

Impact of Carbapenem Heteroresistance Among Multidrug-Resistant ESBL/AmpC-Producing *Klebsiella pneumoniae* Clinical Isolates on Antibiotic Treatment in Experimentally Infected Mice

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Purpose: Antibiotic resistance is a growing health crisis that is further complicated by treatment failures caused by bacteria that exhibit heterogeneous susceptibility to antibiotics. The aim of this study was to describe imipenem (IPM)-heteroresistant strains among multidrug-resistant (MDR) ESBL/AmpC-producing *Klebsiella pneumoniae* clinical isolates, investigate their molecular phenotypic characteristics, and elucidate the outcome of antibiotic treatment in mice infected with the heteroresistant isolates.

Materials and Methods: Antimicrobial susceptibility of *K. pneumoniae* isolates was determined by the disk diffusion and E-test methods. Heteroresistance to IPM was confirmed by population analysis profile (PAP) assays. PCR and sequencing were employed to detect MDR determinants. Molecular differences between the susceptible and resistant subpopulations were evaluated by sequencing and quantitative real-time reverse transcription PCR (qRT-PCR) analysis. The effect of the carbapenem-heteroresistant strains on antibiotic treatment was assessed using a mouse model of peritonitis with heteroresistant *K. pneumoniae* and subsequent treatment with IPM.

Results: In total, 37 MDR ESBL/AmpC-producing clinical isolates of *K. pneumoniae* were identified between September 2018 and December 2019. These strains were notably resistant to conventional antimicrobials other than carbapenems. Among the isolates, three strains exhibited heteroresistance to IPM and carried several ESBL and/or AmpC genes. Mice infected with a lethal dose of any of the three heteroresistant isolates were unable to survive in the presence of IPM treatment, as the percentage of the IPM-resistant subpopulation of each strain was increased in the peritoneum of these mice at 24 h after infection. The resistant subpopulation of the strains presented pulsed-field gel electrophoresis (PFGE) profiles that were identical to those of the susceptible subpopulation, but *ompK36* porin showed a reduction in gene expression (0.09- to 0.50-fold) in the resistant subpopulation.

Conclusion: Carbapenem-heteroresistant strains were present among the MDR *K. pneumoniae* isolates producing ESBL/AmpC β -lactamases, and these heteroresistant strains failed IPM therapy in experimentally infected mice.

Keywords: *Enterobacteriaceae*, imipenem, heterogeneous susceptibility, treatment failure, OmpK porin, in vivo

Introduction

Antibiotic resistance is a major public health threat, and it is predicted to cause 10 million annual deaths worldwide by 2050.¹ *Klebsiella pneumoniae* is widely

distributed in the environment and is increasingly reported as a cause of nosocomial infections in immunocompromised individuals, including urinary tract, bloodstream, and soft tissue infections.^{2,3} *K. pneumoniae* infections have become progressively more difficult to treat due to development of multi-resistance to antibiotics, including β -lactam antibiotics. The most common cause of *K. pneumoniae* resistance to broad-spectrum β -lactam antibiotics is the production of extended-spectrum β -lactamases (ESBLs) and AmpC β -lactamase.⁴ Infections caused by multidrug-resistant (MDR) *Enterobacteriaceae*, including *K. pneumoniae*, are associated with increased morbidity, mortality, long hospital stays, and high health-care costs.^{5,6}

Carbapenems, a class of antibiotics that include imipenem (IPM) and meropenem (MEM), are used widely for the treatment of serious infections involving MDR bacteria.⁷ Carbapenems are effective against *K. pneumoniae*, including strains that produce ESBLs or AmpC cephalosporinases. However, carbapenem-resistant *Enterobacteriaceae* (CRE), such as KPC or NDM-1 β -lactamase-producing *K. pneumoniae*, has emerged in various parts of the world in the last decade.^{8–10} The rate of carbapenem resistance among *K. pneumoniae* in China increased from below 5% in 1998–2012 to 34.9% in 2013–2017.¹¹

In *K. pneumoniae*, the predominant mechanism responsible for carbapenem resistance is the production of carbapenem-hydrolysing β -lactamases such as KPC (*Klebsiella pneumoniae* carbapenemase) (Ambler class A), MBLs (Metallo- β -Lactamases) (Ambler class B), and OXA-48-like (Ambler class D). In addition, nonspecific multidrug-resistance mechanisms, such as the production of an ESBL and/or plasmid-borne AmpC β -lactamases associated with a decrease in permeability of the outer membrane (especially through alteration of OmpK35 and OmpK36 porins) and overexpression of efflux pumps, can result in carbapenem resistance.^{12–14}

The emergence of carbapenem-resistant strains limits treatment options for infections. Moreover, the treatment of some bacterial infections is further complicated by the failure of antibiotic therapy in strains that are classified as heteroresistant.^{15,16} The concept of heteroresistance describes the heterogeneous susceptibility of a microorganism population to an antimicrobial drug, meaning that some subpopulations may be resistant to the antimicrobial, while others are susceptible.¹⁷ Previous studies have shown that heteroresistant *Enterobacteriaceae* misclassified as colistin-susceptible by current diagnostic tests, may cause in vivo colistin treatment

failure.^{18,19} Carbapenem-heteroresistant strains of *K. pneumoniae* have been reported in clinical isolates, but their effect on the outcome of infection and on in vivo antibiotic treatment has yet to be elucidated.²⁰ This study describes the identification of three MDR ESBL/AmpC-producing isolates of *K. pneumoniae* that exhibit IPM heteroresistance. The outcome of antibiotic treatment in a mouse model infected with these heteroresistant isolates was also determined.

Materials and Methods

Bacterial Strains and Phenotypic Assays

K. pneumoniae clinical isolates were obtained from blood samples of different patients in the university-affiliated hospital in Dalian, a 2350-bed tertiary-care hospital with approximately 200,000 patient visits per year, from September 2018 to December 2019. MicroScan WalkAway 96 Plus (Siemens AG, Munich, Germany) was used to confirm bacterial identification and initial susceptibility testing. MDR *K. pneumoniae* isolates (non-susceptibility to at least one agent in three or more antibiotic categories)²¹ and those susceptible to carbapenems (IPM, MEM and ertapenem [ETP]) were included. Phenotypic detection of ESBLs was performed by the double disk synergy test, as guided by the Clinical and Laboratory Standards Institute (CLSI).²² Isolates showing a negative-ESBL phenotype were assayed for AmpC production by three-dimensional test.²³ *Escherichia coli* ATCC25922 and *K. pneumoniae* ATCC13883 (American Type Culture Collection, Manassas, VA, USA) were used as reference strains.

Antimicrobial Susceptibility Testing

Isolates of *K. pneumoniae* showing suspected MDR in the MicroScan system and exhibiting ESBL and/or AmpC production were tested further by the Kirby-Bauer disk diffusion method. The tests were performed according to the CLSI guidelines for the following antimicrobials (Oxoid, Hampshire, England):²² cefotaxime (CTX, 30 μ g), ceftazidime (CAZ, 30 μ g), cefepime (FEP, 30 μ g), cefoxitin (FOX, 30 μ g), aztreonam (ATM, 30 μ g), IPM (10 μ g), MEM (10 μ g), ETP (10 μ g), gentamicin (GEN, 5 μ g), levofloxacin (LEV, 5 μ g), ciprofloxacin (CIP, 5 μ g) and amikacin (AMK, 30 μ g). The minimum inhibitory concentration (MIC) against carbapenems (IPM, MEM, and ETP) was determined using E-test (bioMérieux, Marcy l'Etoile, France) or the standard microdilution broth method. *E. coli* ATCC25922 served as the control strain for susceptibility testing.

Molecular Detection of ESBL, AmpC Genes, and Other Resistance Determinants

Total DNA preparations were obtained by thermolysis of the isolates.²⁴ Polymerase chain reaction (PCR) was performed with previously published primers to amplify the resistance determinants including ESBL genes (*bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, and *bla*_{OXA}),²⁵ AmpC β -lactamase genes (*bla*_{DHA}, *bla*_{CIT} [*bla*_{CMY}], *bla*_{ACC}, *bla*_{EBC} and *bla*_{FOX}),²⁴ and carbapenemases genes (*bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{NDM}, and *bla*_{OXA-48}). Primers used are listed in [Supplementary Table 1](#). In addition, 16S-RMTase-encoding genes involved in aminoglycoside resistance (*armA*, *rmtB*, and *rmtC*) and plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrS*, *qepA*, and *aac(6)-Ib-cr*) were investigated.^{26,27} PCR products were analyzed by electrophoresis in 1.5% agarose gels. Positive and negative controls were included in all PCR assays. Positive amplicons were sequenced and aligned with subtypes of β -lactamase genes by BLAST (<http://blast.ncbi.nlm.nih.gov>).

Multilocus Sequence Typing (MLST)

MLST was performed on all MDR *K. pneumoniae* isolates by amplifying seven standard housekeeping loci (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) according to the protocol described on the *K. pneumoniae* MLST database website (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html>). Sequence types (STs) were assigned using the online database of the Pasteur Institute MLST website (<http://bigsdbs.pasteur.fr/klebsiella/klebsiella.html>).

Population Analysis Profile (PAP)

Heteroresistance was determined by population analysis profile (PAP) for strains displaying small colonies growing within the zone of inhibition surrounding the highest concentrations in the carbapenem E-test. Briefly, cultures were grown overnight in Luria-Bertani (LB) broth and serially diluted in saline. Aliquots (100 μ L) of each dilution were spread on freshly prepared LB plates containing IPM in a range of concentrations from 0.25 to 32 μ g/mL. The plates were incubated overnight at 37°C and the number of growing colonies was counted. The frequency of the heteroresistant subpopulation was calculated by dividing the number of colonies grown on antibiotic-containing plates by the colony counts from the same bacterial

inoculum plated onto antibiotic-free media. Data were presented on a semi-logarithmic graph with relative resistant population frequencies on the vertical axis and drug concentration on the horizontal axis. Isolates that presented heteroresistant colonies were tested in the subsequent methodologies.

Virulence Factor in Heteroresistant Isolates

The presence of virulence factor (VF) genes in the heteroresistant isolates was detected by PCR using specific primers as described previously.^{28,29} These virulence-associated genes included those encoding regulators of exopolysaccharide synthesis (*rmpA*), fimbrial adhesins (*mrkD*), the ferric iron uptake system (*entB*, *ybtS*, *iutA*, and *kfu*), allantoin metabolism (*allS*) and the virulent capsular serotype (K1, K2, K5, K20, K54 and K57), that enable bacteria to overcome host defenses.

Mice, Experimental Design, and Heteroresistant *K. pneumoniae* Infections

A mouse model of peritonitis was used to assess the effect of antibiotic treatment on the heteroresistant isolates.¹⁸ C57/BL6J male and female mice, aged 9–11 weeks and weighing 18–22 g, were obtained from the Laboratory Animal Centre of Dalian Medical University. Experimental groups were matched by age and sex and housed at the Institute of Genome Engineered Animal Models for Human Disease, Dalian, China. All animals were housed in pathogen-free conditions with 12-h light–dark cycles and unlimited access to food and water.

Eighty separately housed mice were allocated to eight experimental groups ([Supplementary Figure 1](#)). A lethal dose (3×10^8 colony-forming units [CFU]) of either of the IPM-heteroresistant *K. pneumoniae* isolates or susceptible *K. pneumoniae* ATCC13883 was inoculated intraperitoneally to each group (n = 10 per group). Imipenem/cilastatin sodium (Zhuhai United Laboratories Co., Ltd, Zhuhai, China) or PBS (as a control) was given intraperitoneally as a treatment at the recommended dosage of 160 mg/kg of body weight at 12 h and 18 h after infection, and then five mice in each group were sacrificed at 24 h after the infection. Peritoneal lavage fluid was collected and plated onto drug-free medium or medium containing 8 μ g/mL IPM to assess the percentages of IPM-susceptible and -resistant subpopulations. The remaining five mice in each group were monitored for survival and

weight loss until 72 h, and were euthanized if their weight decreased to less than 80% of their starting weight. All animal care and use protocols in this study were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China. All animal experimental procedures were approved by the Animal Experimental Ethical Inspection Form of Dalian Medical University (protocol number: AEE 18001).

Quantitative Reverse Transcription PCR (qRT-PCR) Analysis of Molecular Differences Between the IPM-Susceptible and -Resistant Subpopulations of *K. pneumoniae*

To identify the phenotypic differences between the IPM-resistant and -susceptible subpopulations, of *K. pneumoniae*, cultures with a majority resistant or susceptible subpopulations were isolated in MH broth with or without IPM as indicated in Figure 1C. The coding sequences of *ompK35* and *ompK36* porin genes in the original population and the resistant or susceptible subpopulations were amplified and sequenced. The resulting sequences were compared with those of *ompK35* and *ompK36* available in the NCBI database (GenBank accession number NC_016845) using an updated version of the BLAST program.

Subsequently, the expression levels of *ompK35* and *ompK36* genes, efflux pump gene *acrA*, and regulator *ramA*, were determined by real-time quantitative reverse transcription PCR (qRT-PCR). The primers used for PCR and qRT-PCR are listed in Supplementary Table 1. Reactions were performed in triplicate using an Mx3005P qPCR System (Stratagene Agilent, CA, USA) with the TB Green[®] Premix Ex Taq[™] II Kit (TaKaRa, Dalian, China) as we previously reported.²⁵ Relative mRNA expression levels were determined by comparison with expression levels in the reference strain *K. pneumoniae* ATCC13883.

Pulsed-Field Gel Electrophoresis (PFGE)

Clonality among the IPM-resistant or -susceptible subpopulations of the heteroresistant *K. pneumoniae* isolates was demonstrated by comparison of their *Xba*I-digested genomic DNA patterns obtained by pulsed-field gel electrophoresis (PFGE) as described previously.³⁰

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0.1 (GraphPad Software, Ca, USA). Percentages of the resistant subpopulation of the heteroresistant strains in the peritoneal lavage fluid were compared using a Mann-Whitney test. Survival curves were compared using a Gehan-Breslow-Wilcoxon test. Differences in gene expression between the resistant and susceptible subpopulations were compared using Student's *t*-test. Two-tailed *P*-values <0.05 were considered statistically significant.

Ethical Clearance

The collection of *K. pneumoniae* in this study was part of the routine hospital laboratory procedure. This study was performed using samples for secondary use, free of the need for informed consent and ethics committee approval.

Results

Antimicrobial Susceptibility

Thirty-seven MDR isolates of *K. pneumoniae* were confirmed to produce ESBL and/or AmpC β -lactamase and exhibited high resistance rates to cephalosporins other than FOX (CAZ: 97.30%; CTX: 94.59%; FEP: 75.68%; and FOX: 29.73%). These isolates also showed different degrees of resistance to other antibiotics including ATM (86.49%), GEN (51.35%), AMK (91.89%), LEV (89.19%), and CIP (97.30%). All isolates were susceptible to carbapenems, but three isolates of which (*Kp19*, *Kp25*, and *Kp34*) had small colonies that grew within the IPM zone of inhibition (Figure 1A). Antibiotic resistance patterns of the MDR *K. pneumoniae* isolates are shown in Table 1.

Antibiotic Resistance Determinants

The distribution of the antibiotic resistance determinants among the 37 MDR ESBL/AmpC-producing *K. pneumoniae* isolates is presented in Table 1. The most frequently detected ESBL genes were *bla*_{CTX-M-15} (51.35%) followed by *bla*_{CTX-M-22} (10.81%), *bla*_{TEM-53} (8.11%), and *bla*_{CTX-M-14} (8.11%). Regarding β -lactamases other than ESBLs, the *bla*_{SHV-11} and *bla*_{TEM-1} alleles predominated (data not shown). Seventeen of the isolates were designated AmpC producers, and the most frequently detected genes were *bla*_{DHA-1} (45.95%) and *bla*_{CMY-2} (10.81%). Carbapenemase genes (*bla*_{KPC}, *bla*_{VIM}, *bla*_{IMB}, *bla*_{NDM}, and *bla*_{OXA-48}) were not detected in any of the studied isolates. For the PMQR genes, 22 isolates carried *qnrB*, while 28

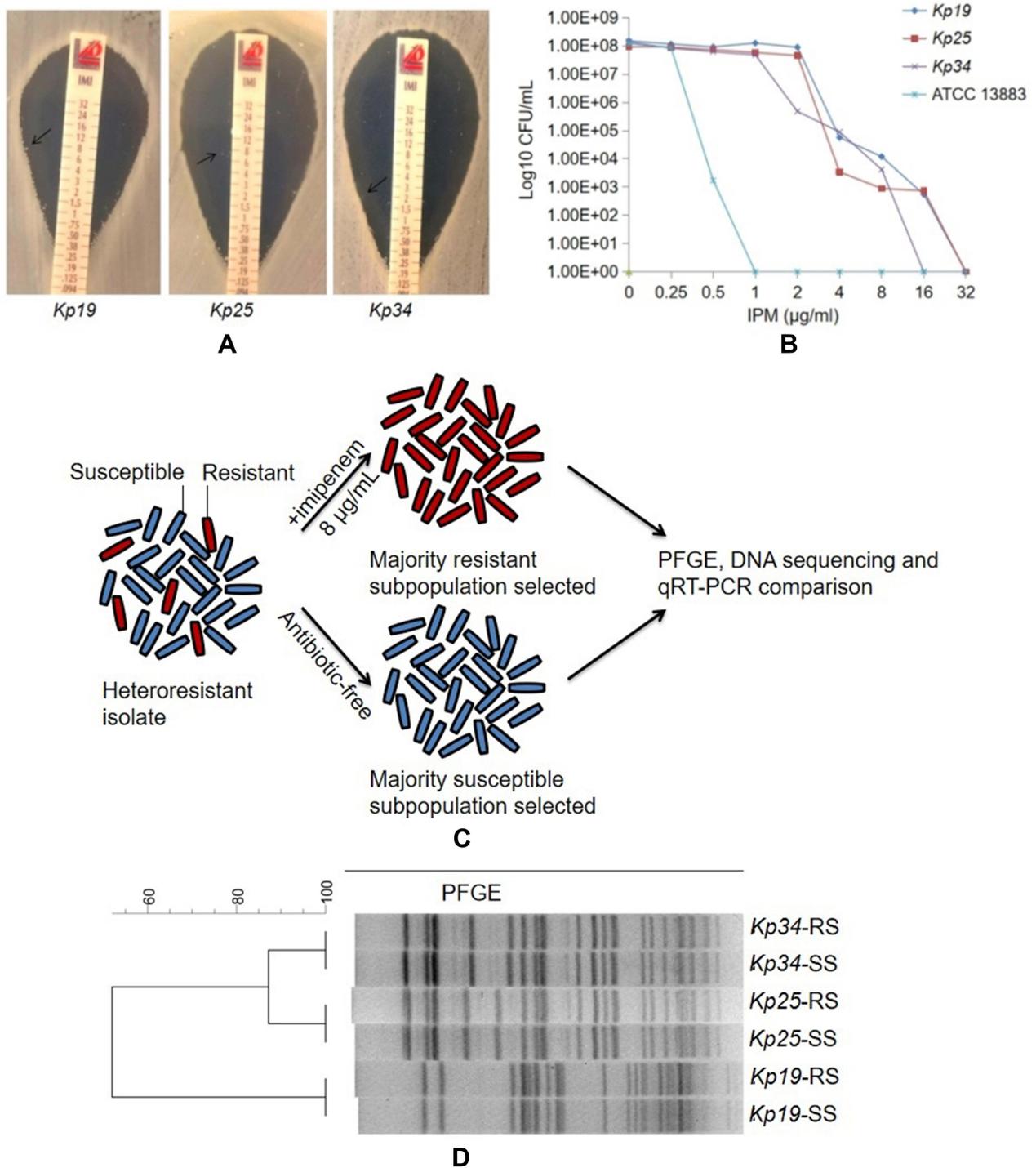


Figure 1 Characteristics of IPM heteroresistance among the *Kp19*, *Kp25* and *Kp34* multidrug-resistant *K. pneumoniae* isolates. **(A)** Satellite colonies in the IPM MIC gradient strips (black arrows) for three *K. pneumoniae* isolates; **(B)** population analysis profiles of the three multidrug-resistant *K. pneumoniae* and control strain; **(C)** workflow for subculture of IPM-susceptible and -resistant subpopulations. Cultures of *Kp19*, *Kp25* and *Kp34* were grown for 18 h in medium containing 8 µg/mL imipenem or drug-free medium, respectively; **(D)** PFGE results of the imipenem-resistant (RS) and -susceptible subpopulations (SS) of heteroresistant strains. IPM, imipenem; ATCC13883, *K. pneumoniae* ATCC13883.

Table 1 Phenotypic and Molecular Characterisation of 37 Multidrug-Resistant *K. pneumoniae* Clinical Isolates

Isolate	Antibiotic Resistance Profile	MLST	ESBLs/ AmpC	Other Resistance Genes
Kp1	CAZ, CTX, FEP, ATM, AMK, LEV, CIP	ST11	<i>bla</i> _{CTX-M-15}	<i>qnrS</i> , <i>rmtB</i>
Kp2	CAZ, CTX, FEP, ATM, GEN, AMK, LEV, CIP	ST304	<i>bla</i> _{SHV-27}	<i>qnrB</i> , <i>qnrS</i> , <i>armA</i>
Kp3	CAZ, CTX, FOX, ATM, AMK, LEV	ST2231	<i>bla</i> _{DHA-1} , <i>bla</i> _{CMY-2}	<i>qnrS</i>
Kp4	CAZ, CTX, FEP, ATM, AMK, LEV, CIP	ST304	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{OXA-10}	<i>qnrB</i> , <i>qnrS</i> , <i>armA</i>
Kp5	CAZ, CTX, FEP, ATM, GEN, AMK, LEV, CIP	ST304	<i>bla</i> _{CTX-M-15}	<i>qnrB</i> , <i>qnrS</i> , <i>armA</i>
Kp6	CAZ, CTX, FEP, FOX, GEN, AMK, LEV, CIP	ST304	<i>bla</i> _{DHA-1} , <i>bla</i> _{CMY-2}	<i>qnrB</i> , <i>qnrS</i> , <i>armA</i>
Kp7	CAZ, CTX, FOX, ATM, GEN, LEV, CIP	ST304	<i>bla</i> _{DHA-1}	<i>qnrA</i> , <i>qnrB</i> , <i>qnrS</i> , <i>armA</i>
Kp8	CAZ, CTX, FEP, ATM, AMK, LEV, CIP	ST37	<i>bla</i> _{CTX-M-15}	<i>qnrB</i> , <i>armA</i>
Kp9	CTX, FEP, GEN, AMK, CIP	ST716	<i>bla</i> _{SHV-27}	<i>qnrS</i>
Kp10	CAZ, ATM, GEN, AMK, LEV, CIP	ST828	<i>bla</i> _{CTX-M-22}	ND
Kp11	CAZ, CTX, GEN, CIP	ST268	<i>bla</i> _{CTX-M-15}	<i>qnrB</i> , <i>qnrS</i> , <i>armA</i>
Kp12	CAZ, ATM, AMK, LEV, CIP	ST65	<i>bla</i> _{CTX-M-15}	ND
Kp13	CAZ, CTX, FOX, ATM, GEN, AMK, LEV, CIP	ST37	<i>bla</i> _{DHA-1}	<i>qnrB</i> , <i>armA</i>
Kp14	CAZ, CTX, FEP, ATM, AMK, LEV, CIP	ST304	<i>bla</i> _{CTX-M-15}	<i>qnrB</i> , <i>qnrS</i> , <i>armA</i>
Kp15	CAZ, CTX, FEP, ATM, AMK, LEV, CIP	ST304	<i>bla</i> _{TEM-53} , <i>bla</i> _{CTX-M-15} , <i>bla</i> _{DHA-1}	<i>qnrS</i> , <i>armA</i>
Kp16	CAZ, CTX, FEP, FOX, ATM, GEN, AMK, LEV, CIP	ST304	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{DHA-1}	<i>qnrB</i> , <i>qnrS</i>
Kp17	CAZ, CTX, FEP, ATM, GEN, LEV, CIP	ST304	<i>bla</i> _{CTX-M-15}	<i>qnrB</i> , <i>qnrS</i>
Kp18	CAZ, CTX, FEP, ATM, AMK, LEV, CIP	ST37	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{DHA-1}	<i>qnrB</i> , <i>armA</i>
Kp19 ^a	CAZ, CTX, FEP, ATM, MEM, ERP, AMK, LEV, CIP	ST2232	<i>bla</i> _{CTX-M-15}	<i>rmtB</i>
Kp20	CAZ, CTX, FEP, AMK, LEV, CIP	ST716	<i>bla</i> _{CTX-M-22}	<i>qnrS</i>
Kp21	CAZ, CTX, FOX, ATM, GEN, AMK, CIP	ST304	<i>bla</i> _{DHA-1}	<i>qnrS</i>
Kp22	CAZ, CTX, FEP, GEN, AMK, LEV, CIP	ST716	<i>bla</i> _{CTX-M-22} , <i>bla</i> _{DHA-1}	<i>qnrS</i>
Kp23	CAZ, CTX, FEP, ATM, GEN, AMK, LEV, CIP	ST304	<i>bla</i> _{CTX-M-15}	<i>qnrB</i> , <i>qnrS</i> , <i>armA</i>
Kp24	CAZ, CTX, ATM, AMK, LEV, CIP	ST304	<i>bla</i> _{CTX-M-22}	<i>qnrB</i> , <i>qnrS</i>
Kp25 ^a	CAZ, CTX, FEP, ATM, ERP, AMK, LEV, CIP	ST304	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{DHA-1}	<i>qnrB</i> , <i>qnrS</i> , <i>armA</i>
Kp26	CAZ, CTX, FEP, ATM, AMK, LEV, CIP	ST304	<i>bla</i> _{TEM-53} , <i>bla</i> _{CTX-M-15}	<i>qnrS</i> , <i>armA</i>
Kp27	CAZ, CTX, FEP, ATM, AMK, LEV, CIP	ST304	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{DHA-1}	<i>qnrS</i>
Kp28	CAZ, CTX, FEP, ATM, AMK, LEV, CIP	ST15	<i>bla</i> _{SHV-28} , <i>bla</i> _{DHA-1}	<i>qnrB</i>
Kp29	CAZ, CTX, FEP, ATM, AMK, CIP	ST1049	<i>bla</i> _{CTX-M-14}	<i>qnrB</i>
Kp30	CAZ, CTX, FEP, ATM, GEN, AMK, LEV, CIP	ST15	<i>bla</i> _{SHV-28}	<i>qnrB</i>
Kp31	CAZ, CTX, FEP, ATM, AMK, LEV, CIP	ST16	<i>bla</i> _{TEM-53}	<i>qnrS</i> , <i>armA</i>
Kp32	CAZ, CTX, FEP, FOX, ATM, GEN, AMK, LEV, CIP	ST17	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{DHA-1}	<i>qnrS</i>
Kp33	CAZ, CTX, FEP, FOX, ATM, GEN, AMK, LEV, CIP	ST18	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{DHA-1} , <i>bla</i> _{CMY-2}	<i>qnrB</i> , <i>qnrS</i> , <i>armA</i>
Kp34 ^a	CAZ, CTX, FEP, FOX, ATM, GEN, AMK, LEV, CIP	ST19	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{DHA-1} , <i>bla</i> _{CMY-2}	<i>qnrB</i> , <i>qnrS</i>
Kp35	CAZ, CTX, FEP, FOX, ATM, GEN, AMK, LEV, CIP	ST660	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{DHA-1}	<i>qnrB</i> , <i>qnrS</i> , <i>armA</i>
Kp36	CAZ, CTX, FOX, ATM, GEN, AMK, LEV, CIP	ST14	<i>bla</i> _{DHA-1}	<i>qnrB</i> , <i>qnrS</i> , <i>armA</i>
Kp37	CAZ, CTX, FEP, ATM, AMK, LEV, CIP	ST15	<i>bla</i> _{CTX-M-14}	<i>qnrS</i>

Note: ^aHad small colonies in the imipenem MIC gradient strips.

Abbreviations: CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; FOX, ceftoxime; ATM, aztreonam; GEN, gentamicin; AMK, amikacin; LEV, levofloxacin; CIP, ciprofloxacin; MLST, multilocus sequence typing; ESBL, extended-spectrum β -lactamase; ST, sequence type; ND, not determined.

isolates contained *qnrS* or other variants such as *qnrA*. The resistance genes for other antibiotics included *armA* in 18 isolates and *rmtB* in two isolates.

Verification of Carbapenem Heteroresistance

Colonies that grew within the zone of inhibition in the IPM E-test were observed in three MDR strains (*Kp19*, *Kp25*, and *Kp34*) (Figure 1A). Heteroresistance in these three

K. pneumoniae isolates was confirmed by PAP assay. IPM-resistant subpopulations were found in these three strains, and they grew in concentrations as high as 16 μ g/mL, 16 μ g/mL and 8 μ g/mL of IPM, respectively (Table 2 and Figure 1B).

Effect of Heteroresistant Isolates on IPM Treatment

Mice were infected with a lethal dose of either IPM-heteroresistant or -susceptible *K. pneumoniae*, treated with

Table 2 Analysis of the Presence of IPM Heteroresistance in Three MDR *K. pneumoniae* Isolates and Sequencing Profiles of *ompK35* and *ompK36* Genes

Isolate	Original Carbapenems MIC ($\mu\text{g/mL}$)			Original Population Porin Sequencing		Population Analysis Profile			Resistant Subpopulations Porin Sequencing	
	IPM E-Test	IPM Microdilution	MEM E-Test	ETP E-Test	<i>ompK35</i>	<i>ompK36</i>	Highest IPM Conc'n Grown ($\mu\text{g/mL}$)	Frequency of Heteroresistant Subpopulation	<i>ompK35</i>	<i>ompK36</i>
<i>Kp19</i>	0.38	0.5	0.047	0.067	WT	WT	16	4.0×10^{-6}	WT	WT
<i>Kp25</i>	0.25	1	0.047	0.047	WT	WT	16	2.7×10^{-6}	WT	WT
<i>Kp34</i>	0.25	0.5	0.064	0.067	WT	WT	8	3.3×10^{-7}	WT	WT

Abbreviations: IPM, imipenem; MEM, meropenem; ETP, ertapenem; WT, wild type.

PBS or IPM after 12 h and 18 h, and then euthanized at 24 h and the peritoneal lavage fluid was collected. The total bacterial load in the peritoneal lavage fluid of mice infected with the susceptible strain (*K. pneumoniae* ATCC13883) was significantly reduced by IPM treatment ($t = 3.495$, $P < 0.01$). In contrast, the heteroresistant strains were refractory to treatment with IPM, with the bacterial levels remaining unchanged between the IPM-treated and PBS-treated groups (Figure 2A). There was a robust increase in the frequency of the resistant subpopulation isolated during in vivo infection with the three heteroresistant isolate (5.08- to 19.35-fold). Interestingly, in the absence of IPM treatment, there was also a significant increase in the frequency of the resistant subpopulation of bacteria isolated from the peritoneum (*Kp19*: pre-infection vs PBS-treated group, $Z = -2.402$, $P = 0.016$; pre-infection vs IPM-treated group, $Z = -2.611$, $P < 0.01$; *Kp25*: pre-infection vs PBS-treated group, $Z = -2.611$, $P < 0.01$; pre-infection vs IPM-treated group, $Z = -2.619$, $P < 0.01$; *Kp34*: pre-infection vs PBS-treated group, $Z = -2.611$, $P < 0.01$; pre-infection vs IPM-treated group, $Z = -2.611$, $P < 0.01$. Figure 2B).

In the survival experiment, both the susceptible and heteroresistant strains led to lethal infections in the absence of IPM (Figure 2C–F). In the presence of IPM, only mice infected with the susceptible strain were rescued ($\chi^2 = 10.000$, $P = 0.0016$, Figure 2C), whereas those infected with the heteroresistant strains of *K. pneumoniae* still succumbed to infection within 72 h (*Kp19*: $\chi^2 = 0.148$, $P = 0.700$; *Kp25*: $\chi^2 = 0.012$, $P = 0.914$; *Kp34*: $\chi^2 = 0.012$, $P = 0.914$, respectively; Figure 2D–F). These data demonstrate the impact of an antibiotic-resistant subpopulation in mediating a lethal infection during antibiotic treatment.

Virulence Factor Detection

PCR analysis for virulence factors demonstrated that *mrkD* genes encoding type 3 fimbrial adhesins were present in all three heteroresistant isolates. The genes *entB*, *ybtS*, *iutA*, and *kfu*, encoding components of the ferric iron uptake system, were detected in three, two, two and two isolates, respectively (Figure 2D–F).

Molecular Differences Between the IPM-Susceptible and -Resistant Subpopulations

The IPM-resistant or -susceptible subpopulations of heteroresistant isolates of *K. pneumoniae* exhibited identical PFGE profiles (Figure 1D). The sequences of *ompK35* and *ompK36*

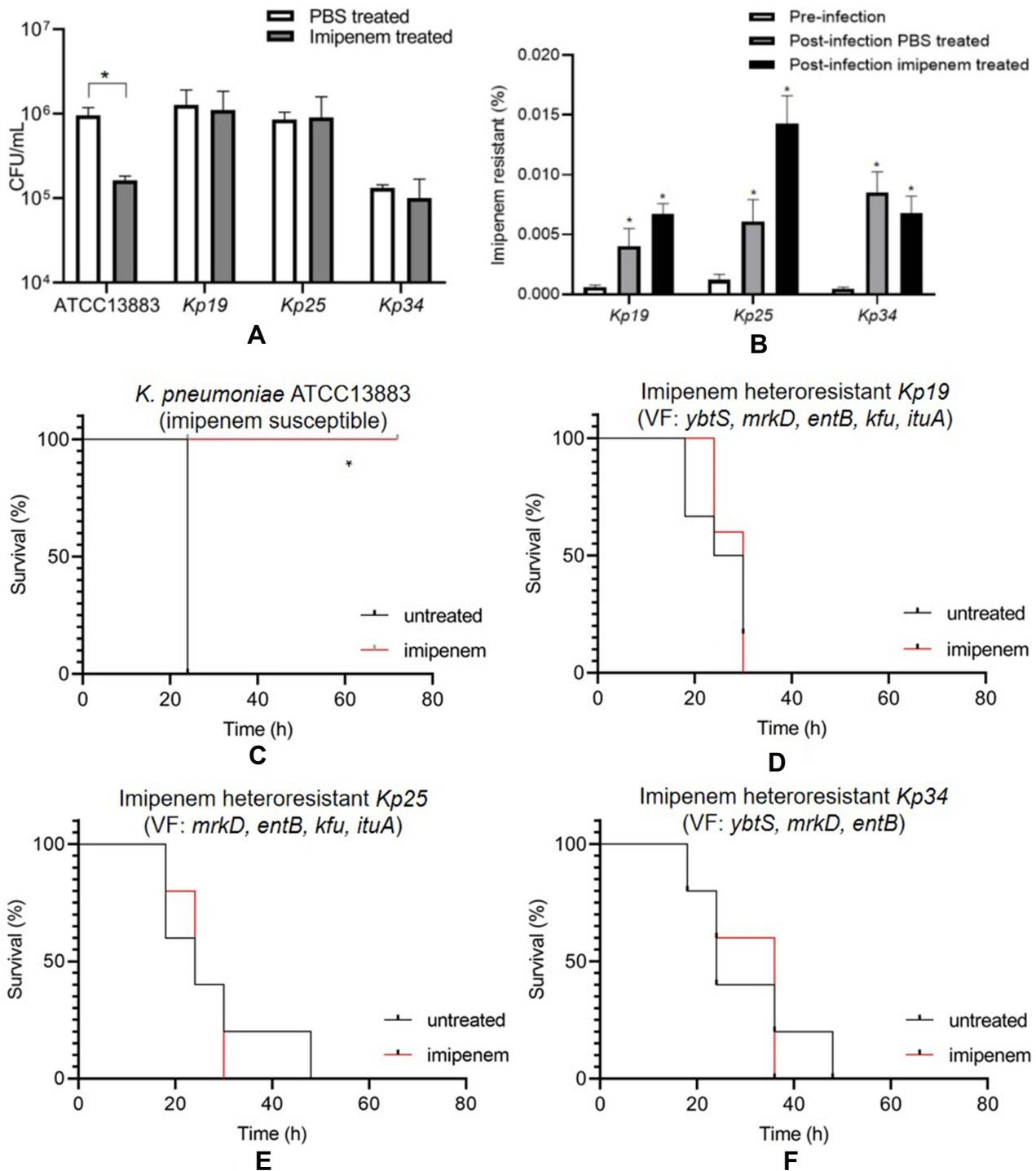


Figure 2 Heteroresistant *K. pneumoniae* isolates lead to IPM treatment failure in vivo. Mice were infected with the IPM-susceptible *K. pneumoniae* ATCC13883 or the IPM-heteroresistant isolates, treated with imipenem/cilastatin sodium or PBS at 12 h and 18 h after infection, and then euthanized at 24 h and the peritoneal lavage fluid was collected. (A) Numbers of CFU were quantified at 24 h in the peritoneal lavage fluid; (B) increase in the frequency of the IPM-resistant subpopulation in the peritoneal lavage fluid; (C) survival of mice infected with the *K. pneumoniae* ATCC13883 and then treated with imipenem or PBS; (D) survival of mice infected with the IPM-heteroresistant *Kp19* and then treated with imipenem or PBS; (E) survival of mice infected with the IPM-heteroresistant *Kp25* and then treated with imipenem or PBS; (F) survival of mice infected with the IPM-heteroresistant *Kp34* and then treated with IPM or PBS. Surviving mice were monitored until day 3 (n = 5). Error bars represent SEM (Mann–Whitney test). VF, virulence factor; ATCC13883, *K. pneumoniae* ATCC13883; *P values are significant (P < 0.05).

in the original heteroresistant *K. pneumoniae* strains, and in resistant or susceptible subpopulations, were identical compared to those available in the GenBank database (Table 2). In addition, real-time qRT-PCR revealed mRNA expression levels of *ompK36* were significantly reduced (0.09- to 0.50-fold) in the IPM-resistant subpopulations compared with the IPM-susceptible subpopulation of each strain (*Kp19*: $t = 12.093$, $P < 0.01$; *Kp25*: $t = 12.616$, $P < 0.01$; *Kp34*: $t = 3.07$, $P = 0.037$, respectively), while the differential expression of *ompK35*, efflux pump gene *acrA* and the regulator gene *ramA* between IPM-resistant and -susceptible subpopulations were not statistically significant (Figure 3A, C and D).

MLST

MLST analysis identified 13 different sequence types (STs) among the 37 isolates, as shown in Table 1. Fifteen isolates (*Kp2*, *Kp4*, *Kp5*, *Kp6*, *Kp7*, *Kp14*, *Kp15*, *Kp16*, *Kp17*, *Kp21*, *Kp23*, *Kp24*, *Kp25*, *Kp26*, and *Kp27*) belonged to ST304, which was the predominant group. Seven isolates (*Kp31*, *Kp32*, *Kp33*, *Kp34*, *Kp37*, *Kp28*, and *Kp30*) belonged to ST15, three isolates (*Kp9*, *Kp20*, and *Kp22*) belonged to ST716, and three isolates (*Kp8*, *Kp13*, and *Kp18*) belonged to ST37. In addition, one strain

of each of ST65, ST11, ST2232, ST828, ST660, ST2231, ST1049, ST268, and ST14 was identified.

Discussion

MDR *K. pneumoniae* infections have become progressively difficult to treat due to the emergence of isolates that are resistant or heteroresistant to carbapenem antibiotics. Carbapenem-resistant *K. pneumoniae* (CRKP), as one of the CRE, is one of the most urgent antibiotic resistance threats according to the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO).^{31,32} In the current study, three IPM-heteroresistant strains were identified among a group of clinical isolates of ESBL/AmpC-producing *K. pneumoniae* and the role of a minor IPM-resistant subpopulation of these heteroresistant strains was highlighted in the promotion of antibiotic treatment failure in vivo.

Heteroresistance to carbapenems occurs in several species of Gram-negative bacterial species, including *K. pneumoniae*.^{33,34} Heterogeneous susceptibility to this antibiotic class among bacterial populations can be attributed to genetic and epigenetic mechanisms.³⁵ Genetic mechanisms may be due to mutations or duplications of key resistance genes or regulatory systems.^{17,36} Epigenetic variation across

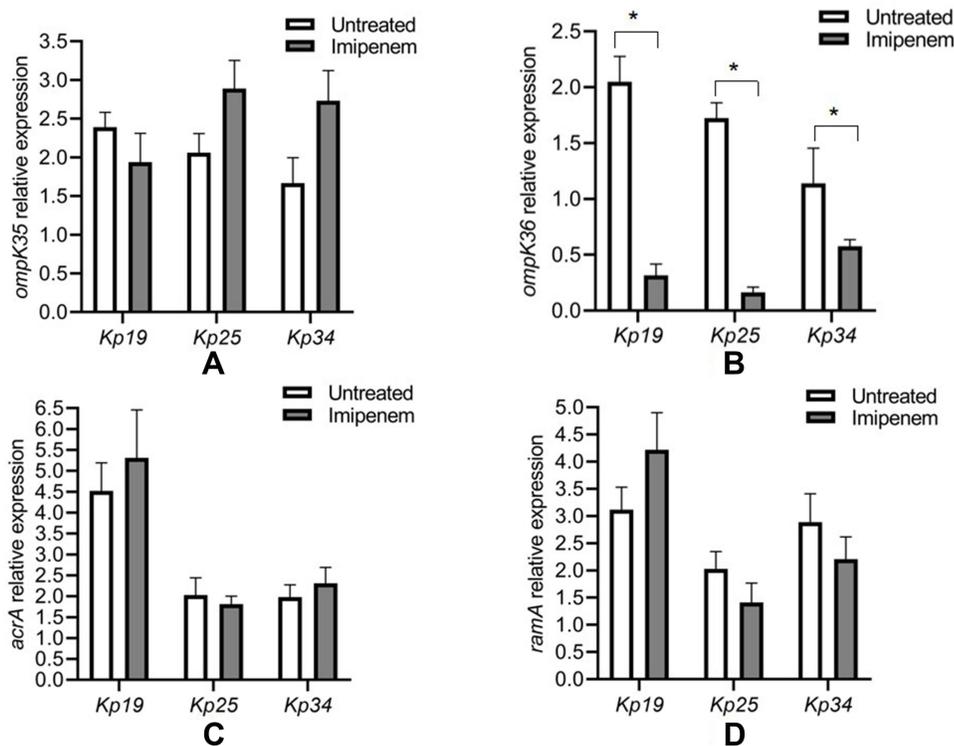


Figure 3 Relative fold gene expressions in the IPM-resistant and -susceptible subpopulations (untreated) of heteroresistant *K. pneumoniae* isolates. (A) Relative expression of *ompK35* gene; (B) relative expression of *ompK36* gene; (C) relative expression of *acrA* gene; (D) relative expression of *ramA* gene. *P values are significant ($P < 0.05$).

the bacterial population can also occur. Differential expression in transcriptional levels of one or more genes, whose products are involved in resistance to antibiotics, may also underlie heteroresistance to carbapenems.^{34,37,38}

By molecular analysis, all three heteroresistant strains identified in the current study were found to carry ESBL and/or AmpC genes and exhibit reduced expression of *ompK36* outer membrane porin gene in the IPM-resistant subpopulation. *K. pneumoniae* expresses two major outer membrane porins (OMPs), OmpK35 and OmpK36, which play an important role in the penetration of antimicrobials into the cell. A decrease in *ompK36* expression was previously shown to contribute to resistance to a wide spectrum of antibiotics, such as CTX, FOX, and carbapenems, which penetrate the outer membrane through the OmpK36 porin.^{39,40} However, OmpK35 may be one of the factors contributing to antimicrobial resistance in *K. pneumoniae* and may favor the selection of additional mechanisms of resistance, including loss of OmpK36 and active efflux.⁴¹ Reduced expression of OmpK36 in the current study correlated with increasing IPM MIC, especially when *K. pneumoniae* also expresses AmpC cephalosporinases or ESBLs, and this was consistent with observations reported by Landman et al and Hamzaoui et al.⁴²⁻⁴⁴

Several important virulence factors, including *mrkD*, *entB*, *ybtS*, *iutA*, and *kfu*, which protect *K. pneumoniae* from lethal serum factors and phagocytosis, were found in the three IPM-heteroresistant isolates. However, *rmpA* and genes encoding K1 and K2, which are highly associated with the hypervirulent (hypermucoviscous) variant of *K. pneumoniae* (hvKP), were not detected,⁴⁵ suggesting that the heteroresistant strains in this study did not present molecular characteristics of hvKP.

It was unclear whether the minor IPM-resistant subpopulations present in the three heteroresistant isolates would have an effect on the outcome of IPM treatment during an in vivo infection. There were few previous studies on exploring the treatment outcome of heteroresistant strains in vivo. In 2016, researchers discovered that colistin heteroresistance can lead to treatment failure in a mouse model.¹⁹ Similarly, it is reported that carbapenem-resistant *K. pneumoniae* exhibiting clinically undetected colistin heteroresistance leads to treatment failure in a murine model of infection.¹⁸ Except for these mouse and rat studies, vancomycin treatment failure associated with two vancomycin-intermediate *Staphylococcus aureus* isolates were also found in a rabbit endocarditis model.⁴⁶

In order to assess the effect of antibiotic therapy on IPM-heteroresistant *K. pneumoniae* strains, we used a mouse model of peritonitis to simulate infection and subsequent treatment upon clinical presentation. We found it was unable to reduce the number of colonies in mice peritoneal lavage fluid of these mice at 24 h after infection and lead to treatment failure when bacteria showed heteroresistant phenotype. Interestingly, even in the absence of IPM, the frequency of the resistant subpopulations of heteroresistant isolates increased following 24 h of in vivo infection compared to the frequency produced by the inoculum. It has been previously shown that the resistant subpopulations could be enriched and the presence of a switch of heteroresistance to homogeneous resistance during antibiotic exposure.^{19,47} Our findings suggest that the process of infection as leading to a significant increase in the frequency of an antibiotic-resistant subpopulation of bacteria. This may be due to cross-resistance of these cells to host innate immune antimicrobials, such as antimicrobial peptides and reactive oxygen species, as has previously been demonstrated.¹⁹ Both the susceptible and heteroresistant strains culminated in lethal infections in mice in the absence of IPM. In the presence of IPM, only mice infected with the susceptible strain were rescued, whereas those infected with the heteroresistant strains were unable to survive. These data demonstrate the impact of an antibiotic-resistant subpopulation in mediating a lethal infection during antibiotic treatment.

There were some limitations to this study in that the role of specific host innate immune components during infection was not evaluated, and the relevance of IPM heteroresistance on the outcome of therapy in human infection was not determined. These points should be addressed in future work.

Conclusion

Previously, limited data were available regarding the effect of carbapenem-heteroresistant clinical isolates on antibiotic treatment of such infections in vivo. This retrospective study is the first report showing that the carbapenem-heteroresistant strains among MDR ESBL- and/or AmpC-producing *K. pneumoniae* clinical isolates fails IPM therapy in a mouse model of peritonitis. Therefore, clinical laboratories should consider testing for carbapenem-heteroresistance if this last-line antibiotic class is required for treatment.

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Disclosure

The authors report no conflicts of interest in this work.

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