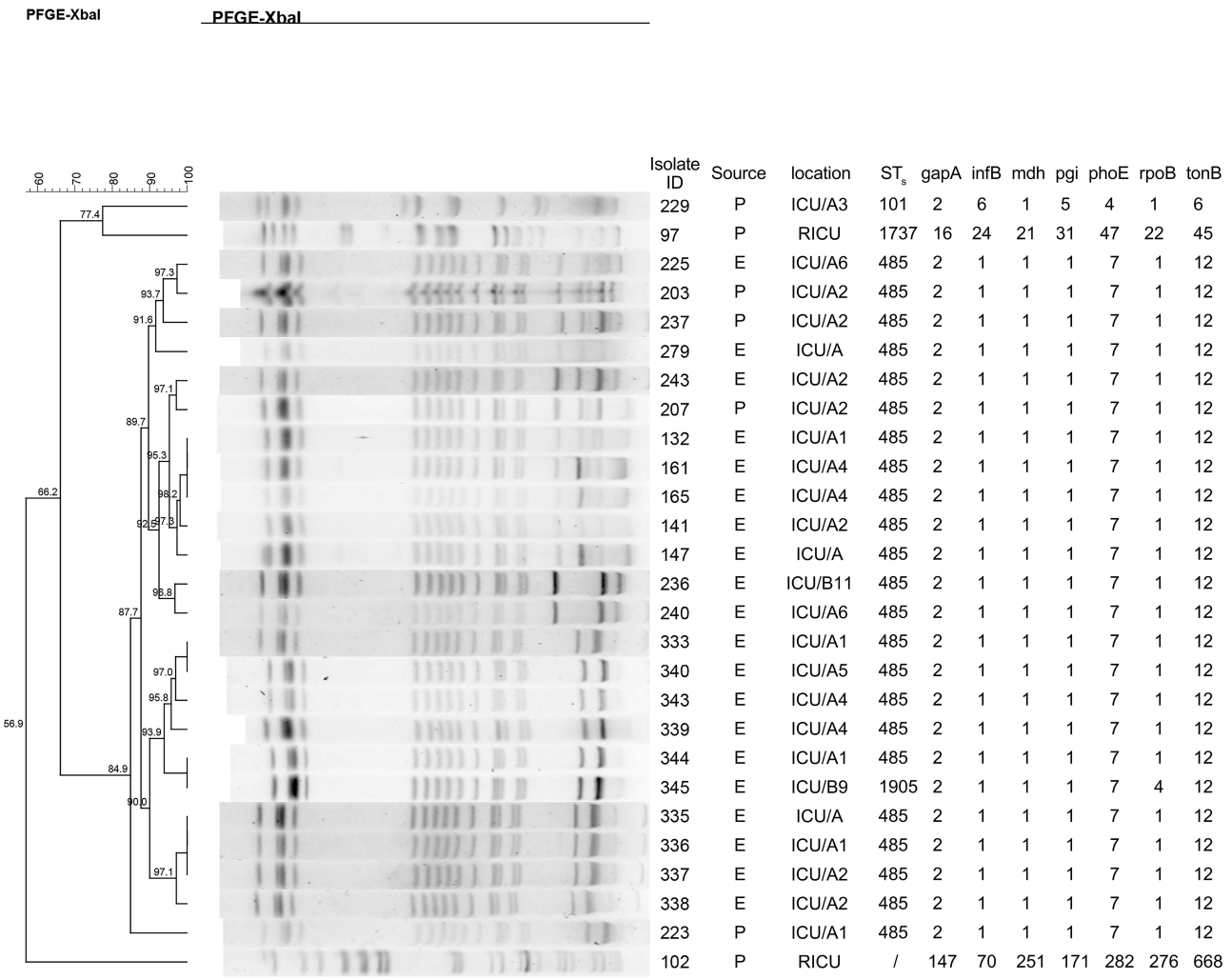
Plasmid Conjugation Test

The plasmid transconjugant test was performed for 20 CR-KP isolates from environment, and one *Klebsiella pneumoniae* resistant plasmid was successfully transferred to E. coli J53. The MIC of the transconjugant for imipenem and meropenem was measured by the agar dilution method (Table 3).

Discussion

The colonization of *Klebsiella pneumoniae* may increase the incidence of corresponding *Klebsiella pneumoniae* infection in critically ill patients in the ICU,[16] which can further markedly prolong the hospital length of stay, increase the medical cost, and the most important hazard is causing high mortality.[17] With the widespread colonization of CR-KP and the wide spread of associated resistant genes around the world,[18] it has become urgent to investigate the molecular characteristics and colonization pathway of carbapenem-resistant *Klebsiella pneumoniae*, which may provide a solid basis for an effective control of carbapenem-resistant *Klebsiella pneumoniae*. To our knowledge, this is the first report of a *Klebsiella pneumoniae* clonal lineage between ICU ward patients and close contact environments.

In recent years, the research on microorganisms in the ICU medical environment has not stopped. Recent studies have found that the surface of the ICU environment is a repository for fungi,[19] and Durán-ManuelEM has found that Acinetobacter baumannii can be clonally spread between medical facilities in the ICU by analyzing 16S rRNA.[20] Even CR-KPs in public settings are reported from time to time,[21] and the potential transmission of this CR-KP in the environment is worrisome. In this study, 20 strains of CR-KP were screened out from 353 ICU environmental samples, with a detection rate



Isolate ID	Source	location	ST_s	gapA	infB	mdh	pgi	phoE	rpoB	tonB
229	P	ICU/A3	101	2	6	1	5	4	1	6
97	P	RICU	1737	16	24	21	31	47	22	45
225	E	ICU/A6	485	2	1	1	1	7	1	12
203	P	ICU/A2	485	2	1	1	1	7	1	12
237	P	ICU/A2	485	2	1	1	1	7	1	12
279	E	ICU/A	485	2	1	1	1	7	1	12
243	E	ICU/A2	485	2	1	1	1	7	1	12
207	P	ICU/A2	485	2	1	1	1	7	1	12
132	E	ICU/A1	485	2	1	1	1	7	1	12
161	E	ICU/A4	485	2	1	1	1	7	1	12
165	E	ICU/A4	485	2	1	1	1	7	1	12
141	E	ICU/A2	485	2	1	1	1	7	1	12
147	E	ICU/A	485	2	1	1	1	7	1	12
236	E	ICU/B11	485	2	1	1	1	7	1	12
240	E	ICU/A6	485	2	1	1	1	7	1	12
333	E	ICU/A1	485	2	1	1	1	7	1	12
340	E	ICU/A5	485	2	1	1	1	7	1	12
343	E	ICU/A4	485	2	1	1	1	7	1	12
339	E	ICU/A4	485	2	1	1	1	7	1	12
344	E	ICU/A1	485	2	1	1	1	7	1	12
345	E	ICU/B9	1905	2	1	1	1	7	4	12
335	E	ICU/A	485	2	1	1	1	7	1	12
336	E	ICU/A1	485	2	1	1	1	7	1	12
337	E	ICU/A2	485	2	1	1	1	7	1	12
338	E	ICU/A2	485	2	1	1	1	7	1	12
223	P	ICU/A1	485	2	1	1	1	7	1	12
102	P	RICU	/	147	70	251	171	282	276	668

Figure 2 Dendrogram showing pulsed-field gel electrophoresis (PFGE) analysis of the 27 carbapenem-resistant *Klebsiella pneumoniae* isolates.

of 5.67% (20/353), including 18 strains in ward A, 2 strains in ward B and no strains in ward C. Comparing the results of intestinal colonization CRE screening, it was found that the high detection rate in ward A may be related to hospitalized patients, because intestinal colonization CRE was screened successively in two patients in two beds in ward A of ICU ward on September 28 and October 10, and CREs were successively detected from the environment in the days before and after this date. Geographically, ward B is connected toward A, while ward C is relatively independent, which may also explain the difference in the detection rate of CR-KP among the three to a certain extent (Figure 1). It is worth noting that several studies[22] have shown that handwashing sinks is the source of transmission of CR-KP in the ICU.[23] In this study, the

Table 3 Minimum Inhibitory Concentrations (MIC) for Imipenem and Meropenem of the Carbapenem-Resistant *Klebsiella pneumoniae* Isolate and Its Transconjugant

Isolate	MIC	
	Impipenem	Meropenem
236	16	8
TC-236	4	4
E coli J53	0.5	≤0.125

Abbreviations: TC, transconjugant strain; E coli J53, recipient strain.

detection rate of stethoscope, bed guard and computer CRE was significantly higher than that of other sampling sites, which suggested that we should focus on strengthening the disinfection measures in these parts to reduce its potential for nosocomial transmission. Implementing a more effective cleaning and disinfection programme may be necessary.

ST11 belongs to the dominant clone in Asia. In the molecular epidemiological study of carbapenem-resistant *Klebsiella pneumoniae* in China, most of the isolates also belong to ST11 and ST15.[24,25] Therefore, it was surprising that K. pneumoniae from the homology analysis between the ward environment and the CR-KP colonized in the patient's intestine, we found that 85.19% (24/27) of the CR-KP belonged to the ST485 type, and PFGE clustering showed that the similarity between them was >85% (Figure 2). These highly homologous strains were all isolated from ward A and B of the comprehensive ICU, and the similarity between the two CR-KP strains from respiratory ICU patients was less than 60%. These results suggest that CR-KPs propagate each other in the environment, especially in close proximity. There is a high degree of homology between the environment and the gut-colonizing CR-KP of the patient in it, which provides strong evidence for the transmission of CR-KP between the patient's gut and the environment. Numerous studies have shown that admission to the ICU is a risk factor for CR-KP infection, and a review of the clinical cases of seven positive patients revealed that two were subsequently infected with CR-KP, which somewhat confirms this conclusion. Therefore, in order to detect and isolate a "superbug" such as CR-KP early, it is necessary to actively screen high-risk patients admitted to the ICU for CRE.

There was a significant correlation between the type and frequency of drug resistance genes in *Klebsiella pneumoniae* and their virulence.[26] There are differences in the risk of infection caused by different drug-resistant genotypes of CR-KP, NDM-KP was associated with increased risk of BSI compared with KPC-KP.[27] Genomic studies of MBLs have shown that NDM and VIM are expressed through genes located on mobile genomic elements and plasmids,[5] and that the rich bacterial environment of the gut provides strong conditions for the horizontal transmission of these drug-resistant genes. In this study two strains of NDM-type CR-KP were identified in respiratory ICU patients, but fortunately VIM was not detected. In China, KPC-2, NDM, and OXA-48-like carbapenemases were predominant among CRE clinical isolates.[28] However, in this study, OXA-48 carbapenem-resistant *Klebsiella pneumoniae* dominated (21/27). In order to explore the possible transmission route of the drug resistance gene, we carried out the plasmid conjugation transfer test. In our experiment, one of the 20 environmental CR-KP strains successfully transferred the drug resistance gene to E. coli J53 (Table 3). Chen's study also highlights how plasmid integration and rearrangements can contribute to the spread of -like genes.[29] Co-existence of a novel plasmid-mediated efflux pump with colistin resistance gene mcr has been previously detected in one plasmid confers transferable multidrug resistance in *Klebsiella pneumoniae*.[30] These results provide important clues for clinical prevention of the spread of carbapenemase-resistant *Klebsiella pneumoniae* strains.

Conclusion

The results of this study suggest that there is a high similarity between CR-KP colonisation in the ICU environment and in the patient's gut, and that the environment may be a potential source of intestinal CR-KP colonisation, suggesting the need for more effective cleaning measures to eliminate such a potential problem. However, it remains to be seen whether it is only the CRE in the gut that contaminates the environment or whether it is the environmental CRE that somehow colonises the patient's gut, or whether it is the bacteria from other patients that pass through the environment and colonise the gut of people in the same ward.

Ethics Approval

The research protocol has been approved by the Medical Research Ethics Review Committee of the General Hospital of Ningxia Medical University (No. 2019-105).

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Disclosure

The authors report no conflicts of interest in this work.

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