Infection and Drug Resistance

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ORIGINAL RESEARCH

Co-occurrence of *Klebsiella variicola* and *Klebsiella pneumoniae* Both Carrying bla_{KPC} from a Respiratory Intensive Care Unit Patient

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Objective: The aim of this study was to use whole-genome sequencing to characterize *Klebsiella pneumoniae* SKp2F and *Klebsiella variicola* SKv2E, both carrying bla_{KPC}, co-isolated from the same sputum specimen.

Methods: Antimicrobial susceptibility testing was performed using microbroth dilution. Biofilm formation was determined by crystal violet staining and virulence was measured by a serum killing assay. Whole-genome sequencing of SKp2F and SKv2E was performed using an Illumina sequencer and the genetic characteristics were analyzed by computer.

Results: SKp2F and SKv2E were sensitive only to tigecycline and polymyxin among the tested antibiotics. The biofilm-forming ability of SKv2E is stronger than that of SKp2F. The grades of serum resistance of SKp2F and SKv2E are 4 and 3. MLST analysis of the 6,115,610 bp and 5,403,687 bp of SKv2E and SKp2F showed associations with ST1615 and ST631, respectively. SKv2E carried 13 resistance genes (bla_{KPC-2}, bla_{TEM-1A}, bla_{LEN17}, *aadA16*, *arr-3*, *qnrB4*, *oqxA/B*, *dfrA27*, *sul1*, *tetD*, *fosA*, *qacEΔ1*) and SKp2F carried 23 (bla_{KPC-2}, $bla_{CTX-M-3}$, bla_{TEM-1B}, $bla_{CTX-M-65}$, bla_{SHV-27}, *aac(6')-IIa*, *rmtB*, *arr-3*, *aph(3')-Ia*, *aadA16*, *qnrS1*, *aac(6')-Ib-cr*, *qnrB91*, *oqxA/B*, *mph(A)*, *tet(A)*, *fosA*, *dfrA27*, and two copies of *qacEΔ1-sul1*). Most of them were carried by various mobile genetic elements, such as IncFIB(K)/IncFII(K)/IncFII(Yp), IncFII(K) plasmid, Tn6338, and In469. Both SKv2E and SKp2F carried a large number of virulence factors, including type 1 and 3 fimbriae, capsule, aerobactin (iutA), ent siderophore (*entABCDEFS*, *fepABCDGfes*), and salmochelin (*iroE/iroEN*). SKv2E also carried type IV pili (*pilW*), fimbrial adherence (*steB*, *stfD*), and capsule biosynthesis gene (*glf*).

Conclusion: bla_{KPC-2}-carrying *K. variicola* and *K. pneumoniae*, which carried multiple resistance genes, virulence factors, and highly similar mobile genetic elements, were identified from the same specimen, indicating that clinical samples may carry multiple bacteria. We should avoid misidentification, and bear in mind that resistance genes carrying mobile genetic elements can be transmitted or integrated between bacteria in the same host.

Keywords: *Klebsiella variicola*, *Klebsiella pneumoniae*, carbapenem-resistant Enterobacteriaceae, CRE, bla_{KPC}

Introduction

Carbapenemase-producing Enterobacteriaceae (CPE) have become a global concern owing to their ability to hydrolyze carbapenems and most β-lactam antibiotics, posing a serious threat to human health and a significant challenge to clinical

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treatment.[1,2] The *Klebsiella pneumoniae* carbapenemase (KPC) and metallo-β-lactamases are the two major groups of carbapenemases produced by the most of the carbapenemase-resistant Enterobacteriaceae (CRE) strains, because they carry the carbapenemase code genes such as bla_{KPC} and bla_{NDM}.[3–6]

The bla_{KPC} and bla_{NDM} gene-carrying strains always co-harbor many other types of resistance genes, such as extended-spectrum β-lactamase (ESBL) genes (bla_{CTX-M}, bla_{SHV}, and bla_{TEM}), fluoroquinolone resistance genes (*qnrA*, *qnrB*, *qnrS*, and*oqxA/B*), and aminoglycoside resistance genes (*rmtA*, *rmtB*, and *rmtC*), resulting in high resistance to almost all kinds of commonly used antibiotics.[7–10] These notorious resistance genes are usually carried by various mobile genetic elements, such as plasmids, integrons, and transposons, which can be transmitted between intraspecific or interspecific microorganisms.[11–13] In recent years, there has been a high incidence of co-infection with more than two different multi-drug-resistan t bacteria in the same patient, which brings a serious threat to patients[14–17] because the variety of bacteria in the co-infection can be misdiagnosed or misidentified.[18,19] For example, many types of *Klebsiella* species or subspecies (eg, *Klebsiella variicola*, *Klebsiella quasipneumoniae* subsp. *quasipneumoniae*, *Klebsiella quasipneumoniae* subsp. *similipneumoniae*, *Klebsiella quasivariicola*, *Klebsiella africanensis*, and *Klebsiella variicola* subsp. *tropicalensis*) have been identified and reported, which make up the *Klebsiella pneumoniae* complex. However, in more and more reports of *K. pneumoniae* infection, in recent years, cases of *Klebsiella variicola* infection are increasingly being found.[18] Because of the morphological similarity between species in the *K. pneumoniae* complex, some *Klebsiella* species are always misidentified as *K. pneumoniae*.[20,21] *Klebsiellapneumoniae* is an opportunistic pathogen that can lead to serious hospital infection and community-acquired infections. *Klebsiella variicola* is also an opportunistic pathogen, responsible for infections such as blood infections, respiratory tract infections, and urinary tract infections (UTIs), and blood infection caused by *K. variicola* has a higher mortality rate than that caused by *K. pneumoniae*.[22] This tells us that a precise diagnosis is important for infection control.

Here, we report and characterize *K. variicola* and *K. pneumoniae* strains that were co-isolated from a sputum sample of a female inpatient, which both carried the carbapenemase-producing gene bla_{KPC}.

Materials and Methods

Bacteria Isolation, Identification, and Antimicrobial Susceptibility Testing

Klebsiella variicola strain SKv2E and *Klebsiella pneumoniae* SKp2F were isolated from the same sputum specimen of a 69-year-old female patient, who was admitted with chronic obstructive pulmonary disease and pulmonary infection to the Department of Respiratory Medicine at The Second Affiliated Hospital of Xiamen Medical College, in November 2020. The species were identified using the VITEK 2 compact system and 16S rRNA and rpoB sequencing.[20] The results of the 16S rRNA and rpoB sequencing displayed overlapping peaks,[17,20] indicating the co-existence of two or more types of bacteria. Thereafter, we purely cultured the colony and chose five colonies randomly to sequence again, which finally confirmed the presence of *K. variicola* strain SKv2E and *K. pneumoniae* SKp2F.

In vitro, antimicrobial susceptibility testing of SKv2E and SKp2F against antimicrobial agents (OXOID), including ampicillin, aztreonam, ceftazidime, ciprofloxacin, ceftriaxone, cefuroxime, cefepime, gentamicin, imipenem, meropenem, polymyxin B, sulfamethoxazole-trimethoprim, and tigecycline, was performed by a broth microdilution method, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, M100-S27) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/).

String, Biofilm Formation Assay, and Serum Killing Activity Testing

To test the mucoviscosity phenotype, the colony of strains SKv2E and SKp2F was cultured on a blood agar plate overnight at 37°C for 24 hours, stretched by an inoculating loop. The strain formed a viscous string of >5 mm which was designated as mucoviscous. The biofilm formation assay was conducted according to our previous method.[23] To address the virulence of the two strains, the human serum killing activity was defined using a previously described method.[6]

Whole Genome Sequencing and Analysis

Genomic DNA of *K. variicola* strain SKv2E and *K. pneumoniae* strain SKp2F was extracted using a DNA extraction kit (Sangong, China). The 300-bp paired-end library was constructed using the standard Illumina DNA

sample preparation instructions. Then, it was sequenced on an Illumina MiSeq systems sequencer (Majorbio, China). The readings were assembled de novo and gene prediction was performed with a Glimmer 3.02 (http://www.cbcb.umd.edu/software/glimmer/). Annotation of the *K. variicola* SKv2E and *K. pneumoniae* SKp2F genomes was achieved using the NCBI Prokaryotic Genome Annotation Pipeline. The pairwise alignment was performed by a blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The resistome was identified using ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/) (minimum threshold for identity, 85%; minimum coverage, 60%).[24] The virulence factors were predicted using the VFanalyzer of VFDB (http://www.mgc.ac.cn/VFs/).[25]

Conjugation Assay

To determine whether the bla_{KPC} was carried by a conjugative plasmid, *K. variicola* SKv2E and *K. pneumoniae* SKp2F were cultured in Luria–Bertani (LB) broth as the donor, and azide-resistant *E. coli* strain J53 was used as the recipient. The transconjugants were selected on LB agar plates containing sodium azide (100 μg mL^{-1}) and meropenem (1 μg mL^{-1}). The presence of the bla_{KPC} resistance gene in transconjugants was confirmed by PCR.[6] The antimicrobial susceptibility of transconjugants was determined by the microbroth dilution method.[14] The replicon F of the transconjugants was determined according to the previous method, based on the whole genome sequencing (WGS) analysis.[26]

Results

In Vitro Assay of Antimicrobial Susceptibility, Hypermucoviscosity, Biofilm, and Serum Resistance Assay

As shown in Table 1, SKv2E and SKp2F were resistant to all of the test antibiotics except for polymyxin B and tigecycline. String testing showed that SKv2E and SKp2F were non-hypermucoviscous strains. The two strains were biofilm-forming isolates, with SKv2E and SKp2F having optical density values (OD_{595}) of 1.93 and 1.65, respectively. In the serum killing assay, the grades of SKv2E and SKp2F were 4 and 3, respectively (Table 2).

Genome Characteristics of Strains SKv2E and SKp2F

The assembled WGS of *K. variicola* SKv2E and *K. pneumoniae* SKp2F produced 126 and 45 scaffolds,

Table 1 Determination of Minimum Inhibitory Concentration (MIC) for *K. variicola* SKv2E and *K. pneumoniae* SKp2F and Their bla_{KPC} Transconjugants

MIC (μg/mL)	AMP	ATM	CAZ	CIP	CRO	CXM	SAM	FEP	GEN	IPM	MEM	SXT	TGC	PB
SKv2E	≥32	≥32	≥64	≥4	≥16	≥32	≥32	≥64	≥16	≥8	≥8	≥160	0.5	1
SKp2F	≥32	≥32	≥64	≥4	≥16	≥32	≥32	≥64	≥16	≥8	≥8	≥320	0.5	0.5
J53-pSKv2E-bla_{KPC}	≥32	≥32	≥32	≥4	≥16	≥32	≥32	≥32	≥16	≥8	≥8	160	0.25	1
J53-pSKp2F-bla_{KPC}	≥32	≥32	≥32	≥4	≥16	≥32	≥32	≥32	≥16	≥8	≥8	160	0.25	0.5
J53	1	0.125	0.25	0.125	1	0.25	0.5	0.25	0.5	0.125	0.25	4	0.125	0.5

Abbreviations: AMP, ampicillin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, cefriaxone; CXM, cefuroxime; SAM, ampicillin–sulbactam; FEP, cefepime; GEN, gentamicin; IPM, imipenem; MEM, meropenem; SXT, sulfamethoxazole–trimethoprim; TGC, tigecycline; PB, polymyxin B.

Table 2 Genome Characteristics of *K. variicola* SKv2E and *K. pneumoniae* SKp2F

Isolate	SKv2E	SKp2F
Genome length (bp)	6,115,610	5,403,687
No of scaffolds	126	45
No of tRNA	79	84
No of rRNA	15	14
No of ncRNA	10	14
No of CDs	5089	5231
MLST	ST1615	ST631
Resistance genes	bla_{KPC-2}, bla_{TEM-1A}, bla_{LEN17}, *aadA16*, *arr-3*, *qnrB4*, *oqxA/B*, *dfrA27*, *sul1*, *tetD*, *fosA*, *qacEΔ1*	bla_{KPC-2}, $bla_{CTX-M-3}$, bla_{TEM-1B}, $bla_{CTX-M-65}$, bla_{SHV-27}, *aac(6')-IIa*, *rmtB*, *arr-3*, *aph(3')-Ia*, *aadA16*, *qnrS1*, *aac(6')-Ib-cr*, *qnrB91*, *oqxA/B*, *mph(A)*, *tet(A)*, *fosA*, *dfrA27*, two copies of *qacEΔ1-sul1*
Grade of human serum resistance	4	3
String testing	Non-hypermucoviscous	Non-hypermucoviscous
Mean biofilm formation (OD_{595})	1.93	1.65
Plasmid replicons	Col(pHAD28), Col440I, IncFIB(K), IncFII(K), IncFII(Yp), IncHI1B	IncFII(K)

respectively, which resulted in estimated draft genomes 6,115,610 bp and 5,403,687 bp in length, with a total of 5130 and 4740 coding sequences (Table 2). Multi-locus sequence typing (MLST) analysis of the WGS data indicated that SKv2E belongs to ST1615, while SKp2F was found to be associated with ST631.

Resistome and Virulence Factors of SKv2E and SKp2F

The WGS data confirmed the presence of bla_{KPC-2} carried by SKv2E; in addition, other resistance genes related to resistance to β-lactams (bla_{TEM-1A}, bla_{LEN17}), aminoglycosides (*aadA16*, *arr-3*), fluoroquinolones (*qnrB4*, *oqxA/B*), trimethoprim (*dfrA27*, *sul1*), tetracycline (*tetD*), fosfomycin (*fosA*), and benzylkonium (*qacEΔ1*) were identified. SKp2F also carried bla_{KPC-2}, along with other resistance genes related to resistance to β-lactams ($bla_{CTX-M-3}$, bla_{KPC-2}, bla_{TEM-1B}, $bla_{CTX-M-65}$, bla_{SHV-27}), aminoglycosides (*aac(6')-IIa*, *rmtB*, *aph(3')-Ia*, *aadA16)*, fluoroquinolones (*qnrS1*, *aac(6')-Ib-cr*, *qnrB91*, and *oqxA/B*), phenicol (*floR*), rifamycin (*arr-3*), macrolide (*mph(A)*), tetracycline (*tet(A)*), fosfomycin (*fosA*), and trimethoprim (*dfrA27*, *sul1*). Furthermore, both SKv2E and SKp2F carried a large number of virulence factors, including type 3 fimbriae (*mrkABCDFHIJ*), type 1 fimbriae (*fimABCDEFGHIK*), capsule coding genes, rscAB (virulence regulation genes), aerobactin (iutA), ent siderophore (*entABCDEFS* and *fepABCDGfes*), and salmochelin (iroE/iroEN). Type IV pili (pilW), fimbrial adherence determinants (*steB*, *stfD*), and capsule biosynthesis and transport genes (*glf*) were also identified from SKv2E (Supplementary Table S1).

Plasmid Transferability of bla_{KPC-2}

Conjugation assays showed that both of the bla_{KPC} genes were successfully transferred to azide-resistant *E. coli* J53. It was found that the bla_{KPC} genes were carried by plasmid, designated pSKv2E-KPC and pSKp2F-KPC, respectively. The transconjugants of SKv2E and SKp2F were named J53-pSKv2E-bla_{KPC} and J53-pSKp2F-bla_{KPC}. Plasmid replicon typing showed that the replicons of the bla_{KPC}-carrying plasmid of SKv2E are IncFIB(K), IncFII(K), and IncFII(Yp), and the replicon of SKp2F is IncFII(K).

Genetic Context of the Resistance Gene-Carrying Regions

For *K. variicola* strain SKv2E, 10 of the 13 resistance genes were carried by scaffold27, scaffold32, and scaffold41. Sequence analysis showed that bla_{KPC} was found in the 65,049-bp-long scaffold32, with a G+C content of 54.16%. The bla_{KPC} gene was carried by *klcA-korC-ISKpn6-*bla_{KPC-2}*-*bla_{TEM}*-IS*Kpn27-Tn3. This region is the same bla_{KPC}-carrying region in plasmids pPUTH2 (CP024709.1) and pKPC2_130002 (CP064852.1) (Figure 1). Furthermore, the 44,418-bp-long scaffold41 of SKv2E carried six resistance genes (*qnrB4*, *arr-3*, *dfrA27*, *qacEΔ1*, *sul1*, and *aadA16*), which were harbored by the partial integron In469 (Figure 2).

For *K. pneumoniae* strain SKp2F, the WGS data confirmed that bla_{KPC} was found in the 21,330-bp-long

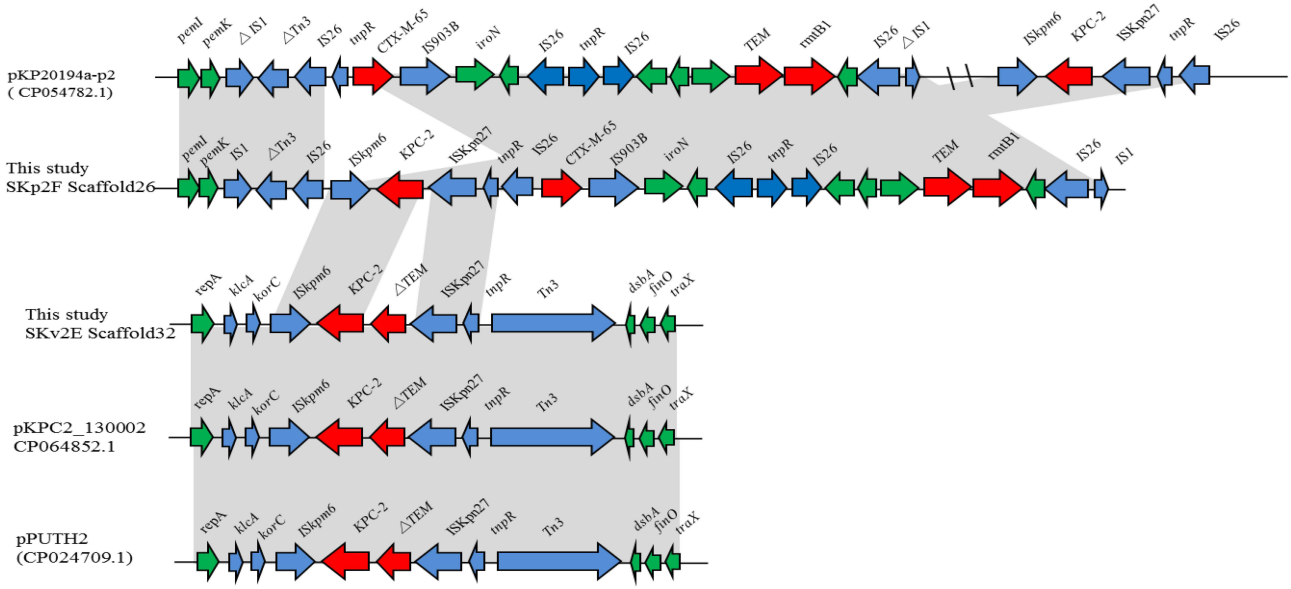

Figure 1 Schematic mapping of the genetic characteristics of the resistance gene (bla_{KPC-2})-carrying region in strain *K. variicola* SKv2E and *K. pneumoniae* SKp2F. The construction of the sequence comparison was performed using blast (http://blast.ncbi.nlm.nih.gov). Genes are shown as arrows, and their orientations of transcription are indicated by the arrowheads.

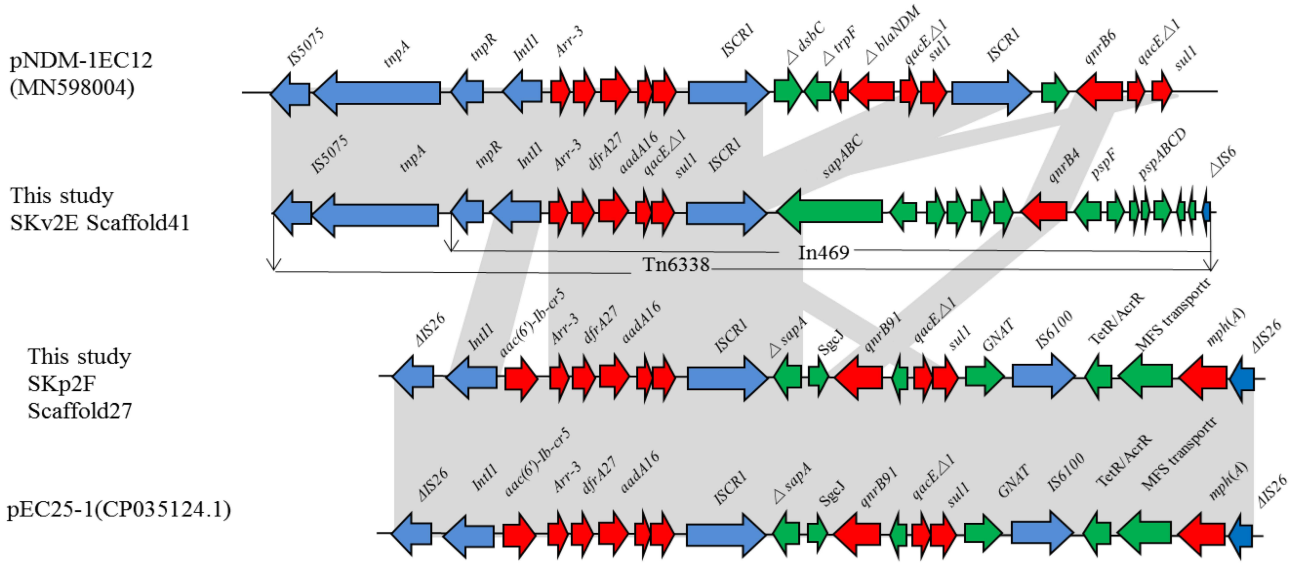

Figure 2 Schematic mapping of the genetic characteristics of resistance gene (*arr-3*, *dfrA27*, *aadA16*, *qnrB*)-carrying region in strain *K. variicola* SKv2E and *K. pneumoniae* SKp2F. The construction of the sequence comparison was performed using blast (http://blast.ncbi.nlm.nih.gov). Genes are shown as arrows, and their orientations of transcription are indicated by the arrowheads.

scaffold26, along with several other resistance genes (*bla*$_{CTX-M-65}$, *bla*$_{TEM-1}$, *rmtB*), with a G+C content of 51.77%. The *bla*$_{KPC}$ gene-carrying context (*IS*Kpn6-*bla*$_{KPC-2}$–*IS*Kpn27) and (*bla*$_{CTX-M-65}$, *bla*$_{TEM-1}$, *rmtB*) gene-harboring regions were both the same as the corresponding region of plasmid pKP20194a-p2 (CP054782.1). The 16,914-bp-long scaffold27 of SKp2F carried 10 resistance genes (*arr-3*, *dfrA27*, *aadA16*, *aac(6')-Ib-cr*, *qnrB91*, *mph(A)*, and two copies of *qacEΔ1-sul1*). The linear structure of this resistance gene-carrying region is similar to several plasmids, such as pKSH203-CTX-M-3 (CP034325.1), pEC25-1 (CP035124.1), pM297-1.2 (CP051492.1), and pHC139-5copy (CP061843.1) (Figure 2). Furthermore, the resistance region (*IntI1-aac(6')-Ib-cr5-arr-3-dfrA27-aadA16-qacEΔ1-sul1-IS*CR1) in this scaffold was similar to the *arr-3*, *dfrA27*, *aadA16*, *qacEΔ1-sul1*-carrying scaffold41 of SKv2E, with the difference of a resistance gene *aac(6')-Ib-cr5* inserted between *IntI1* and *arr-3* (Figure 2). Four other resistance genes (*tet(A)*, *floR,* *bla*$_{TEM-1B}$, and *bla*$_{CTX-M-3}$) were carried by the 14,502-bp-long scaffold28, which is the same as in many plasmids, such as pHKU49_CIP (MN543570.1) and pRGF99-1-75k (CP075554.1).

Discussion

Misidentification of bacterial infections from the same sample is a serious problem, which often affects the infection control and the therapeutic outcome.[17,20] In recent years, several *Klebsiella* species or subspecies (eg, *K. variicola*, *K. quasipneumoniae* subsp., *K. quasivariicola*, and *K. africanensis*) have been increasingly identified from clinical samples.[18] Because of the morphological similarity between these *Klebsiella* species, some other non-*K. pneumoniae* species are being misidentified as *K. pneumoniae*.[20,21] It is well known that these *Klebsiella* species, as well as *K. pneumoniae*, are opportunistic pathogens responsible for infections, and blood infection has also been shown to be caused by other *Klebsiella* species; for example, *K. variicola* has a higher pathogenicity than *K. pneumoniae*.[22] This tells us that precise diagnosis is important in infection control. In this study, we isolated *K. variicola* and *K. pneumoniae,* which both carry *bla*$_{KPC}$ and other resistance genes, from the same patient using the VITEK 2 compact system and 16S rRNA and rpoB sequencing.

These *Klebsiella* species carry many types of carbapenemase-coding genes, such as *bla*$_{KPC}$, *bla*$_{NDM}$, and *bla*$_{OXA48}$, leading to resistance to most commonly used antimicrobial agents and which causing serious threats to public health.[5,27–31] With no exception for *K. variicola* SKv2E and *K. pneumoniae* SKp2F, antimicrobial susceptibility testing showed that these two strains were resistant to most commonly used antibiotics, such as the β-lactam antibiotics, fluoroquinolones, aminoglycosides, and others. For the virulence assay, we proved that *K. variicola* SKv2E has a higher pathogenicity than *K. pneumoniae* SKp2F via human serum killing testing, which was similar to previous research.[20] This is because the type IV pili coding gene (*pilW*), colonization and immune evasion gene (*glf*), and fimbrial adherence determinant genes (*steB, stfD*) were determined from SKv2E, which may increase the grade of serum resistance or virulence.[32,33] In addition, we identified the gene *pilW*, which encodes type IV pili, from *K. variicola* SKv2E, which may be beneficial to the formation of biofilm,[34,35] and this may be a reason for *K. variicola* SKv2E having stronger biofilm-forming capability than *K. pneumoniae* SKp2F.

The transmission of antibiotic resistance genes and/or virulence factors by various mobile genetic elements (plasmids, integrons, and transposons)[36,37] among the bacterial community is one of the major threats to human health. In this study, we found that the carbapenemase-coding *bla*$_{KPC}$ genes of SKv2E and SKp2F were carried by similar linear structures, *IS*Kpn6-*bla*$_{KPC-2}$-*bla*$_{TEM}$-*IS*Kpn27 and *IS*Kpn6-*bla*$_{KPC-2}$-*IS*Kpn27, which had a high incidence in the *bla*$_{KPC}$-carrying *Klebsiella* isolates.[38–40] Moreover, other resistance genes (*arr-3*, *dfrA27*, *aadA16*, and *qacEΔ1-sul1*) were carried by the transposon Tn6338 and were confirmed in the genomes of both SKv2E and SKp2F. These results indicate that resistance genes carrying mobile genetic elements can be transmitted or integrated between bacteria in the same host.

Conclusions

We identified *bla*$_{KPC}$-harboring *K. variicola* and *K. pneumoniae* from the same sample, and both carried multiple resistance genes, virulence factors, and various mobile genetic elements. Our results demonstrate that we should pay more attention to the bacteria identified. We also found that some mobile genetic elements from *K. variicola* and *K. pneumoniae* were highly similar. This indicates that these resistance genes carrying mobile genetic elements can be transmitted or integrated between bacteria in the same host.

Nucleotide Sequence Accession Numbers

These Whole Genome Shotgun projects have been deposited in DDBJ/EMBL/GenBank under the sequence accession numbers JAHRXK000000000 and JAHRXL000000000 for *Klebsiella pneumoniae* strain SKp2F and *Klebsiella variicola* strain SKv2E, respectively.

Ethical Approval

This study was conducted after agreement from the local ethics committee (no. 20180309059) and with the patient's informed consent.

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Disclosure

The authors have no conflicts of interest to declare.

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