Drivers of Emergence and Spread of Antibiotic Resistance in Carbapenem-Resistant *Klebsiella pneumoniae*

by

Kyle James Gontjes

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Doctoral Committee:

Associate Professor Evan S. Snitkin, Chair
Professor Michael A. Bachman
Professor Adam S. Lauring
Associate Professor Krishna Rao
Associate Professor Jennifer A. Smith

Kyle J. Gontjes

kgontjes@umich.edu

ORCID iD: 0000-0001-8671-7990

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Dedication

To my family

Acknowledgements

My interest in infectious diseases started in middle school during the Science Olympiad. Inspired and encouraged by Mr. and Mrs. Niemann, I quickly learned that I wanted to become a disease detective, a calling that still guides my career path. I brought this passion to Calvin College, where I gained an appreciation for the intersection of microbiology and public health. My interest in microbiology burgeoned during my participation in the SEA-PHAGES program. Conducting research alongside my college lab partner, Rae House, including a study of metabolite cross-feeding under the tutelage of our fantastic mentor, Dr. John T. Wertz, instilled in me a sense of joy that I still associate with science. I always enjoy returning to Grand Rapids to swap science and life stories with these excellent scientists and friends. My interest in public health was stimulated through internships with the Kent County Health Department and Bronson Healthcare's Infection Prevention & Epidemiology department.

My passion for infection prevention was brought to Ann Arbor, where I pursued an MPH in Hospital & Molecular Epidemiology at the University of Michigan School of Public Health. During my MPH, I worked in Dr. Lona Mody's laboratory in the Division of Geriatric & Palliative Medicine. Through the mentorship of Dr. Mody and their team, I learned how to leverage clinical studies to understand the epidemiology and transmission of bacteria in vulnerable populations. More importantly, their encouragement to pursue interesting research questions sparked a curiosity that has greatly benefited my research and life. The emboldening to foster collaborations led to a project with the superstar clinician and mentor, Dr. Payal K. Patel, who taught me everything that I know about antibiotic stewardship, the importance of accessible, translational research, and the power of enthusiasm.

Wanting to grow my knowledge in molecular epidemiology, I pursued a PhD in Microbiology & Immunology at the University of Michigan Medical School. Having learned about how whole-genome sequencing improves our understanding of bacterial transmission, I was excited to join Dr. Evan Snitkin's laboratory for my PhD training. I wish to sincerely thank Dr. Snitkin for their mentorship. I have greatly valued the supportive environment that he

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Abstract

The discovery of antibiotics revolutionized our ability to treat infectious diseases. The development of antibiotic resistance significantly undermines this achievement, as infections caused by resistant bacteria often have marked clinical complexity and high case-fatality rates. Compounding this problem is the emergence and global dissemination of multidrug-resistant bacteria, such as carbapenem-resistant *Klebsiella pneumoniae* (CRKP). Efforts to reduce the burden of resistance are hindered by a limited understanding of the factors driving its emergence and spread. Of utmost importance is developing a better understanding of the factors driving resistance to recently introduced CRKP-targeting antibiotics, particularly in high-risk populations, such as residents of long-term acute care hospitals (LTACHs).

In this dissertation, I leveraged clinical metadata, antibiotic susceptibility testing, and bacterial whole-genome sequencing to study the drivers of the emergence and spread of antibiotic resistance. Access to two dense samplings of clinical CRKP isolates from an LTACH network enabled the unique opportunity to investigate the molecular epidemiology and evolutionary dynamics of resistance.

First, I developed a phylogenetic methodology that leverages ancestral state reconstruction to characterize the emergence and spread of genome-influenced traits. Applying this framework to a rich collection of clinical CRKP isolates improved our understanding of antibiotic resistance by providing more precise insights into the relative contributions of *de novo* evolution and cross-transmission to resistance to five clinically important antibiotics. Phylogenetic contextualization strengthened clinical risk factor modeling by illuminating the unique roles of patient characteristics, antibiotic exposures, indwelling devices, and bacterial genetic backgrounds in driving the emergence and spread of resistance.

Next, I extended this framework to investigate how patient and bacterial features drive the emergence and spread of resistance to β-lactam/β-lactamase inhibitor (BL/BLI) combinations in isolates collected several years before the clinical deployment of these promising antibiotics. Genomic and experimental investigations revealed considerable clade-specific differences in the

evolution, cross-transmission, and genetic pathways underlying resistance in the epidemic CRKP lineage sequence type 258. In clade II, resistance was predominantly driven by convergent mutation at a small number of loci, with resistant isolates rarely spreading among patients. Conversely, resistance in clade I was potentiated by a lineage-defining insertion in loop 3 of the major porin, OmpK36, thereby making high-level resistance accessible by transposition of the *K. pneumoniae* carbapenemase, bla_{KPC}, to a higher-copy-number plasmid, which was observed to spread among patients.

Finally, I used a contemporary collection of clinical CRKP isolates, obtained in the same facilities nearly a decade later, to investigate changes in molecular epidemiology and resistance patterns following the introduction of several promising CRKP-targeting antibiotics. A more diverse CRKP population was observed, predominantly driven by the importation of numerous epidemic lineages and remarkable variability in the long-term success of pre-existing lineages. These changes in population diversity were strongly correlated with differences in circulating resistance-associated genotypes and resistance patterns.

Together, this body of work demonstrates how integrating clinical surveillance, whole-genome sequencing, and wet lab experimentation can improve understanding of the molecular epidemiology and evolution of multidrug-resistant pathogens. This dissertation highlights the diverse roles of patient characteristics, medical practices, and bacterial genetic background in driving the emergence and spread of resistant bacterial lineages in high-risk patient populations. Furthermore, this research exemplifies how interdisciplinary, translational research can be leveraged to identify drivers of resistance dynamics and inform the development of innovative interventions to slow the development and dissemination of antibiotic resistance.

Chapter 1 Introduction

1.1 Antibiotic Resistance Is an Urgent Public Health Threat With a Serious Clinical Burden

The identification of molecules that inhibit bacterial growth was one of the most important scientific discoveries that shaped public health and modern medicine[1]. Screening environmental microorganisms for pathogen inhibition uncovered numerous molecules that target critical pathways, including deoxyribonucleic acid (DNA) replication, protein biosynthesis, cell wall formation, and folic acid metabolism[2,3]. The clinical deployment of antibiotics, alongside improvements in quality of life and hygiene, coincided with a large reduction in morbidity and mortality from infectious diseases in the 20th century[4-6].

Amidst the excitement of this breakthrough were concerns about the development of resistance, broadly defined as the ability to withstand exposure to an antibiotic regimen[7]. Early studies detailing the inhibitory effects of promising compounds often speculated about the reasons for insensitivity[8]. Indeed, the mechanism of enzymatic resistance to penicillin, the first β-lactam antibiotic, was identified before clinical deployment[9]. Resistance has often been detected rapidly after the clinical deployment of an antibiotic[10]. For example, widespread reports of treatment failure were detailed in healthcare settings in the years following penicillin's clinical introduction in the 1940s[11]. Foreshadowing the role of community transmission in shaping the epidemiology of resistance, high rates of penicillin resistance were also observed outside healthcare settings[11].

By the late 1960s, the declining efficacy of natural product discovery challenged the antibiotic research and development pipeline[2]. Environmental screening repeatedly rediscovered compounds with already described mechanisms of action[2]. In response, researchers started employing high-throughput screening of libraries of synthetic compounds and approved pharmaceuticals to identify off-target antibiotic activity or synergy[2,3]. Advanced statistical modeling of molecular structures and phenotypic data has identified molecules with promising

inhibitory profiles and novel mechanisms, such as halicin and dynobactin[12,13]. Whether such novel antibiotics will succeed in clinical trials and reach the market remains to be seen.

Unfortunately, the clinical deployment of novel antibiotics has been outpaced by the evolution and dissemination of antibiotic-resistant bacteria. In the 21st century, resistance is a significant public health threat with a serious clinical burden. Nearly 178 million disability-adjusted life years and five million deaths were associated with resistance in 2021[14]. In the United States, the Centers for Disease Control & Prevention estimated an annual burden of 2.8 million resistant infections and over 35,000 deaths[15]. Population growth and aging, alongside the global spread of resistant lineages, have been implicated in the increasing burden of resistance[15]. Without adequate clinical and public health intervention, current forecasts estimate that over eight million deaths will be associated with resistance in 2050[14].

1.2 Genomic Pathways to the Emergence and Spread of Antibiotic Resistance

To develop novel pharmaceuticals and design innovative interventions, researchers must understand the molecular epidemiology and evolution of antibiotic resistance[16].

Observational studies have described how host characteristics, genomic pathways, and selective pressures drive the evolution of antibiotic resistance in human populations. Descriptive and interventional studies have shown that selective pressures, such as antibiotic exposures, can induce marked changes in population allelic frequencies[17]. These population sweeps can facilitate the outgrowth of pre-existing resistant subpopulations, lead to the fixation of resistance-conferring mutations, and increase bacterial fitness in the presence of antibiotics[17,18]. Environmental stressors can further drive bacterial evolution by promoting the exchange of adaptive genes, including those that confer antibiotic resistance[19,20].

To understand the genomic pathways driving antibiotic resistance, researchers have employed experimental evolution to select for resistance under tightly controlled laboratory conditions[21]. Measurement of resistance evolution enables comparison of resistance rates across antibiotics and bacterial genetic backgrounds[21]. When comparing evolved strains and ancestral strains, individual genomic mutations and multi-step pathways underlying resistance can be identified[21]. Although such experiments predominantly explore mutational space, alterations in plasmid content and the movement of insertion sequences have also been implicated in adaptation to environmental stressors[22,23]. Collectively, observational and experimental studies

have illuminated how mutational changes and horizontal gene transfer drive the evolution and spread of antibiotic resistance. In this section, we will explore these mechanisms in more detail.

1.2.1 Mutational Resistance

Heritable mutations in the bacterial genome arise through many pathways, including DNA replication errors, transcription-replication conflicts, template switching, DNA mismatches, single-strand DNA (ssDNA) deamination, and ssDNA oxidation[24]. Alongside point mutations, genomic insertions, deletions, and translocations also shape genetic function. These mutations have diverse effects on the bacterial genetic code, including silent (synonymous) mutations, non-synonymous mutations that modify amino acid sequences, frameshift mutations, and duplications or losses of genetic content[24]. Through their effects on gene expression and protein function, mutations can dramatically impact bacterial physiology, behavior, and genome-influenced traits, such as virulence and antibiotic resistance.

The spontaneous accumulation of mutations facilitates the evolution of antibiotic resistance through diverse mechanisms[25]. First, mutations can directly modify the antibiotic's target. For example, non-synonymous mutations in penicillin-binding proteins influence the binding affinity of β-lactams[26,27]. Second, mutations can disrupt the cellular entry of antibiotics. For example, modification of major membrane transport channels can reduce antibiotic entry[28], as seen with the loss of porins leading to elevated levels of β-lactam resistance[32]. Insertions in porins, such as the loop 3 insertions in OmpK36, can reduce channel width and thereby decrease antibiotic entry, facilitating a moderate increase in resistance with a more modest fitness cost[32]. Third, mutations can alter antibiotic efflux rates. For example, mutations in efflux pump regulators can increase the cellular exit of molecules[29,30] Fourth, mutations can modify the efficacy of enzymes that inactivate antibiotics. Mutations in β-lactamases with varying ability to hydrolyze β-lactam antibiotics and evade inhibitors continue to emerge and disseminate worldwide[25,31]. Finally, mutations can alter the expression of resistance genes. For example, mutations in the promoter region of β-lactamases can upregulate gene expression and influence phenotypic penetrance[32,33].

Mutations can also influence the propensity for the evolution of resistance. Pre-existing mutations impact resistance evolution rates and the phenotypic penetrance of an acquired genotype[34,35]. In *Mycobacterium tuberculosis*, genomic mutations, such as deletions in the lipoprotein *lppP*, can increase the risk of resistance evolution in Lineage [34]. First-step mutations

that confer low-level resistance can also facilitate easier transitions to clinical resistance. For example, mutations in *parC*, which confer low-level resistance, increase the propensity to acquire *gyrA* mutations, subsequently inducing high-level quinolone resistance[36]. Mutations can also alleviate the fitness cost of acquired resistance[37]. Compensatory chromosomal mutations can alleviate the cost of acquiring resistance plasmids[37-39]. Finally, spontaneous mutation can also stimulate reversion to susceptibility[40]. Long-term cultivation of resistance mutants in the absence of selective pressure exemplifies the reversibility of resistance, often through spontaneous mutation or gene transposition into other genetic contexts[40,41].

1.2.2 Horizontal Gene Transfer

The acquisition of foreign genetic material is an important evolutionary mechanism[42]. Bacteria can uptake extracellular environmental DNA[43]. Natural transformation of environmental DNA, often triggered by environmental perturbation, enables the acquisition of adaptive genes[43]. Transformation also enables the acquisition of genes with alternative alleles, such as the interchange of penicillin-binding protein mutants that confer resistance to β-lactams[44].

Infection with bacteriophages, broadly defined as viruses that infect bacteria, can drive the evolution of bacterial populations[45]. Integration of phage DNA into the host's genome can promote the acquisition of adaptive genes, such as extracellular toxins and resistance genes[46]. Moreover, the co-association between phages and bacterial gene clusters suggests that lysogenic conversion can create evolutionary and functional linkages[45]. Bacteriophage integration, like other genetic elements, can also induce insertional mutagenesis by disrupting the genetic content at its insertion site, further driving bacterial evolution[47].

The transfer of mobile genetic elements between bacterial hosts via direct cell-to-cell contact enables the acquisition of diverse traits, including cooperative genes, virulence factors, and antibiotic resistance[42,48]. The horizontal and vertical transfer of plasmids, broadly defined as extrachromosomal DNA molecules capable of autonomous replication, enables the acquisition and spread of resistance. Plasmids can harbor transposable elements, such as insertion sequences and transposons, which can facilitate adaptation through insertional mutagenesis and the transfer of resistance genes, such as the carbapenemase-containing *tn4401* transposon[41]. These mobilizable elements serve as adaptive strategies during environmental stress, as antibiotic and non-antibiotic pharmaceuticals can increase the transmission of resistance plasmids[49,50]. Plasmids can also exhibit dynamic copy number, which influences gene dosage and modulates responses

to environmental pressures[51]. Experimental studies have indicated that antibiotic exposure can drive changes in plasmid copy number and amplify resistance gene expression[51-53]. Moreover, the movement of resistance genes across plasmid and chromosomal genetic backgrounds can shape both fitness costs and gene dosage effects[54]. Experimental evolution has consistently shown that plasmid modification and the hopping of transposable elements can mediate the evolution of resistance to carbapenems and promising β-lactam/β-lactamase inhibitor (BL/BLI) combinations[54].

Plasmids have played an important role in the emergence and spread of resistant bacterial lineages, such as carbapenem-resistant *Klebsiella pneumoniae* (CRKP)[55,56]. Resistance genes, such as carbapenemases, can be found across diverse genetic backgrounds with varying fitness costs and impacts on resistance[56]. The circulation of carbapenemases across diverse genetic backgrounds facilitated the emergence and spread of many successful, high-risk healthcare-associated CRKP lineages[56]. Although tight associations between resistance plasmids and genetic backgrounds have been detected, genomic investigations have revealed that resistance plasmids vary in diversity and host range, with some bacterial lineages exhibiting flexibility in which plasmids they can acquire and maintain[57,58]. Collectively, these observations exemplify the importance of understanding how resistance genotypes, plasmids, and bacterial genetic background interact to drive the emergence and spread of antibiotic resistance.

1.3 Bacterial Genetic Background Influences the Dynamics of Antibiotic Resistance

To characterize the diversity of the natural world, organisms are arranged into an ordered, hierarchical classification based on genealogical relationships[59]. Whole-genome sequencing has enhanced our ability to subtype bacteria efficiently and accurately beyond species boundaries[60]. Multilocus sequence typing is one such successful strategy that uses allelic differences in conserved housekeeping genes to subtype bacteria[61]. More precise methodologies, such as single-nucleotide polymorphism distance and core genome multilocus sequence typing, have improved our appraisal of bacterial genetic diversity[62].

Genetic background has an important mechanistic role in bacterial evolution. Genome-scale evolutionary rates differ across species and sequence types (STs)[63]. Genetic content also varies across taxonomic partitions[64]. The acceptance of foreign genetic material varies with a population's propensity for horizontal gene transfer, population size, the presence of genome

defense systems, and selective pressures[64]. The rate of homologous recombination, a mechanism for integrating and exchanging genetic material, also varies across bacterial populations[65].

Bacterial genetic background has been implicated in shaping the epidemiology and evolution of antibiotic resistance. Experimental and genomic studies have identified differences in the evolution of resistance across genetic backgrounds[41,66]. The accessibility of mutational pathways and the potential for acquiring plasmids are directly influenced by mutational epistasis, the presence of other genes, mutations, and plasmids[67]. The pre-existing mutational landscape can modify the fitness costs and phenotypic impacts of beneficial resistance-conferring mutations[68]. Mutational modeling of dual-mutant permutations in *gyrA* and *rpoB* detailed the positive and negative pairwise fitness impacts relative to the parental strain[69].

The prevalence and diversity of resistance genotypes and phenotypes also vary across genetic backgrounds[70,71]. Fitness costs, transcriptional responses, and phenotypic penetrance of acquired genotypes differ across populations, reflecting a tight interplay between bacterial chromosome and plasmid genetic backgrounds[67,72,73]. Selection experiments using distinct evolved lineages have revealed that the mutational pathways of resistance evolution can vary across antibiotics and genetic backgrounds[74]. The differential influence of selection and within-host dynamics has also been implicated in the epidemiological success of resistance lineages and plasmids. Survival analysis of the longitudinal dynamics of *Streptococcus pneumoniae* colonization has shown that antibiotic-resistant bacteria can exhibit variable fitness rates, particularly in the presence of competing strains and antibiotic exposures[75]. Collectively, these findings underscore the importance of comprehensive, genomic investigations into the epidemiology and evolution of resistance across diverse bacterial populations and environments.

1.4 Ancestral State Reconstruction to Understand Bacterial Evolution

To investigate genealogical relationships in the natural world, researchers often use phylogenetic comparative methods to infer evolutionary history. Phylogenies are hierarchical, tree-shaped representations of evolutionary relationships, in which sampled taxa are connected to ancestral nodes by edges[76]. Evolutionary relationships among sampled taxa are inferred using distance-based or character-based statistical algorithms applied to genotypic or phenotypic data[76,77]. Sampled taxa are represented at the phylogeny's tips, while internal nodes represent unobserved ancestral taxa. Branches connecting nodes, which can vary in length, represent inferred evolutionary distance among sampled taxa. Groups of sampled taxa sharing an ancestral

node are referred to as clades. As these taxa are inferred to share evolutionary history, clade members can have similar genetic backgrounds and corresponding phenotypes[78].

Although phylogenetic reconstruction provides powerful inferences about evolutionary history, it relies on several assumptions and carries important limitations[79]. First, reconstruction relies on accurate, informative measurements of phenotypic or genotypic diversity[80]. Second, processes that shape genomic diversity and content among populations, such as recombination and horizontal gene transfer, affect the accuracy of phylogenetic inferences[81]. Third, mitigating sampling bias is necessary to ensure an accurate representation of evolutionary history[80]. Finally, the selection of an appropriate substitution model is often integral to ensuring accurate reconstructions[82].

Phylogenies enable researchers to study the evolution and spatiotemporal distribution of populations and genome-influenced traits[83]. To characterize evolutionary histories, comparative biologists often rely on ancestral state reconstruction[77]. By integrating phylogenetic reconstruction with trait data from sampled taxa, statistical algorithms, such as Markov *k*-state or hidden Markov models, can be used to estimate the states at ancestral nodes[77,84]. Ancestral states can be inferred using local, node-wise (marginal) or global (joint) reconstruction algorithms[77]. Marginal reconstructions estimate state probabilities at each node, whereas joint reconstruction identifies the most representative sequence of states across the phylogeny[85]. State transition matrices enable the specification of assumed evolutionary processes, such as modeling equal or different evolutionary rates among states[85]. Furthermore, innovative methodologies, such as modeling multiple rate classes and testing for heterogeneous rates across a phylogeny, have strengthened hypothesis testing and evolutionary inferences[84,86].

Phylogenetic comparative methods, such as ancestral state reconstruction, have been widely applied to study population diversity and trait evolution. Such methodologies have investigated gene orthology[87], the ecology and evolution of infectious diseases[88], animal behavior and geographic dispersal[89], and large-scale evolutionary relationships across the natural world[90]. Phylogenetic methods have become increasingly important in studying microbial epidemiology and evolution. Ancestral state reconstruction is foundational to convergence-based, genotype-phenotype association testing[91,92]. Phylogenetics enables characterization of the spatiotemporal dynamics and regional transmission of pathogens[93,94]. Comparative methods have also been employed to study the emergence and spread of genome-influenced phenotypes,

including antibiotic resistance[95]. Collectively, phylogenetic comparative methods represent an analytical framework with growing capacity to characterize the epidemiology and evolution of antibiotic resistance, informing clinical and public health interventions to reduce the burden of multidrug-resistant pathogens.

Although ancestral state reconstruction is a widely used and powerful methodology, violations of its assumptions undermine its accuracy and interpretability[96]. First, this methodology assumes that the phylogeny accurately reflects the evolutionary relationships among sampled taxa[97]. Second, trait measurement can shape the efficacy and biological interpretability of ancestral state reconstructions. Third, sampling must accurately reflect population diversity. Biased sampling can distort evolutionary reconstructions and impact ecological and epidemiological inferences. Finally, as in phylogenetic reconstruction, choosing an appropriate reconstruction algorithm and transition rate matrices is necessary to ensure accurate and informative ancestral state reconstructions[77].

Despite their widespread use and statistical rigor, these methodologies infer unobserved ancestral states and must therefore be interpreted with appropriate caution. While joint reconstruction is often more accurate than marginal reconstruction, a recent phylogenetic investigation that accounted for reconstruction uncertainty uncovered alternative plausible reconstructions that present competing hypotheses about the evolution of antibiotic resistance[84]. As these models generally assume vertical inheritance of traits, violations introduced by horizontal gene transfer and recombination complicate reconstruction accuracy and biological inferences. In turn, phylogenetic comparative methods require integration with additional sources of evidence, including clinical metadata, additional genomic investigations, and coordinated experimental validation of evolutionary inferences. The development of biologically informed analytical frameworks will be essential to improving the utility of phylogenetic comparative methods for studying the epidemiology and evolution of antibiotic resistance.

1.5 Genomic Epidemiology Illuminates Drivers of the Emergence and Spread of Resistance

The integration of clinical metadata, bacterial phenotyping, and whole-genome sequencing provides a powerful framework for improving our understanding of the epidemiology and evolution of antibiotic resistance. This section examines how genomic epidemiology methods can be leveraged to characterize the emergence of resistance, describe the circulation of resistant lineages, and identify risk factors for resistance.

1.5.1 Characterizing the Emergence of Antibiotic Resistance

Genomic investigations of *in vivo* evolution have shaped our understanding of bacterial adaptation, especially during persistent infections[98,99]. Longitudinal surveillance can distinguish between the persistence of an infecting strain and the acquisition of novel strains[98]. It also enables the identification of bacterial genotypes and mutational pathways that correlate with clinically relevant outcomes, including antibiotic resistance[100]. Integrating clinical metadata and phenotypic data can illuminate selective pressures and investigate genotype-phenotype associations[100]. Mechanistic insights into the evolutionary pathways to resistance have been identified through observational studies, including the roles of selective sweeps, hypermutation, and alterations to chromosomal and plasmid backbones[17,98]. The complementation of such studies with experimentation enables researchers to directly test observed patterns of resistance evolution, selective pressures, and genotype-phenotype associations[101,102].

1.5.2 Describing the Circulation of Resistant Lineages

Molecular subtyping enhances our ability to characterize evolutionary relationships among bacterial isolates[61]. Partitioning species into subgroups, based on evolutionary relationships, enables a more precise description of population diversity. Coupling these methods with rich spatiotemporal data enables researchers to better understand the distribution and transmission of resistant bacterial lineages[94]. Such information can be used to model evolutionary relationships among resistant isolates and investigate longitudinal lineage dynamics[103].

Whole-genome sequencing sharpened our ability to subtype bacteria. Recombination-filtered single-nucleotide polymorphisms characterize nucleotide-level diversity, which is useful for describing the persistence and cross-transmission of bacterial strains[62]. Researchers have also employed phylogenetic methods to describe the evolutionary dynamics of a population. The phylogenetic description of evolutionary history can cluster isolates based on shared history, enabling the identification of emergent and circulating lineages within a population[104].

Advanced subtyping methods have improved our understanding of the emergence and spread of antibiotic resistance. Previous studies have described the composition, diversity, and geographic spread of resistant, clonal populations[58,105,106]. Investigations of sub-lineage dynamics have revealed roles for adaptive mutations, accessory gene content, the importation of competing lineages, and plasmid stability in shaping the longitudinal dynamics of resistant lineages[58,105,106].

Integrating advanced subtyping methods into epidemiological studies can enhance our ability to track the emergence and spread of antibiotic resistance. Phylogenetic modeling enables moving from describing resistance patterns and resistance-associated genotypes to modeling the evolutionary dynamics and genomic pathways of resistance. Phylogenetic inferences, often obtained through ancestral state reconstruction, can more precisely describe how resistance is gained, lost, and maintained within a population. Such methodologies have been implemented, often in conjunction with genotypic investigations, to illuminate the frequency and dynamics of resistance emergence and the persistence of resistant lineages across the phylogeny[107,108]. These findings can improve our ability to predict the emergence of resistance and to identify resistance-conferring genotypes with a heightened capacity to spread to other hosts.

1.5.3 Identification of Risk Factors for Antibiotic Resistance

Central to understanding the epidemiology and evolution of antibiotic resistance is the identification of the bacterial genetic determinants and genomic pathways that drive resistance[109]. The increasing amount of whole-genome sequencing data has enabled the identification and validation of resistance-associated genotypes. While traditional regression modeling approaches have improved our understanding of resistance-associated traits, convergent genome-wide association studies and ancestral state reconstruction permit more precise modeling of the evolutionary relationships between bacterial genotypes and phenotypes[91,109,110].

Researchers have sought to identify host characteristics associated with antibiotic resistance. Epidemiological studies have identified risk factors for harboring resistant pathogens by comparing affected populations to hosts harboring susceptible bacteria and to matched unaffected controls[111-113]. Meta-analyses of case reports have also quantified variable rates of resistance emergence among patient populations[113]. Risk factors for harboring resistant pathogens include older age, multiple comorbidities, immunological disorders, recent antibiotic use, and the presence of indwelling devices[114]. Prolonged exposure to settings with high colonization burden and transmission potential is also an important risk factor for acquiring resistant bacteria[115,116].

Ecological investigations have identified that population-level differences contribute to the emergence and spread of antibiotic resistance. Antibiotic prescribing patterns, crowding, and infection prevention practices influence the burden of resistant bacteria[114,117]. Facilities with high

colonization pressure and transmission potential have been implicated in the regional circulation of multidrug-resistant bacteria[118]. Such analyses have identified at-risk populations, such as post-acute care patients and those with indwelling devices, which have been integral to the design and implementation of interventions that reduce the burden of multidrug-resistant bacteria[119-121].

Although traditional investigations have illuminated the burden of antibiotic resistance, these studies overlook the genomic pathways underlying resistance, notably *de novo* evolution and the acquisition of circulating resistant bacteria. Genomic studies have shown that the propensity for resistance to be acquired through *de novo* evolution and cross-transmission can vary across populations[108]. The identification of distinct and shared risk factors for the emergence and spread of resistance is critical because these pathways require different interventions[122]. Antibiotic stewardship interventions, including cycling and de-escalation, have been proposed to limit intra-host evolution of resistance or even steer bacteria toward favorable susceptibility profiles[122]. Conversely, cross-transmission can be reduced by cohorting, decolonization interventions, and infection prevention precautions[122]. A deeper understanding of these pathways is critical for designing innovative, tailored strategies to slow the emergence and spread of resistance in vulnerable populations.

1.6 *Klebsiella pneumoniae* is a Bacterial Pathogen With an Important Role in the Emergence and Global Dissemination of Antibiotic Resistance

Klebsiella pneumoniae is an important bacterial pathogen that plays an integral role in driving the worldwide dissemination of antibiotic resistance[123]. This Gram-negative bacterium belongs to the Enterobacteriaceae family, which includes human pathogens from the *Enterobacter*, *Escherichia*, *Salmonella*, and *Shigella* genera[124]. Its genome is approximately five million base pairs, with roughly five thousand genes[125]. Considerable genomic diversity has been reported, as only two-fifths of the genome is conserved across species members[70,125]. The permissibility of acquiring accessory genes and plasmids enables bacterial adaptation to diverse environments[126]. In turn, this bacterium is ubiquitous in nature and known to colonize human mucosal surfaces, such as the nasopharynx and gastrointestinal tract[127]. An opportunistic pathogen, this bacterium is a leading cause of infections in the respiratory tract, urinary tract, liver, and bloodstream[127].

Of particular concern is the development of resistance to carbapenems, an effective broad-spectrum β-lactam antibiotic that disrupts cell wall synthesis by inactivating penicillin-

binding proteins[128]. As carbapenem antibiotics are commonly reserved for treating infections caused by multidrug-resistant, β-lactamase-producing Gram-negative bacteria, infections with carbapenem-resistant bacteria often have increased clinical complexity, as the acquisition of carbapenem resistance is frequently accompanied by resistance to other antibiotics[129,130]. In turn, these infections can have high case-fatality rates, increased healthcare utilization, and high costs[131-133].

The emergence and dissemination of CRKP lineages have compounded the burden of antibiotic resistance[20]. Since the detection of carbapenemases that hydrolyze carbapenems in the 1990s, many CRKP lineages have emerged and disseminated worldwide[70,71,118]. This global dissemination was associated with frequent antibiotic use in humans and the environment, healthcare-associated transmission, and increasing international travel[123]. Early populations were dominated by a few successful epidemic lineages, notably ST258 and ST11; however, in recent years, the population structure has become more polyclonal[56]. The presence of carbapenemases on mobile genetic elements has facilitated the transfer of resistance to novel hosts, promoting the emergence of novel resistant strains with varying epidemic success. As such, the previous dominance of classical lineages, notably ST258, has been superseded by a more diverse collection of epidemic lineages[134]. The prevalence of circulating carbapenemases has changed over time, with decreases in the once-dominant bla_{KPC} carbapenemase and increases in inhibitor-resistant metallo-β-lactamase carbapenemases, such as the New Delhi metallo-β-lactamases[135].

Before the introduction of promising CRKP-targeting antibiotics, treating carbapenem-resistant Gram-negative bacteria was challenging[130]. Historically, these infections were often managed with high-dose carbapenem therapy or treatment with polymyxins, tigecycline, fosfomycin, or aminoglycosides. Although these antibiotics represented valuable last-resort strategies, toxicity and pharmacological concerns limited their clinical utility[130]. The rise of carbapenem resistance spurred significant investment in the research and development of CRKP-targeting antibiotics[136]. In the past decade, several promising antibiotics have been clinically deployed, including cefiderocol and multiple BL/BLI combinations[136]. Accompanying these deployments are shifting recommendations for treating carbapenem-resistant infections, with novel antibiotics supplanting nephrotoxic polymyxins[137]. To guide the development of interventions to slow the emergence and spread of resistance to these antibiotics, researchers must better characterize the prevalence and evolution of resistance in CRKP, especially in high-

risk populations. Only by identifying the drivers of the emergence and spread of resistance will the long-term efficacy of CRKP-targeting antibiotics be preserved.

1.7 Long-Term Acute Care Hospitals Are Important Regional Hotspots for Antibiotic Resistance

Long-term acute care hospitals (LTACHs) are important regional hotspots for the emergence and spread of antibiotic resistance. These facilities admit patients requiring intensive care with anticipated lengths of stay over 25 days[138]. Common indications for admission to LTACHs include indwelling device weaning, treatment of chronic infection, and wound maintenance[138]. Due to their high-acuity patient population, long lengths of stay, and frequent antibiotic prescribing, the burden of multidrug-resistant bacteria is often higher in LTACHs than in intensive care units[139]. Simulation studies have implicated these facilities as integral regional hotspots and valuable intervention points for reducing the burden of resistant bacteria[140,141] In turn, understanding the epidemiology and evolutionary dynamics of last-resort resistance in these facilities represents an invaluable opportunity to inform the design of interventions that maintain our ability to treat CRKP and reduce the regional transmission of resistant subpopulations.

1.8 Dissertation Datasets

To study the epidemiology of CRKP in LTACHs, collaborators conducted prospective clinical surveillance across two time periods within a healthcare network. Clinical isolates obtained from regional microbiology laboratories were subjected to antibiotic susceptibility testing to important last-resort and novel antibiotics[129]. Whole-genome sequencing, rich clinical metadata, and patient transfer data enabled innovative examinations into the prevalence and risk factors for resistance. The ascertainment of two dense samplings separated by nearly a decade also permits investigation of how the molecular epidemiology and resistance patterns of CRKP changed during a period of considerable shifts in antibiotic prescribing.

1.8.1 Study Period 1: Collection of Clinical Isolates From 2014-15

This dissertation leverages a comprehensive collection of clinical CRKP isolates obtained from 21 LTACHs within the same network from August 1, 2014, to July 25, 2015[142]. A total of 451 isolates were obtained from 386 patients. As virtually all isolates were collected from California facilities and belonged to ST258, this dataset provides the unique opportunity to explore the epidemiology and dynamics of resistance in an epidemic lineage.

1.8.2 Study Period 2: Collection in the Same California Hospital Network From 2021-23

To study the changing epidemiology of resistance in CRKP, collaborators returned to the California LTACHs and performed another round of comprehensive surveillance. Isolates were collected from July 7, 2021, to December 30, 2023. A total of 507 CRKP isolates were collected from 394 patients across 13 facilities. Clinical metadata extraction, antibiotic susceptibility testing, and whole-genome sequencing were performed using the same specifications.

1.9 Dissertation Outline

The goal of this dissertation is to characterize the emergence and spread of resistance to last-resort and novel antibiotics across a healthcare network. To address this goal, a genomic epidemiology framework was implemented to characterize the epidemiology of CRKP isolates and understand the drivers of resistance evolution and spread. In Chapter 2, a phylogenetic methodology was developed and deployed to characterize the evolutionary history of resistance to five antibiotic phenotypes in our 2014-15 CRKP isolate collection. In Chapter 3, this framework is extended to study the epidemiology of resistance to two promising BL/BLI antibiotics, meropenem-vaborbactam and imipenem-relebactam. Advanced phylogenetics and regression modeling identified putative patient characteristics, medical practices, and bacterial genomic features that drive the emergence and spread of BL/BLI resistance. In Chapter 4, the molecular epidemiology and resistance patterns in the 2014-15 study were compared with those of a 2021-21 CRKP collection obtained from the same California LTACH network. In Chapter 5, I discuss how this research extends current understanding of how antibiotic resistance evolves and spreads across a healthcare network. I also argue that integrating phylogenetic context into epidemiological investigations will sharpen our understanding of antibiotic resistance and guide the development of innovative public health surveillance and clinical interventions.

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Chapter 2 Phylogenetic Context of Antibiotic Resistance Provides Insights Into the Dynamics of Resistance Emergence and Spread

2.1 Preamble

This chapter evaluates the hypothesis that whole-genome sequencing improves understanding of the epidemiology of antibiotic resistance. In this chapter, I performed all data analyses and generated all tables and figures. Other co-authors performed isolate collection, clinical metadata extraction, antibiotic susceptibility testing, and contributed their expertise to the formulation of this work.

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The methods developed in this chapter were implemented in the open-source R package, *phyloAMR*, with help from my undergraduate mentee, Aryan Singh. In addition to its application in this dissertation, this tool has been used in several other publications and ongoing projects. This package is hosted on GitHub at https://github.com/kylegontjes/phyloAMR.

2.2 Introduction

Antibiotic resistance is a significant public health challenge[1]. The development of infections with antibiotic-resistant pathogens significantly reduces treatment options and clinical cure rates. Of greatest concern are multidrug-resistant lineages that have evolved resistance to multiple classes of antibiotics and disseminated worldwide[2].

The increasing problem of antibiotic resistance demands urgent clarity on the precise drivers of resistance proliferation. To this end, previous studies have sought to identify risk factors associated with harboring a pathogen that exhibits resistance[3-5]. This approach has a significant limitation—it overlooks the distinct routes to the acquisition of resistance, notably *de*

novo evolution (i.e., the emergence of a unique resistant strain spontaneously in an individual via chromosomal mutation or the acquisition of mobile genetic elements) and cross-transmission of circulating resistant lineages (i.e., the acquisition of a resistant strain that is epidemiologically linked to other individuals)[6-8]. These pathways call for distinct intervention strategies. For example, judicious antimicrobial stewardship may be useful to reduce selection for *de novo* resistance evolution, whereas measures to minimize cross-transmission include patient cohorting, hand hygiene compliance, and contact isolation[9-12]. A deeper understanding of these pathways to resistance acquisition will facilitate the application of tailored prevention strategies and sharpen our comprehension of how bacterial genetic background, patient characteristics, and medical practices drive the emergence and spread of antibiotic resistance.

Here, we developed an approach that uses phylogenetic context to partition antibiotic-resistant isolates into those putatively derived from *de novo* evolution versus cross-transmission. We then applied this method to understand drivers of these 2 pathways to resistance in the epidemic carbapenem-resistant *Klebsiella pneumoniae* (CRKP) sequence type 258 (ST258) lineage, using a comprehensive collection of clinical isolates and patient metadata collected over 1 year from a network of long-term acute care hospitals (LTACHs). The emergence and spread of 5 critical antibiotic resistance phenotypes were identified, with the CRKP ST258 sublineage, antibiotic exposures, and patient clinical characteristics being differentially associated with the emergence and spread of each phenotype.

2.3 Methods

2.3.1 Sample Collection

Clinical CRKP isolates were obtained from 1 August 2014, to 25 July 2015, at 12 southern California LTACHs[13]. Bacterial isolates from blood, respiratory, urine, and wound cultures identified as *K. pneumoniae* were tested for phenotypic carbapenem resistance using the 2015 Centers for Disease Control and Prevention criteria[13].

2.3.2 Clinical Metadata Collection

Clinical metadata were extracted from electronic medical records, as described previously[13]. Patient demographics, medical comorbidities, and indwelling medical devices were recorded at specimen collection, along with antibiotic exposures up to 30 days prior to specimen collection. Antibiotic exposure data were available only during a patient's stay in the LTACH.

2.3.3 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed for 2 aminoglycosides, colistin, trimethoprim–sulfamethoxazole (TMP–SMX), and 2 recently approved β-lactam/β-lactamase inhibitor (BL/BLI) combinations that were not clinically available at the time of specimen collection. Susceptibility profiles for TMP–SMX and gentamicin were extracted from regional microbiology laboratory records. Susceptibility to amikacin, colistin, imipenem–relebactam, and meropenem–vaborbactam was determined using broth microdilution (SENSITITRE, Thermo Scientific)[14]. Antibiotic resistance was defined as a minimum inhibitory concentration within the intermediate or resistant category by the 2021 Clinical and Laboratory Standards Institute interpretative criteria for all antibiotics except TMP–SMX[14], for which resistance was defined as a minimum inhibitory concentration ≥16/80 µg/mL (**Appendix A Table 1**). Resistance to BL/BLI agents was defined as resistance to imipenem–relebactam and/or meropenem–vaborbactam.

2.3.4 Whole-Genome Sequencing

Whole-genome sequencing of bacterial isolates was performed, as described previously[13]. The maximum-likelihood phylogeny was reconstructed from Gubbins-recombination filtered polymorphic sites using a custom, in-house Snakemake pipeline (https://github.com/Snitkin-Lab-Umich/phylokit)[15-24]. Details on whole-genome sequencing, variant calling, and phylogenetic tree reconstruction are present in **Appendix A**.

2.3.5 Phylogenetic Analysis of Antibiotic Resistance Using Joint Ancestral State Reconstruction

We developed the open-source R package, *phyloAMR*, to study the emergence and spread of antibiotic resistance (https://github.com/kylegontjes/phyloAMR).

PhyloAMR's core function, *asr*, leveraged ancestral state reconstruction to characterize the evolution of resistance across the phylogeny. The model with the lowest sample size–corrected Akaike information criterion (AICc) was used for joint ancestral state reconstruction using *corHMM*[25]. Edges on the phylogeny were evaluated to determine episodes where the trait continued (i.e., susceptible -> susceptible or resistant -> resistant), was gained (i.e., susceptible -> resistant), or was lost (i.e., resistant -> susceptible).

PhyloAMR's phylogenetic tree traversal algorithm, *asr_cluster_detection*, inferred the evolutionary history of antibiotic resistance. This algorithm classifies trait-containing isolates as phylogenetic singletons (i.e., evidence of *de novo* evolution of a trait) or members of a phylogenetic cluster of the trait (i.e., evidence that the trait was inherited from a common ancestor of circulating trait-containing lineage). Resistant isolates with gain events inferred at the tip were classified as phylogenetic singletons. However, these isolates were eligible for classification as members of a phylogenetic cluster if a reversion event was detected at its parental node. Resistant isolates were classified as members of a phylogenetic cluster if their ancestral gain event was shared with at least one additional resistant isolate. Resistant isolates that did not share an ancestral gain event were classified as phylogenetic singletons. Phylogenetic clusters where all isolates belonged to one patient were reclassified as redundant phylogenetic singletons.

To describe the evolutionary history of antibiotic resistance, the transitional data and phylogenetic clustering of each phenotype were characterized. Specifically, the frequency of resistance gain, loss, and continuation events was determined using phyloAMR's *asr_transition_analysis* function. Descriptive statistics for phylogenetic clustering were generated using *phyloAMR*'s *asr_cluster_analysis* function (see **Appendix A**).

2.3.6 Testing for Heterogeneous Rates of Antibiotic Resistance Across Clades

To further evaluate the influence of genetic background on the evolution of antibiotic resistance, we tested whether differences in rates of resistance evolution exist across the 2 clades of ST258. To achieve this, we utilized *phytool*'s *fitmultiMK* function, which implements a modified Markov model that allows for heterogeneous rates of discrete character evolution across user-specified regions on the phylogeny[26,27]. For each phenotype, a *fitmultiMK* model was constructed with a single regime, indicative of uniform rates of resistance evolution across the phylogeny. Next, a *fitmultiMK* model was constructed with 2 regimes, permitting each clade to have distinct rates of resistance evolution. The fit of the single- and multi-regime models was compared using the likelihood ratio test. Finally, hidden-rate modeling using *corHMM*, a data-driven approach, was performed to validate the presence of 2 distinct rate classes. Support for the existence of 2 rate classes was determined by comparing the AICc of the models. For these analyses, we fit 2 model structures: equal rates and all rates different.

2.3.7 Identification of Risk Factors for Resistance Emergence and Spread

Logistic regression modeling was performed to evaluate the association between genetic background, patient demographics, and clinical variables with the primary resistance outcomes of crude resistance and our 2 phylogenetically informed classifications of resistance. For each phenotype, patients with resistant isolates were partitioned as acquiring resistance via putative in vivo emergence (i.e., phylogenetic singletons) or the acquisition of a strain belonging to a resistant lineage (i.e., members of a phylogenetic cluster), presumably due to a cross-transmission event. For patients with more than one isolate, their first isolate was selected unless they contributed a later resistant isolate, in which case the resistant isolate was chosen. Patients contributing only susceptible isolates served as the reference group.

First, logistic regression was performed to evaluate the association between these outcomes and 3 explanatory variables: prior exposure to the resistance phenotype's cognate antibiotic, exposure to dysbiotic antibiotics, and having an isolate belonging to clade I of ST258. Exposure to dysbiotic agents—drugs that increase the risk for disruption of the gut microbiota and *Clostridioides difficile* infection—was defined as having a history of exposure to at least one of the following antibiotics: cefepime, ceftaroline, ceftazidime, ceftriaxone, ciprofloxacin, ertapenem, metronidazole, levofloxacin, meropenem, imipenem, and piperacillin/tazobactam[28,29]. Antibiotic exposure history was binarized as the receipt of ≥1 day of therapy in the past 30 days. Regression models evaluating the association between our resistance outcomes and antimicrobial exposures were adjusted for patient age and sex.

Next, we performed data-driven, multivariable regression modeling to identify shared and unique risk factors for our resistance outcomes[30]. Eligible variables included patient demographics, clinical comorbidities, indwelling medical devices, and antibiotic exposure histories (**Appendix A Table 2**). First, unadjusted logistic regression was performed. Next, all variables with an unadjusted *P*-value of <.20 were included in a logistic regression model. An iterative process removed variables if their *P*-value was >.10. After compiling this model, all initially ineligible variables were iteratively included in the model and retained if their *P*-value was <.10.

2.3.8 Data Analysis and Visualization

All data analysis and visualization, unless otherwise stated, were performed using R version 4.5.0[31]. The *ggplot2* and *ggnewscale* packages were used to generate common

figures[32,33]. Phylogenetic visualizations were generated using *ape*, *phytools*, and *ggtree*[26,34,35]. Descriptive tables were created using *tableone* [36]. Forest plots were generated using *forestplot*[37]. Multi-panel figures were constructed using *cowplot*[38]. Code for this project is available at https://github.com/kylegontjes/phylogenetic-resistance-ms/.

2.4 Results

2.4.1 Study Population

A total of 386 clinical CRKP ST258 isolates were collected from 312 patients across 12 California LTACHs over nearly 12 months (**Appendix A Figure 1A** and **1B**). Fifty-five patients (17.6%) contributed more than one isolate during the study period (**Appendix A Figure 1C**). On first isolate collection, the median age was 72.7 years (interquartile range [IQR], 18.2 years), and 149 (47.8%) were female. Most patients had at least one indwelling medical device (87.8%), multiple medical comorbidities (64.4%), and received antibiotics in the 30 days preceding isolate collection (72.8%). The median LTACH length of stay before clinical culture was 21 days (IQR, 37 days). Cohort characteristics are provided in **Appendix A Table 3**.

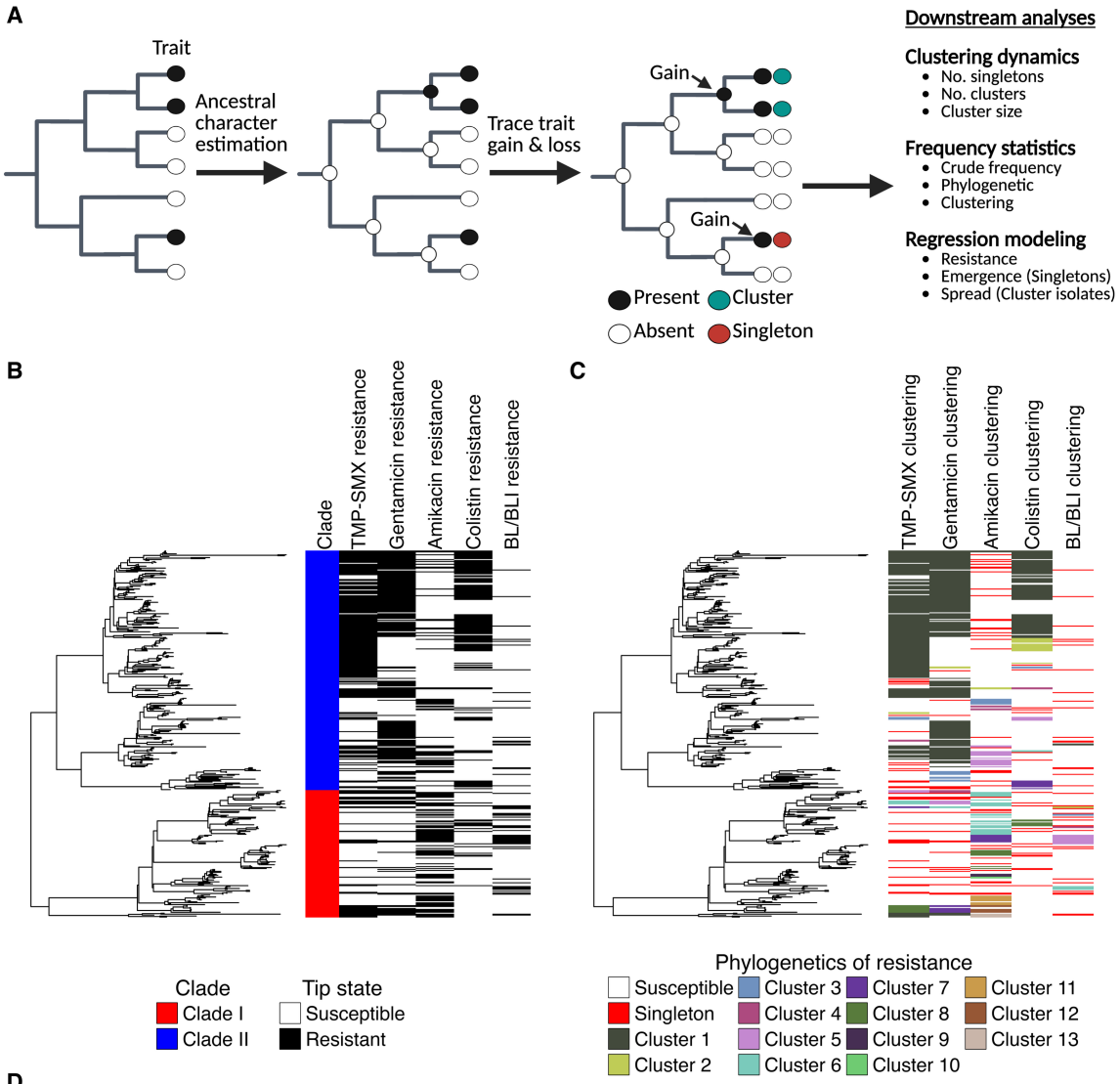
2.4.2 Antibiotic Susceptibility Profiles

On application of clinical breakpoints, resistance was observed in 207 (53.6%) to TMP-SMX, 199 (51.6%) to gentamicin, 132 (34.2%) to amikacin, 129 (33.4%) to colistin, and 56 (14.5%) to novel BL/BLI combinations (meropenem–vaborbactam, 39 [10.1%]; imipenem–relebactam, 36 [9.3%]) (**Appendix A Figure 1D**). Minimum-inhibitory concentration distributions are reported in **Appendix A Figure 2**.

2.4.3 Whole-Genome Sequencing Revealed Variable Phylogenetic Clustering of Resistance

We implemented a phylogenetic algorithm to characterize the emergence and spread of antibiotic resistance in this densely sampled population (**Figure 2.1A**). Overlaying resistance on the phylogeny revealed numerous independent episodes of resistance emergence and spread for each antibiotic (**Figure 2.1B**). Quantification using our ancestral state reconstruction-informed algorithm revealed differences in the number, size, and spatiotemporal overlap of resistant clusters for each antibiotic (**Figure 2C-E**; **Appendix A Figures 3** and **4**), suggesting differential propensities for resistance to emerge and spread. Indeed, ancestral state reconstruction produced evolutionary models with evolutionary rates that varied in magnitude (**Appendix A Table 4**),

further supporting differences in emergence rates across antibiotics. The ancestral states and phylogenetic clustering of each phenotype were overlaid on the phylogeny in **Appendix A Figure 5**.



D

Phenotype	Transitions	Gains	Gains (tip)	Losses	Losses (tip)	Continuations	Continuations (resistance)	Continuations (susceptible)
TMP-SMX	54	35	25	19	13	716	361	355
Gentamicin	50	25	16	25	18	720	371	349
Amikacin	76	49	32	27	14	694	193	501
Colistin	41	28	20	13	7	729	215	514
BL/BLI	39	38	30	1	1	731	37	694

E

Phenotype	Resistant isolates	Resistance frequency	Phylogenetic events	Singletons	Singleton isolates	Clusters	Cluster isolates	Cluster size, median (range)	Phylogenetic frequency	Clustering frequency
TMP-SMX	207	53.63%	36	28	32	8	175	3 (2-151)	8.35%	22.22%
Gentamicin	199	51.55%	26	19	23	7	176	4 (2-150)	6.58%	26.92%
Amikacin	132	34.20%	47	34	38	13	94	6 (2-25)	5.43%	27.66%
Colistin	129	33.42%	28	20	20	8	109	5 (2-71)	5.82%	28.57%
BL/BLI	56	14.51%	38	32	34	6	22	2 (2-10)	9.36%	15.79%

Figure 2.1 Phylogenetic Characterization of Resistance Revealed Differential Patterns of Resistance Emergence and Spread for 5 Antibiotic Phenotypes Among Clinical Carbapenem-Resistant *Klebsiella pneumoniae* Sequence Type 258 Isolates

(A) Ancestral character estimation was performed to characterize the phylogenetic clustering of antibiotic resistance. Phenotypes were binarized into susceptible and resistant using clinical breakpoints. Resistance was inferred at internal nodes using corHMM's joint ancestral reconstruction algorithm. The phylogenetic tree was traversed from parent to child node to determine episodes of trait continuation (i.e., susceptible -> susceptible and resistant -> resistant), gain events (e.g., susceptible -> resistant), and loss events (i.e., resistant -> susceptible). Resistant isolates were inferred as phylogenetic singletons if the resistant gain event was inferred at the tip with no history of susceptible reversion at their parental node or the resistant isolate did not share its gain event with another isolate. Resistant isolates were classified as members of a phylogenetic cluster if their gain event was shared with at least one additional resistant isolate. Lineages where all resistant isolates belonged to one patient were reclassified as redundant resistant singletons. This schematic was generated using BioRender. (B) Resistance overlaid across the K. pneumoniae sequence type 258 phylogeny. (C) Phylogenetic clustering of resistance, as inferred from the ancestral state reconstruction algorithm, was overlaid across the phylogenetic tree. (D) Phylogenetic transition and (E) clustering statistics for each antibiotic. The frequency of phylogenetic occurrence accounts for the number of episodes a trait occurs across the phylogenetic tree (i.e., clusters + singleton events) relative to the total number of possible events (i.e., clusters + singleton events + isolates without the trait). The frequency of clustering characterizes the proportion of phylogenetic events that are phylogenetic clusters. Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; NS, resistance; TMP-SMX, trimethoprim-sulfamethoxazole.

In addition to differences across antibiotics, we also observed differences in phylogenetic clustering between the 2 major ST258 clades (**Figure 2.2A**; **Appendix A Table 5**). Testing for heterogeneity in evolutionary rates supported a role for the genetic background of ST258 in shaping the emergence and spread of antibacterial resistance, with differences between the 2 clades in the rates of resistance evolution and reversion to susceptibility observed for gentamicin, amikacin, colistin, and BL/BLI agents (**Appendix A Table 6**). Data-driven modeling of rate heterogeneity using *corHMM* also supported the existence of distinct evolutionary rates of resistance across this phylogeny (**Appendix A Table 7**). Lastly, we employed logistic regression to evaluate the association of ST258 clade with the emergence and spread of resistance. On unadjusted regression, clade I was positively associated with the following outcomes: amikacin resistance emergence (odds ratio [OR], 2.49; 95% CI, 1.10–5.47), amikacin resistance spread (OR, 9.53; 95% CI, 5.39–17.30), BL/BLI resistance emergence (OR, 2.34; 95% CI, 1.09–4.99) and BL/BLI resistance spread (OR, 21.19; 95% CI, 5.89–135.76). Conversely, clade II was positively associated with the following outcomes: TMP-SMX resistance spread (OR, 7.50; 95% CI, 4.22–13.91), gentamicin resistance spread (OR, 10.99; 95% CI, 6.01–21.24), and colistin resistance spread (OR, 13.39; 95% CI, 5.72–39.27).

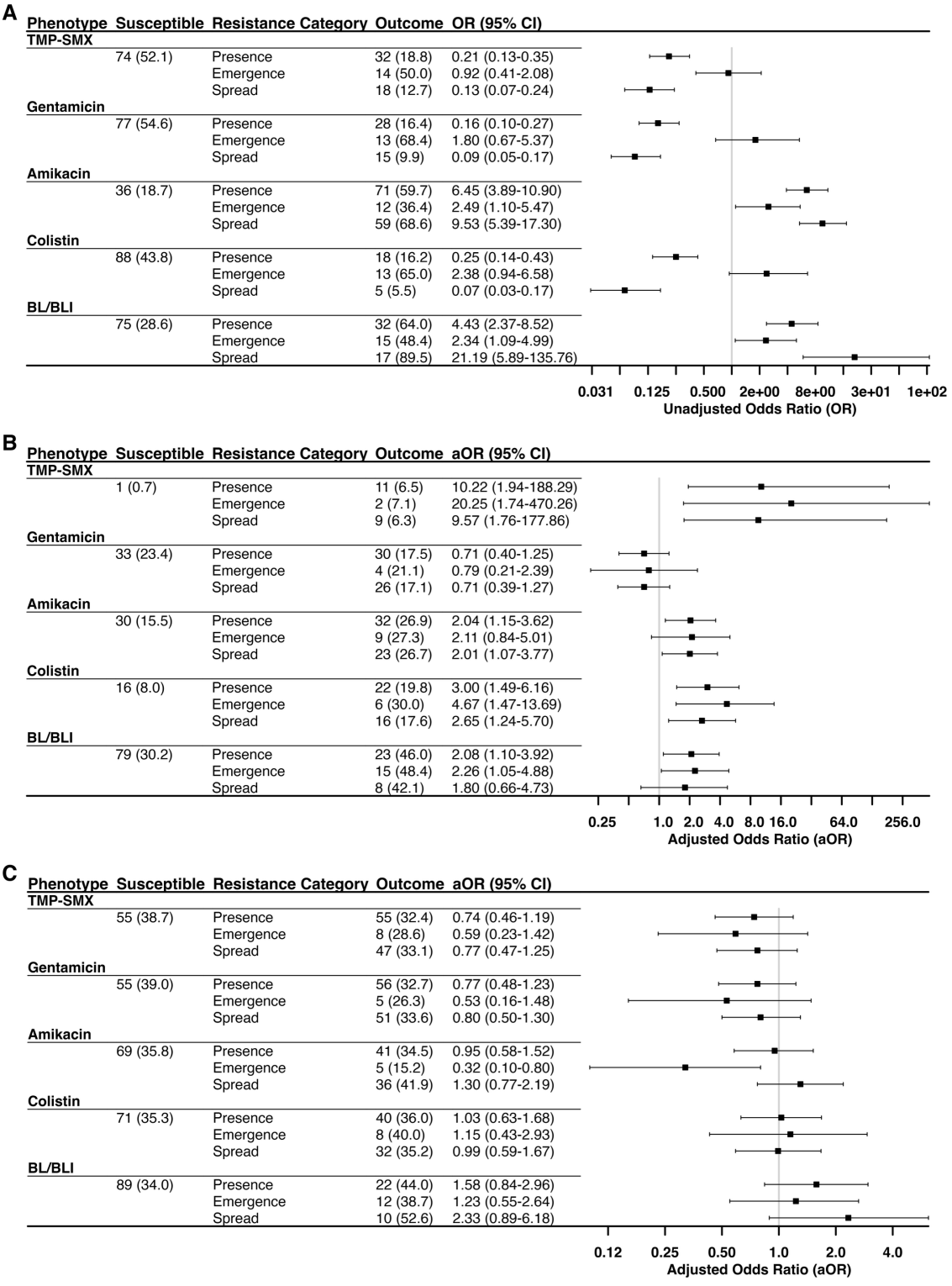


Figure 2.2 Influence of Genetic Background and Antibiotic Exposures on the Emergence and Spread of Resistant Lineages

Logistic regression was used to analyze the association between our resistance outcomes and (A) having an isolate belonging to clade I of the sequence type 258 phylogeny, (B) exposure to cognate antibiotics, and (C) exposure to

dysbiotic antibiotics. For patients with more than one isolate, the first resistant isolate was retained. Each outcome (resistant, emergence, and spread) was compared with susceptible isolates. Exposure to an outcome's cognate antibiotic was defined as follows: TMP-SMX for TMP-SMX, aminoglycoside exposure for gentamicin and amikacin, polymyxin exposure for colistin, and carbapenem exposure for resistance to BL/BLI agents. Exposure to a dysbiotic agent was defined as a history of exposure to at least one of the following antibiotics: cefepime, ceftaroline, ceftazidime, ceftriaxone, ciprofloxacin, ertapenem, metronidazole, levofloxacin, meropenem, imipenem, and piperacillin/tazobactam. Models for antibiotic exposures were adjusted for patient sex and age at culture collection. Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; OR, odds ratio; TMP-SMX, trimethoprim-sulfamethoxazole.

2.4.4 Phylogenetically Informed Risk Factor Analysis Revealed Shared and Unique Risk Factors Associated With the Emergence and Spread of Antibiotic Resistance

Having identified cases of resistance emergence and spread, we next explored whether differential risk factors exist across these 2 groups: phylogenetic singletons (i.e., resistance emergence) and clusters (i.e., spread of circulating resistant lineages). Initially focusing on antibiotic use, we postulated that exposure to cognate antibiotics would be preferentially associated with selection for resistance emergence. After adjusting for age and sex, cognate antibiotic exposure was positively associated with resistance emergence for TMP-SMX (adjusted OR [aOR], 20.25; 95% CI, 1.74–470.26), colistin (aOR, 4.67; 95% CI, 1.47–13.69), and BL/BLI agents (aOR, 2.26; 95% CI, 1.05–4.88) (**Figure 2.2B**). Interestingly, cognate antibiotic exposure was also positively associated with resistance spread for TMP–SMX (aOR, 9.57, 95% CI, 1.76–177.86), colistin (aOR, 2.65, 95% CI, 1.24–5.70), and amikacin (aOR, 2.01, 95% CI, 1.07–3.77). As microbiome-disrupting antibiotics can increase susceptibility to colonization with multidrug-resistant organisms, we tested whether these antibiotics were associated with the spread of antibiotic-resistant lineages. No statistically significant, positive association was detected (**Figure 2.2C**).

To more broadly identify clinical and patient factors that drive the emergence and spread of antibiotic resistance, we performed data-driven, multivariable regression modeling (**Table 2.1**; **Appendix A Table 8**). In addition, models were constructed that did not consider phylogenetic context (i.e., phenotypic resistance) to understand how phylogenetic contextualization might provide nuance beyond the standard approach. Inspection of multivariable models revealed risk factors with positive associations for the emergence and spread of resistance (**Figure 2.3**). Cognate antibiotic exposures were detected in both resistance emergence and spread models. Trimethoprim–sulfamethoxazole exposure was associated with the emergence and spread of resistance to TMP–SMX. Tobramycin and gentamicin, 2 aminoglycoside antibiotics, were associated with the emergence and spread of amikacin resistance. Exposure to polymyxin

antibiotics was associated with colistin emergence and spread. Increased age was positively associated with the emergence and spread of resistance to BL/BLI agents.

Phenotype	Multivariable								
	Present (n = 170)	**OR (95% CI)**	**P Value**	**Emergence (n = 28)**	**OR (95% CI)**	**P Value**	**Spread (n = 142)**	**OR (95% CI)**	**P Value**
TMP-SMX	Cephalosporin exposure	0.40 (.22–.72)	.0022	TMP-SMX exposure	119.13 (5.95–5891.04)	.0041	Acute kidney injury	0.41 (.23–.70)	.0014
	TMP-SMX exposure	24.97 (4.02–504.50)	.0044	Female sex	0.35 (.13–.86)	.0284	Cephalosporin exposure	0.40 (.22–.73)	.0031
	Acute kidney injury	0.49 (.29–.81)	.006	Tigecycline exposure	3.66 (1.10–11.75)	.0292	TMP-SMX exposure	18.93 (3.13–368.12)	.0077
	Gastrostomy tube	1.86 (1.11–3.18)	.0203				Fluoroquinolone exposure	0.42 (.20–.82)	.0132
	Decubitus ulcer stage IV/V	2.08 (1.12–3.96)	.0227				Decubitus ulcer stage IV/V	2.10 (1.11–4.06)	.0247
	Tigecycline exposure	2.34 (1.12–5.11)	.0278				Gastrostomy tube	1.89 (1.09–3.33)	.0256
	Gentamicin exposure	0.17 (.02–.76)	.0346				Gentamicin exposure	0.09 (.00–.54)	.0286
	Amikacin exposure	0.46 (.21–.98)	.0465						
Gentamicin	**Present (n = 171)**	**OR (95% CI)**	**P Value**	**Emergence (n = 19)**	**OR (95% CI)**	**P Value**	**Spread (n = 152)**	**OR (95% CI)**	**P Value**
	Decubitus ulcer stage IV/V	2.23 (1.22–4.20)	.0105	Congestive heart failure	3.61 (1.21–10.75)	.0199	Decubitus ulcer stage IV/V	2.57 (1.37–4.96)	.0038
	Cephalosporin exposure	0.49 (.28–.84)	.0108				Chronic kidney disease	0.47 (.27–.82)	.0082
	Chronic kidney disease	0.52 (.31–.88)	.0152				TMP-SMX exposure	10.85 (1.96–203.63)	.0259
	TMP-SMX exposure	12.95 (2.22–249.77)	.0193				Cephalosporin exposure	0.56 (.32–.98)	.0438
Amikacin	**Present (n = 119)**	**OR (95% CI)**	**P Value**	**Emergence (n = 33)**	**OR (95% CI)**	**P Value**	**Spread (n = 86)**	**OR (95% CI)**	**P Value**
	Tobramycin exposure	2.82 (1.14–7.40)	.0279	Tobramycin exposure	3.79 (1.03–12.65)	.0334	Cephalosporin exposure	1.88 (1.06–3.33)	.0297
	Gentamicin exposure	4.44 (1.24–20.80)	.0318	Gentamicin exposure	5.61 (.94–33.04)	.0474	Gentamicin exposure	4.61 (1.08–23.30)	.0424
Colistin	**Present (n = 111)**	**OR (95% CI)**	**P Value**	**Emergence (n = 20)**	**OR (95% CI)**	**P Value**	**Spread (n = 91)**	**OR (95% CI)**	**P Value**
	Polymyxin exposure	3.85 (1.85–8.28)	4e−04	Indwelling urinary catheter	0.15 (.04–.49)	.0039	Polymyxin exposure	3.19 (1.45–7.11)	.0039
	Ventilator-dependent respiratory failure	0.54 (.30–.95)	.0353	Polymyxin exposure	7.87 (1.92–34.78)	.0042	Underweight or malnourished	0.47 (.24–.87)	.0208
	Underweight or malnourished	0.53 (.29–.95)	.0361	COPD or chronic bronchitis	4.60 (1.54–14.26)	.0064			
				Tigecycline exposure	3.95 (1.11–13.41)	.0277			
BL/BLI	**Present (n = 50)**	**OR (95% CI)**	**P Value**	**Emergence (n = 31)**	**OR (95% CI)**	**P Value**	**Spread (n = 19)**	**OR (95% CI)**	**P Value**
	Age, 1 y unit	1.04 (1.01–1.06)	.0036	Age, 1 y unit	1.03 (1.01–1.06)	.025	Cephalosporin exposure	5.74 (2.03–17.14)	.0011
	Cephalosporin exposure	2.21 (1.13–4.27)	.0186	Carbapenem exposure	2.41 (1.08–5.37)	.0299	COPD or chronic bronchitis	4.16 (1.38–12.54)	.0102
	Carbapenem exposure	1.94 (1.02–3.69)	.0424				Age, 1 y unit	1.04 (1.00–1.09)	.0383
	Underweight or malnourished	0.42 (.16–.95)	.0498				Indwelling urinary catheter	3.10 (1.07–10.49)	.0478

Table 2.1 Multivariable Logistic Regression Modeling Identified Distinct Statistically Significant Risk Factors for Resistance Emergence and Spread

Data-driven regression modeling was performed to identify risk factors for antibiotic resistance in our long-term acute care hospital population. For each phenotype, patients with resistant isolates were partitioned as acquiring resistance via (1) putative in vivo emergence (i.e., phylogenetic singletons) or (2) the acquisition of a strain belonging to a resistant lineage (i.e., members of a phylogenetic cluster), presumably due to a cross-transmission event. For patients with more than one isolate, their first isolate was selected unless they contributed a later resistant isolate, in which case the resistant isolate was chosen. Patients contributing only susceptible isolates served as the reference group for each analysis. Alongside risk factor modeling for resistance emergence and spread, analysis was also performed to identify risk factors for harboring a resistant isolate. This table includes all model components with a *P*-value <.05. Full multivariable regression modeling results are found in **Appendix A Table 8**.
Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; COPD, chronic obstructive pulmonary disease; OR, odds ratio; TMP-SMX, trimethoprim-sulfamethoxazole.

Independent risk factors in resistant models tended to segregate into emergence or spread models, supporting the hypothesis that the standard approach imprecisely merges 2 distinct populations (**Figure 2.3**). Several variables were identified as independent risk factors for the emergence of resistance, but not its spread. Antibiotic exposures were often associated with the emergence of resistance. Tigecycline was associated with emergence of resistance to TMP–SMX, colistin, and BL/BLI agents. Exposure to carbapenems was associated with the emergence of resistance to BL/BLI agents but not spread. Additional risk factors that were unique to

resistance emergence were detected, notably associations between congestive heart failure and the emergence of gentamicin resistance and malignancy with the emergence of amikacin resistance.

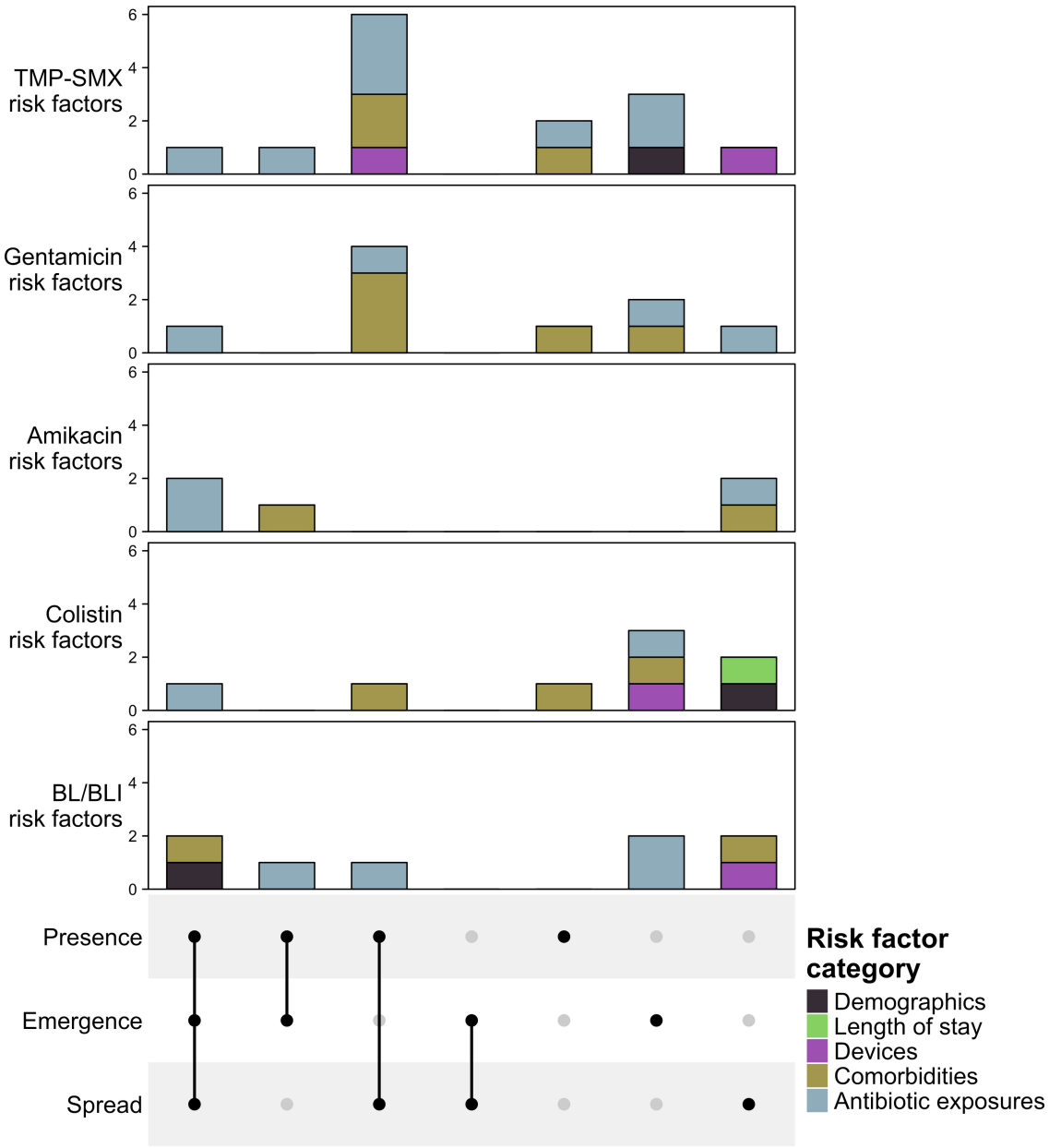


Figure 2.3 Shared and Unique Risk Factors for the Emergence and Spread of Antibiotic Resistance

Logistic regression identified risk factors for harboring a resistant strain, harboring a resistant strain inferred to be due to *de novo* evolution, and harboring a resistant strain inferred to be acquired due to cross-transmission. The sharing of model components across these 3 regression analyses is visualized for resistance to trimethoprim-sulfamethoxazole, gentamicin, amikacin, colistin, and β-lactam/β-lactamase inhibitor agents. Represented as an UpSet plot, the heatmap indicates the possible membership patterns for an identified risk factor. For each phenotype,

the bar plot indicates the number of variables in each intersection, while the fill colors indicate the variable category. Full multivariable regression models are reported in **Appendix A Table 8**. Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; OR, odds ratio; TMP-SMX, trimethoprim-sulfamethoxazole.

While models for resistance spread included exposure to antibiotics, additional proxies for complexity of care, notably patient comorbidities and indwelling medical devices, were uniquely associated with resistance spread. The presence of stage IV+ decubitus ulcers was associated with the spread of resistance to TMP–SMX and gentamicin. Chronic respiratory comorbidities, specifically the presence of chronic obstructive pulmonary disorder or chronic bronchitis, were associated with the spread of amikacin and BL/BLI resistance. This comorbidity was also associated with the emergence of colistin resistance. Indwelling medical devices were also often associated with the spread of resistant lineages, with presence of a gastrostomy and tracheostomy associated with the spread of TMP–SMX resistance, while presence of an indwelling urinary catheter was associated with the spread of BL/BLI resistance. Highlighting its potentially complex role in resistance dynamics, cephalosporin exposure was positively associated with spread of amikacin and BL/BLI resistance but negatively associated with spread of TMP–SMX resistance, alongside the emergence and spread of resistance to gentamicin. Collectively, these analyses suggest that the traditional modeling approaches may mask important differences in the patient characteristics and clinical practices that drive the emergence and spread of antibiotic resistance.

2.5 Discussion

To test the hypothesis that more refined insights into the drivers of antibiotic resistance could be attained by considering the distinct pathways to resistance acquisition, we developed a phylogenetically informed approach, compiled as the open-source R package *phyloAMR*. This easy-to-use tool enables the partitioning of patients from a densely sampled cohort into those putatively acquiring resistant strains via *de novo* evolution or cross-transmission events.

Applying *phyloAMR* to a regional collection of CRKP ST258 isolates revealed differences in the relative roles of emergence and spread for 5 critical antibiotics. We also identified differential impacts of ST258 clade, antibiotic exposures, and clinical characteristics on the emergence and spread of each resistance phenotype.

Modeling the acquisition of antibiotic resistance as a binary state of phenotypic resistance (i.e., resistant or susceptible) oversimplifies and masks complexity. Our phylogenetic characterization revealed considerable differences in the contribution of *de novo* evolution and

cross-transmission to resistance proliferation in a regionally disseminating bacterial lineage, even among antibiotics with comparable frequencies of resistance. Differences in the dynamics of resistance evolution may reflect differences in magnitudes of selection, the fitness and transmissibility of resistant strains, or the contribution of within-host dynamics[39]. These findings highlight the potential for whole-genome sequencing to enhance traditional epidemiological investigations into precise microbial and host-associated factors that influence the evolution and spread of resistance. Furthermore, phylogenetic contextualization can be leveraged to inform the development of innovative antimicrobial stewardship and infection prevention strategies to account for the dynamics of resistance evolution and spread to prolong the long-term efficacy of an antibiotic.

Our analysis revealed striking differences in the rates of resistance emergence and spread across clades of epidemic lineage CRKP ST258. Prior in vitro experimentation and epidemiological studies have observed that resistance-conferring genotypes can impose varying fitness costs across distinct genetic backgrounds and that genetic background influences the propensity for mutational resistance[40-43]. Genetic background's influence on cross-transmission, as observed with colistin resistance, may also influence the dynamics of resistance evolution[44]. Our analysis also identified clade-specific differences in the dynamics of resistance to meropenem–vaborbactam and imipenem–relebactam, 2 recently approved BL/BLI agents that were not yet clinically available in this population. Investigations of phylogenetic clustering, like those presented in this manuscript, could inform surveillance efforts by identifying genetic backgrounds with a high propensity for resistance development or spread across healthcare systems, as well as distinguishing microbial risk factors for resistance emergence and spread.

Integrating phylogenetic context into risk factor modeling supports the emerging hypothesis that patient comorbidities and medical practices exert distinct selective pressures for the evolution[45] and cross-transmission of antibiotic-resistant lineages[46]. Like prior studies[3-5], we observed that cognate antibiotic exposure was often an independent risk factor for acquisition of antibiotic resistance, potentially through selective pressure. Our modeling additionally suggests that other noncognate antibiotic exposures can also differentially drive the evolution of resistance. While we observed that antibiotic exposures were predominantly identified as risk factors when modeling resistance emergence, distinct antibiotic exposures and markers of clinical severity, notably comorbidities and indwelling medical devices, also contribute to the

spread of resistant lineages. Therefore, we postulate that antimicrobial stewardship interventions could be tailored to the specific dynamics for each antibiotic-resistant lineage while also considering the underlying patient population. Leveraging the rich phylogenetic contextualization of antibiotic resistance provides novel insights for development of innovative strategies to detect and prevent the emergence and spread of antibiotic-resistant organisms.

Our study has several limitations. First, our collection only included clinical CRKP isolates, but not those from asymptomatic active surveillance, which could have facilitated a more comprehensive view of resistance dynamics. This limitation is anticipated to be mitigated due to the LTACH setting, in which dense culture sampling is frequently performed for clinical evaluation[47-49]. Second, we analyzed only select clinical factors that may select for resistance or increase the susceptibility for acquiring a resistant strain. Future investigations should evaluate additional medical comorbidities and exposure to nonantibiotic medications. Third, our analysis was performed on a complex, high-acuity LTACH population with a high frequency of antibiotic exposures and use of indwelling medical devices, which may limit the generalizability of our risk factor analyses. Nonetheless, identifying the drivers of antibiotic resistance in this setting is critical due to their high colonization burden and the role these facilities play in the regional transmission of antibiotic-resistant organisms[48-50]. Fourth, our ability to detect precise and robust statistical associations between patient characteristics and our phylogenetically informed resistance outcomes, notably the emergence of resistance, was limited by a small sample size. Finally, our study did not seek to directly resolve the evolutionary predictions of resistance with genotypic data. Prior work in this population of resistance to colistin and BL/BLI combinations suggests concordance between our evolutionary predictions and the chromosomal and plasmid-mediated mechanisms of resistance[7,8], underscoring the feasibility of our phylogenetic framework to generate inferences regarding the evolution of antibiotic resistance.

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Chapter 3 Identifying Drivers of β-lactam/β-lactamase Inhibitor Resistance Before Their Clinical Deployment

3.1 Preamble

This chapter uses whole-genome sequencing, regression modeling, and experimental evolution to study the epidemiology and evolution of resistance to two promising new antibiotics in a clinical isolate collection obtained several years before their clinical deployment. In this chapter, I performed all data analysis and generated all data tables and figures. Other co-authors performed isolate collection, clinical metadata extraction, antibiotic susceptibility testing, wet lab experimentation, and contributed their expertise to the formulation of this work.

This work has been submitted to a peer-reviewed journal. A version of this work is hosted as a pre-print: Kyle J. Gontjes, Ali Pirani, Dhatri Badri Narayanan, Lisa J. Lojek, Jennifer H. Han, Pam Tolomeo, Ellie J.C. Goldstein, Ebbing Lautenbach, and Evan Snitkin. "Identifying drivers of β-lactam/β-lactamase inhibitor resistance emergence and spread before their clinical deployment." *medRxiv* (2025). https://doi.org/10.1101/2025.07.29.25331838.

3.2 Introduction

The emergence and spread of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) poses a significant public health threat[1]. Of greatest concern are epidemic lineages that have acquired multidrug resistance, rendering standard therapies ineffective and increasing mortality rates[2]. The rising prevalence of multidrug-resistant CRKP, especially among critically ill patients, heightens the risk of poor outcomes in vulnerable populations[3].

Several antibiotics were recently approved and introduced to treat these highly resistant infections[4]. Among the most notable therapeutics are β-lactam/β-lactamase inhibitor (BL/BLI) combinations, such as imipenem-relebactam (IR) and meropenem-vaborbactam (MVB)[5]. Despite promising initial reports of clinical effectiveness, concerns about their long-term viability have arisen due to resistance in high-risk patient populations, even before these antibiotics reached the market[6-14].

Our ability to limit the emergence and spread of resistance as antibiotics come to market is hindered by an incomplete understanding of the bacterial and clinical factors that drive resistance emergence. Previous reports have, unsurprisingly, demonstrated that antibiotic exposure exerts selective pressure for resistance[14-17]. Whether other patient characteristics or exposures contribute to the emergence of antibiotic-resistant bacteria, particularly those with epidemic potential, is less clear. High rates of resistance emergence have been reported among patients with increased acuity[17,18]. In addition, variation in rates of resistance emergence across healthcare settings has also been reported, but this may be due to differences in case mix, transmission risk, or antibiotic stewardship and infection prevention practices[19,20].

Even less is understood about how bacterial genetic background potentiates the emergence and spread of antibiotic resistance. Support for the role of genetic background comes from observed lineage-specific differences in the prevalence and fitness costs of resistance-conferring genotypes and plasmids[21,22]. Further support comes from strictly *in vitro* studies, which have shown that chromosomal and plasmid backgrounds can affect strain fitness and transcriptional responses upon the acquisition of resistance-conferring plasmids[23-25]. Pre-existing chromosomal mutations have also been postulated to influence not just fitness costs but also potentiate the emergence of antibiotic resistance[26-28]. Understanding how bacterial genetic background and patient clinical characteristics jointly influence resistance emergence and spread could strengthen our ability to predict and prevent resistance development.

To better understand how resistance to BL/BLI combinations emerges and spreads, we conducted a genomic epidemiology investigation using a comprehensive set of CRKP clinical isolates collected from a long-term acute care hospital (LTACH) network between August 2014 and July 2015, several years before the clinical deployment of MVB and IR. Genomic analysis provided evidence of repeated emergence and spread of resistant strains belonging to the epidemic lineage sequence type 258 (ST258). Genome-wide association studies (GWAS), phylogenetic analysis, and experimental evolution demonstrated that the genetic background within ST258 influenced baseline resistance levels, the likelihood of resistance crossing clinical breakpoints, and the spread of resistant strains. Finally, risk factor analysis for patients whose resistant isolate was predicted to be acquired via carbapenemase or non-carbapenemase mechanisms uncovered that patient characteristics and antibiotic exposures may differentially contribute to these distinct routes to BL/BLI resistance. Together, these findings highlight the

importance of surveillance before an antibiotic is clinically deployed in identifying both bacterial genetic and clinical drivers of resistance, which can inform molecular surveillance and interventions to prolong the efficacy of novel antibiotics.

3.3 Results

3.3.1 Detection of Resistance to β-lactam/β-lactamase Inhibitor Combinations Before Their Clinical Deployment

We collected 412 CRKP ST258 isolates from August 2014 to July 2015 across 21 LTACHs, including 232 respiratory, 139 urinary, 35 blood, and 6 wound isolates. When performing susceptibility testing for several last-line and novel antibiotics[6], we observed that the median minimum inhibitory concentration (MIC) was 0.50 µg/mL (log_2 interquartile range [IQR], 1.0) for IR and 0.25 µg/mL (log_2 IQR, 2.0) for MVB, both in the susceptible range according to 2021 Clinical & Laboratory Standards Institute breakpoints (**Figure 3.1**). However, when comparing strict susceptibility, 58 (14.1%) isolates were resistant to at least one BL/BLI combination: 39 (9.5%) to MVB and 38 (9.2%) to IR. Due to cross-resistance and a strong positive Spearman's correlation between MICs (**Appendix B Figures 1-2**), downstream analyses evaluated BL/BLI resistance as a composite outcome.

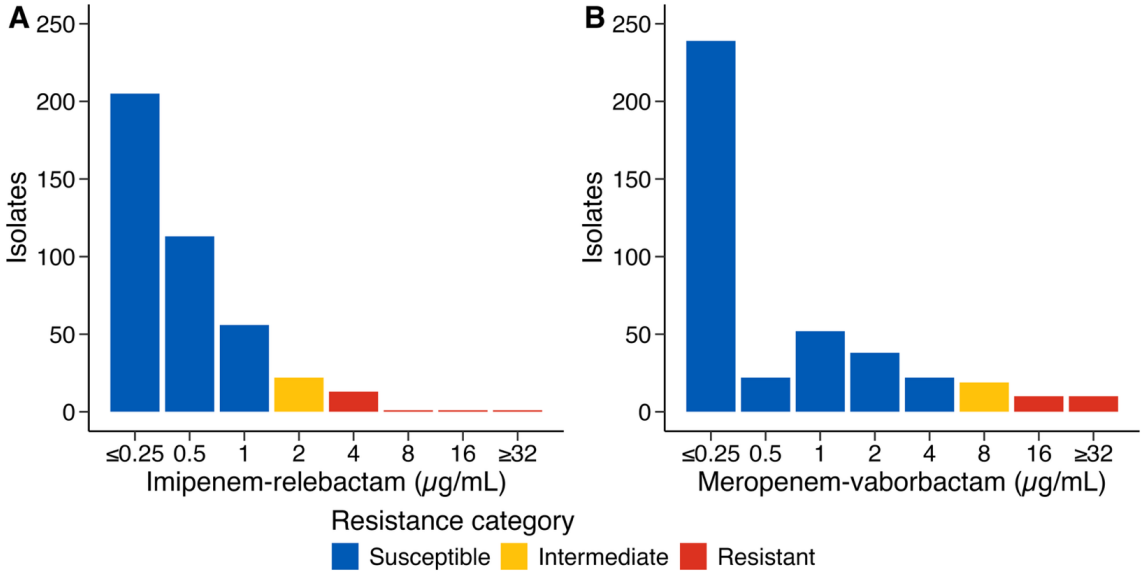


Figure 3.1 Minimum Inhibitory Concentrations for Imipenem-Relebactam and Meropenem-Vaborbactam in Carbapenem-Resistant *Klebsiella pneumoniae* Sequence Type 258 Isolates

Distribution of minimum inhibitory concentrations for (A) imipenem-relebactam and (B) meropenem-vaborbactam, as measured using a custom broth microdilution panel (SENSITITRE, Thermo Fisher Scientific). The 2021 Clinical and Laboratory Standards Institute breakpoints were used to categorize the susceptibility profiles of these isolates.

The β-lactamase inhibitor concentrations were 4 μg/mL and 8 μg/mL for imipenem-relebactam and meropenem-vaborbactam, respectively.

3.3.2 Differences in Resistance Emergence and Spread Exist Across Clades of Epidemic Lineage Sequence Type 258

As previously reported[29,30], our ST258 phylogeny had two clades: clade I (145 isolates [35.2%]) and clade II (267 isolates [64.8%]). Overlaying resistance phenotypes on the phylogeny revealed striking differences in resistance between the two clades (**Figure 3.2**). First, baseline MICs were significantly elevated in clade I for both IR and MVB (Wilcoxon rank-sum test p-values <0.001). Moreover, resistance, with MICs above clinical breakpoints, was significantly more frequent in clade I (39 [26.9%]) than in clade II (19 [7.1%]) (X^2 test p-value <0.001).

To better understand the historical basis for elevated resistance in clade I, ancestral state reconstruction of BL/BLI resistance was performed to identify independent emergence events of resistance. Using this approach, the 58 resistant isolates were partitioned into 36 emergence events. Twenty-nine emergence events involved a single patient, indicating repeated *de novo* evolution of resistance without subsequent spread. Of note, these 29 emergence events were split between the two clades, with 15 in clade I and 14 in clade II, despite there being almost twice as many clade II isolates. Consistent with the increased frequency of resistance emergence in clade I observed with ancestral reconstruction, we detected significant differences in the phylogenetic rates of resistance emergence (likelihood ratio test p-value < 0.001), with higher rates of phenotypic transition in clade I ($q_{transition}$, 20965.15) than in clade II ($q_{transition}$, 3881.492). In addition to resistant singletons, seven phylogenetic clusters of resistance involving multiple patients were detected, consistent with the emergence and spread of resistant strains: five in clade I and two in clade II. Although not statistically significant (Fisher's exact test p-value = 0.43), the proportion of emergence events that spread among patients was also elevated in clade I (5 clusters / 20 events [25.0%]), as compared to clade II (2 clusters / 16 events [12.5%]). These results suggest that ST258 clade I had an increased propensity for both the evolution and cross-transmission of BL/BLI resistance, supporting a role for bacterial genetic background in the dynamics of resistance to these therapeutics.

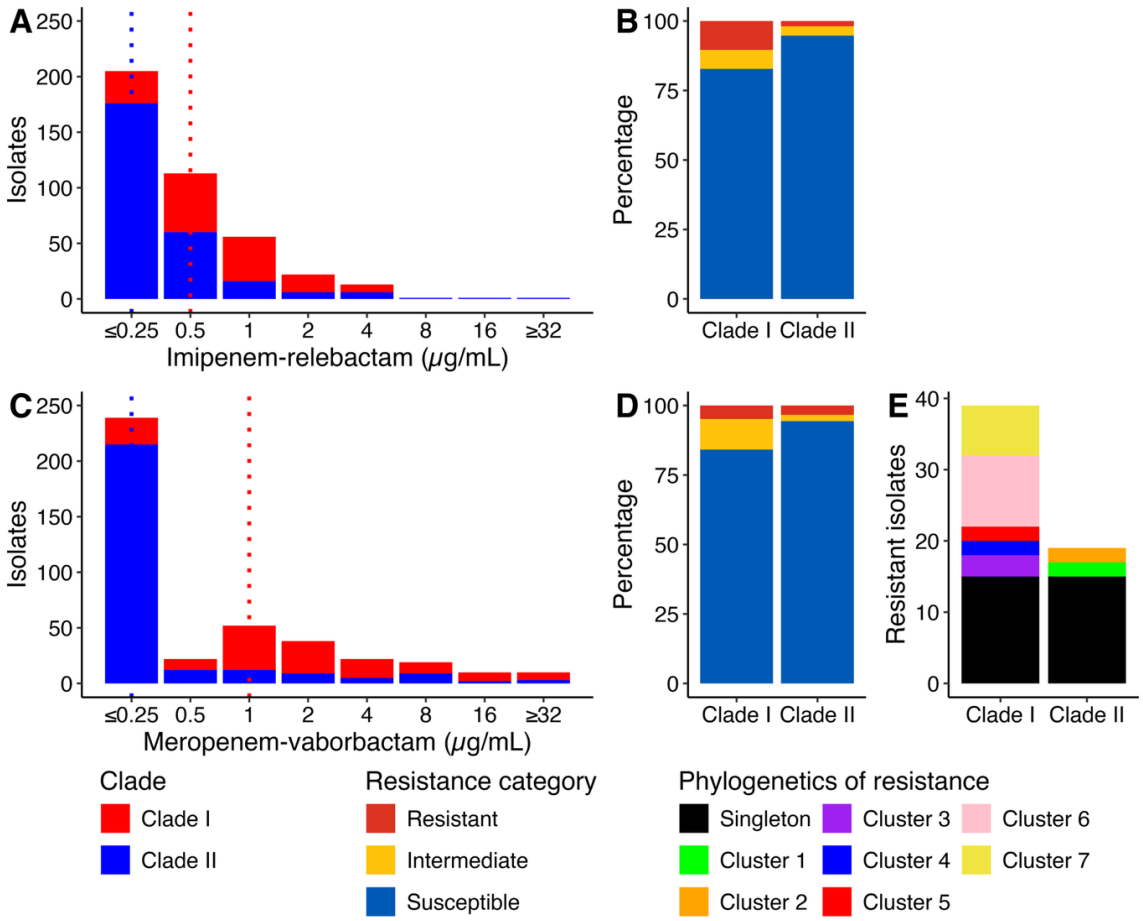


Figure 3.2 Differences in β-lactam/β-lactamase Inhibitor Combination Resistance Across Clades of Epidemic Lineage Sequence Type 258

Minimum inhibitory concentration (MIC) distributions stratified by clades of sequence type 258 for (A) imipenem-relebactam and (C) meropenem-vaborbactam. Dashed vertical lines indicate the median MIC values for each clade. The 2021 Clinical and Laboratory Standards Institute breakpoints were used to categorize the susceptibility profile for (B) imipenem-relebactam and (D) meropenem-vaborbactam. The membership of resistant isolates in phylogenetic clusters and singletons (E).

3.3.3 Mutations in Chromosomal Carbapenem Resistance-Associated Genes Explain BL/BLI Resistance in a Clade-Specific Manner

We next sought to understand the genetic basis for BL/BLI resistance across the two ST258 clades. As both β-lactamase inhibitors inhibit the *bla*$_{KPC}$ carbapenemase, which is ubiquitous in ST258 (408/412 [99.0%]), and reports of *bla*$_{KPC}$ mutations conferring resistance remain limited[31], we hypothesized that resistance was driven by mutations in chromosomal carbapenem resistance-associated genes[5,32-34]. To test this hypothesis, genotypes involved in carbapenem entry, efflux, and target modification were overlaid on the phylogeny in **Appendix B Figure 3,** and their frequencies in resistant isolates are reported in **Table 3.1.**

Mechanism of interest	Overall (n=412)	BL/BLI Resistant (n=58)	BL/BLI Susceptible (n=354)	p-value	Nearest-neighbor criterion	Discriminatory criterion
Carbapenem entry						
ompK35 porin truncation at 25%	412 (100.0)	58 (100.0)	354 (100.0)	NA	NA	NA
ompK36 porin						
Cytosine-to-thymine transition at position 25	131 (31.8)	37 (63.8)	94 (26.6)	<0.001	Pass	Fail
Loop 3 insertion	124 (30.1)	37 (63.8)	87 (24.6)	<0.001	Pass	Fail
Glycine-Aspartate (GD) insertion	119 (28.9)	35 (60.3)	84 (23.7)	<0.001	Pass	Fail
Threonine-Aspartate (TD) insertion	5 (1.2)	2 (3.4)	3 (0.8)	0.147	Fail	Pass
Non-synonymous mutation	22 (5.3)	10 (17.2)	12 (3.4)	<0.001	Pass	Pass
Putative function-altering variants	20 (4.9)	10 (17.2)	10 (2.8)	<0.001	Pass	Pass
Truncation	8 (1.9)	6 (10.3)	2 (0.6)	<0.001	Pass	Pass
ompK36 5' intergenic region						
Any mutation	31 (7.5)	8 (13.8)	23 (6.5)	0.061	Fail	Pass
Insertion sequences	24 (5.8)	6 (10.3)	18 (5.1)	0.128	Pass	Pass
Carbapenem efflux						
acrAB-tolC efflux pump mutant	2 (0.5)	0 (0.0)	2 (0.6)	1.000	Fail	Pass
ramR efflux pump regulator putative-function altering variants	27 (6.6)	7 (12.1)	20 (5.6)	0.083	Fail	Pass
ramA efflux pump activator putative-function altering variants	10 (2.4)	6 (10.3)	4 (1.1)	0.001	Pass	Pass
Carbapenem target modification						
Non-synonymous mutations in penicillin-binding-proteins	9 (2.2)	6 (10.3)	3 (0.8)	<0.001	Pass	Pass
Penicillin-binding protein-2	8 (1.9)	6 (10.3)	2 (0.6)	<0.001	Pass	Pass
Penicillin-binding protein-4	2 (0.5)	1 (1.7)	1 (0.3)	0.262	Pass	Pass

Table 3.1 Presence of Non-Carbapenemase Mechanisms of Carbapenem Resistance Stratified by Resistance to β-lactam/β-lactamase Inhibitor Combinations

The frequency of non-carbapenemase mechanisms of carbapenem resistance was stratified by resistance to at least one β-lactam/β-lactamase inhibitors (BL/BLI) combination: imipenem-relebactam and meropenem-vaborbactam. Statistical significance was evaluated using Fisher's exact test due to a small number of events, except for the presence of any loop 3 insertion or the Glycine-Aspartate (GD) insertion in loop 3 of OmpK36, where the X^2 test was performed. To account for confounding by genetic background, the MICs of isolates with the genotype were compared to their nearest phylogenetic neighbor without the genotype. Genotypes with a median $\log_2$ fold change in MIC > 1 passed our nearest-neighbor criterion. Genotypes with discriminatory potential (specificity > 92.5% and accuracy > 80%) passed the discriminatory potential criterion. Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; GD, Glycine-Aspartate; NA, not applicable; TD, Threonine-Aspartate.

To account for confounding by genetic background and identify associations more likely to be causal, discriminatory genotypes were identified by comparing the MICs of isolates with each putative resistance genotype to their nearest phylogenetic neighbor lacking that genotype (**Appendix B Figure 3C**). The following genotypes had discriminatory potential (92.5%+ specificity and 80% accuracy) and a median $\log_2$ fold change in MIC > 1, rendering them eligible for inclusion into a panel of explanatory genotypes: mutations in *ompK36* (truncations and putative function-altering variants), insertion sequences near the *ompK36* promoter, putative function-altering variants in *ramA*, and non-synonymous mutations in penicillin-binding protein (PBP) genes. Genotypes passing these criteria were identified in 22 of 58 (37.9%) BL/BLI-resistant isolates. Notably, there were marked differences in both the frequency and explanatory power of carbapenem resistance-associated genotypes across clades (**Appendix B Table 1**).

Resistance in clade II was almost completely accounted for by variation in this curated gene set (15/19 [78.9%]), whereas resistance in clade I was left largely unexplained (7/39 [17.9%]).

3.3.4 Convergence-Based Genome-Wide Association Studies Improved the Explanation of Resistance and Illuminated Distinct Pathways to Resistance Across Clades

As most resistant isolates were not explained by the evaluated non-carbapenemase mechanisms of carbapenem resistance, we next sought to identify alternative genetic mechanisms. To identify novel determinants of resistance, convergence-based GWAS were performed on continuous $\log_2$ MIC and discrete BL/BLI resistance. GWAS performed on continuous MIC values sought to identify modulators of resistance, while GWAS performed on discrete resistance categorization sought determinants of transitions across the clinical breakpoint. Ten significant hits were detected (**Appendix B Figure 4**), eight of which passed the nearest-neighbor criterion and were considered further (four continuous and four discrete, **Figure 3.3**).

A

Significant hit	Gene	Product	Significant tests	Significant models	Phenotypes	Explanatory genotype
KPNIH1_RS02940		hypothetical protein	Continuous	All variants & Non-synonymous	MVB	Yes
KPNIH1_RS18100		MdtA/MuxA family multidrug efflux RND transporter periplasmic adaptor subunit	Continuous	Putative function-altering variants	IR	No
Loop 3 insertion in OmpK36	*ompK36*	porin OmpC	Continuous	Kleborate	MVB	No
GD loop 3 insertion in OmpK36	*ompK36*	porin OmpC	Continuous	Kleborate	MVB	No
PFAV in *ompK36*	*ompK36*	porin OmpC	All tests	All variant models	BL/BLI & IR & MVB	Yes
Truncation of *ompK36*	*ompK36*	porin OmpC	All tests	Kleborate	BL/BLI & IR & MVB	Yes
AA018 bla_{KPC}-containing plasmid		bla_{KPC}-containing plasmid cluster	Synchronous	bla_{KPC}-containing plasmids	BL/BLI	No
AA552 bla_{KPC}-containing plasmid		bla_{KPC}-containing plasmid cluster	PhyC & Synchronous	bla_{KPC}-containing plasmids	BL/BLI	Yes

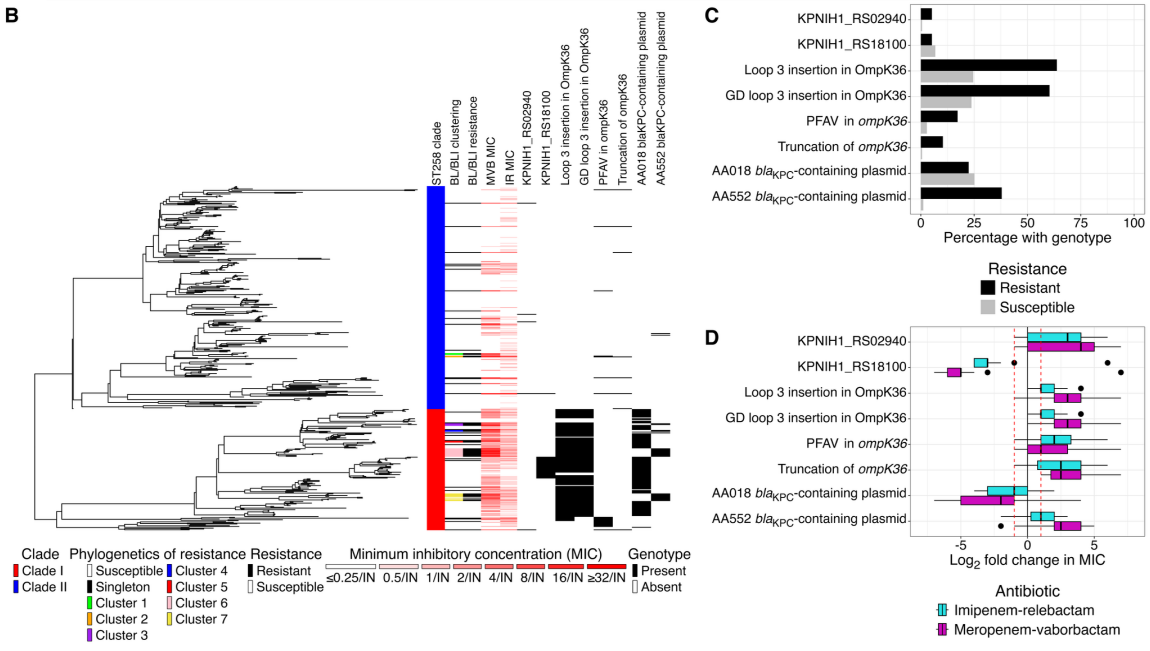


Figure 3.3 Qualifying Genome-Wide Association Study Hits for β-lactam/β-lactamase Inhibitor Resistance

Convergence-based genome-wide association study was performed on log_2 minimum inhibitory concentration (MIC) and resistance to β-lactam/β-lactamase inhibitor (BL/BLI) combinations using hogwash v1.2.6. Hits that passed the nearest-neighbor criterion (median log_2 fold change in MIC > |1|) were eligible for downstream analysis and included in this figure. (A) Information about each significant genome-wide association study hit. Genotypes were classified as explanatory if their median log_2 fold change in MIC > 1 and had diagnostic potential (specificity > 92.5% and accuracy > 80%). (B) The significant hits are overlaid on the phylogeny. (C) The frequency of genotypes in β-lactam/β-lactamase inhibitor combination susceptible and resistant isolates. (D) The log_2 fold change in imipenem-relebactam and meropenem-vaborbactam minimum inhibitory concentration for isolates with the genotype relative to their nearest phylogenetic neighbor without the genotype. Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; GD, Glycine-Aspartate; IR, imipenem-relebactam; MVB, meropenem-vaborbactam; PFAV, putative function-altering variant.

The continuous GWAS identified two novel genotypes associated with large changes to MIC. First, there were putative function-altering variants in KPNIH1_RS18100, which encodes a putative efflux protein. While associated with consistent (96.3%) and large changes in MVB MIC (median log_2 change [range], -5 [-7,7]), isolates harboring these variants had overall lower MICs than their nearest phylogenetic neighbors, leading us to conclude that KPNIH1_RS18100 is not an explanatory genotype. Second, there were mutations in the hypothetical protein KPNIH1_RS02940, which were associated with consistent (3/5, 60%) increases in IR MIC (IR median log_2 change [range], 3 [-1,6]). In addition to the two novel genotypes, the continuous GWAS also identified insertions in *ompK36* mapping to loop 3 of the protein, including both a composite outcome for any insertion and a specific association with Glycine-Aspartate (GD) insertions. The presence of loop 3 insertions in OmpK36, previously reported to confer elevated carbapenem resistance[35,36], was associated with consistent increases in MVB MIC (MVB, 93.5%) of moderate magnitude (median log_2 fold change [range], 3 [-1,7]).

The discrete GWAS identified mutations in *ompK36* (truncation and putative function-altering variants), both of which were identified in the curated genotypic panel above. In addition, two *bla*$_{KPC}$-containing plasmid clusters, AA552 and AA018, were identified as being associated with resistance. The AA552 plasmid cluster, an IncN plasmid, was associated with the gain of resistance. Conversely, the AA018 plasmid cluster, an IncFIB/IncFII plasmid, was associated with the loss of resistance. Characteristics of *bla*$_{KPC}$-associated plasmid clusters and their distribution across the phylogeny are detailed in **Appendix B Figures 5 and 6**, respectively.

Inspection of the presence of these two plasmids on the ST258 phylogeny revealed that the gain of the *bla*$_{KPC}$-containing AA552 plasmid was consistently associated with the loss of the AA018 plasmid, and in turn, the gain of BL/BLI resistance (**Appendix B Figure 7**). Hybrid sequencing of representative isolates revealed that this pattern was due to the *bla*$_{KPC}$ transposon,

tn4401, hopping from the AA018 plasmid to a pre-existing AA552 plasmid, followed by loss of the AA018 plasmid backbone (**Appendix B Figure 8**). We hypothesized that this change in plasmid context mediated resistance by increasing bla_{KPC} copy number in the AA552 plasmid, a hypothesis confirmed by comparing bla_{KPC} copy number across plasmid clusters (**Appendix B Figure 7)**. Evaluation of bla_{KPC} copy number and resistance across plasmid clusters revealed that the association between elevated copy number and transitions to resistance was exclusive to the AA552 plasmid backbone (**Appendix B Figure 9).**

Having identified additional putative genetic mediators of resistance, we next evaluated overall explanatory power when adding these variants to the curated set. While present in three resistant isolates, KPNIH1_RS02940 mutations did not improve the explanation of resistance, as all three isolates also contained putative disruptions in *ompK36*. Loop 3 insertions in OmpK36, while associated with an increase in MIC, were lineage-defining in clade I, and therefore necessary, but not sufficient to explain resistance, and therefore not considered as part of the explanatory panel. In contrast, the bla_{KPC}-containing AA552 was both enriched in resistant isolates (22/58 [37.9%] vs. 4/354 [1.1%]; Fisher's exact test p-value < 0.001) and accounted for previously unexplained resistance. Addition of this plasmid to our explanatory panel markedly improved the explanation of resistance (37.9% to 72.4%), an effect observed exclusively in clade I (17.9% to 69.2%). The diagnostic statistics for our genotypic panel and BL/BLI resistance are reported in **Appendix B Figure 10**.

3.3.5 Interaction Between Carbapenemase Copy Number and Loop 3 Insertions in OmpK36

The restriction of the AA552 plasmid as a resistance-associated genotype to clade I led us to hypothesize that its associated increase in bla_{KPC} copy number modulated MICs exclusively in the clade I genetic background. Moreover, based on the observed increase in baseline MIC associated with loop 3 insertions in OmpK36 and their restriction to clade I, we hypothesized that loop 3 insertions were necessary for AA552 to influence MIC. To test this hypothesis, we examined the association between $\log_2$ bla_{KPC} copy number and $\log_2$ BL/BLI MIC (**Figure 4**). Positive associations between bla_{KPC} copy number and MVB and IR MICs were exclusive to isolates with loop 3 insertions in OmpK36, indicating that the restriction of porin size associated with these insertions enables the modulation of MIC through bla_{KPC} copy number variation. Linear regression models are reported in **Supplementary Table 2**. Together, these results suggest that the clade I lineage-defining loop 3 insertions in OmpK36 both increased baseline

MICs and made accessible the evolution of resistance above the clinical breakpoint via hopping of the *bla*$_{KPC}$-containing transposon to the higher-copy-number AA552 plasmid.

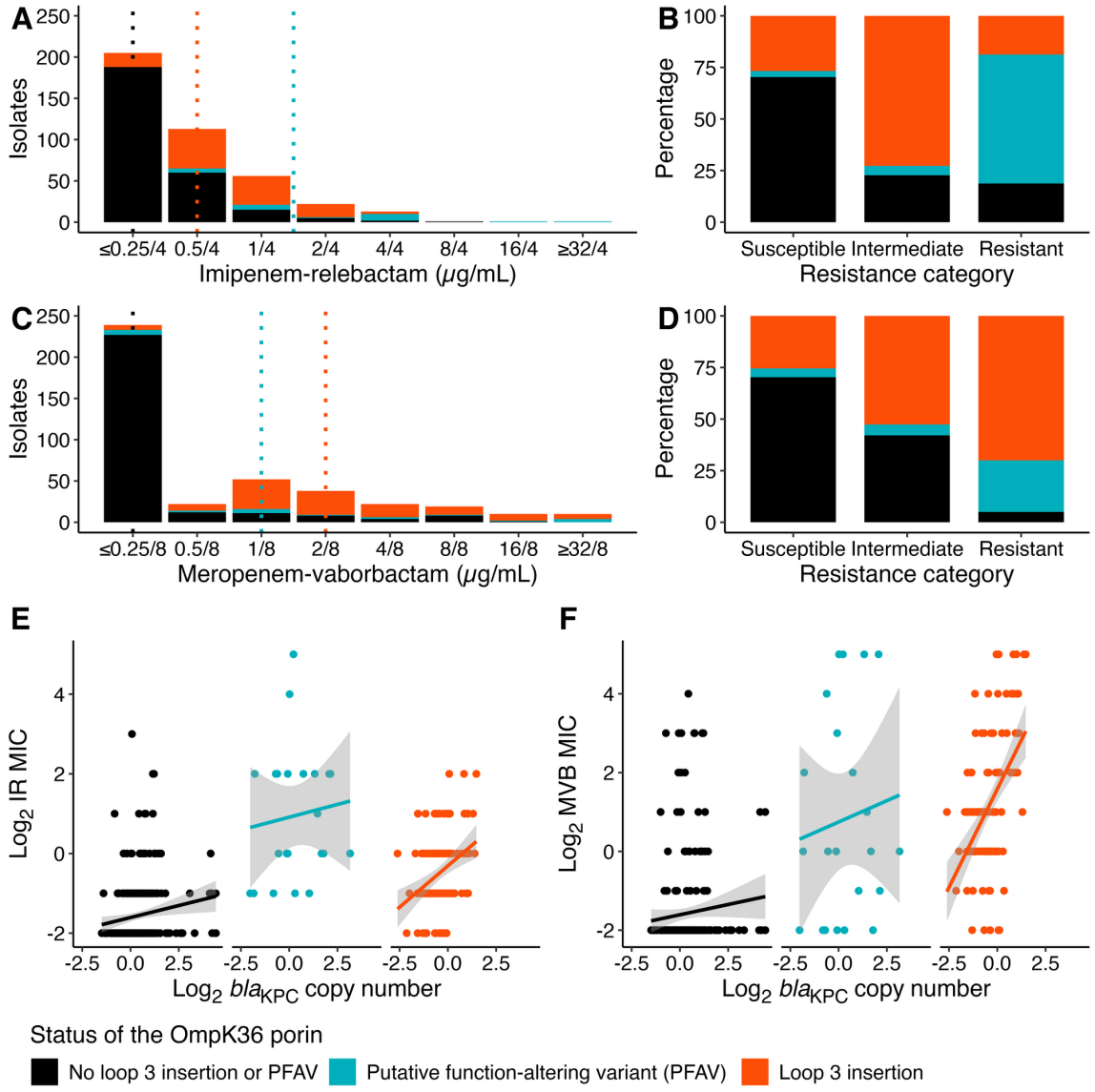


Figure 3.4 Interaction of Loop 3 Insertions in OmpK36 Porin with *bla*$_{KPC}$ Copy Number

Minimum inhibitory concentration histogram stratified by the presence of mutations in the major OmpK36 porin for (A) imipenem-relebactam and (C) meropenem-vaborbactam. The 2021 Clinical and Laboratory Standards Institute breakpoints were used to categorize the susceptibility profiles for (B) imipenem-relebactam and (D) meropenem-vaborbactam, stratified by the presence of mutations in the major porin, OmpK36. The presence of mutations in *ompK36* porin influences the relationship between $\log_2$ *bla*$_{KPC}$ copy number and $\log_2$ minimum inhibitory concentration for imipenem-relebactam (E) and meropenem-vaborbactam (F).

3.3.6 Experimental Evolution Corroborated Clade-Specific Differences in Resistance

To test the hypothesis that the genetic background of ST258 influenced the dynamics of resistance evolution, we performed experimental evolution of MVB on 12 representative isolates

(**Figure 3.5**). Eight isolates consistently developed detectable levels of clinical resistance to MVB. Among isolates with detectable resistance, the resistance frequency varied (ANOVA p-value < 0.001). The resistance frequency was elevated in clade I (β, 1.62; confidence interval [CI], 0.36-2.87; p-value, 0.012). Supporting the role of porin modification in elevated resistance evolution, the presence of loop 3 insertions in the OmpK36 porin were positively associated with resistance frequency when modeled among all isolates (β, 1.76; confidence interval [CI], 0.77-2.76; p-value, 0.001) and upon restriction to clade I isolates (β, 1.34; confidence interval [CI], 0.90-1.77; p-value, <0.001). In support of the interaction between carbapenemase content and loop 3 insertions in OmpK36, no evolved resistance was detected in the GD-insertion-harboring clade I isolate that did not harbor *bla*$_{KPC-2}$.

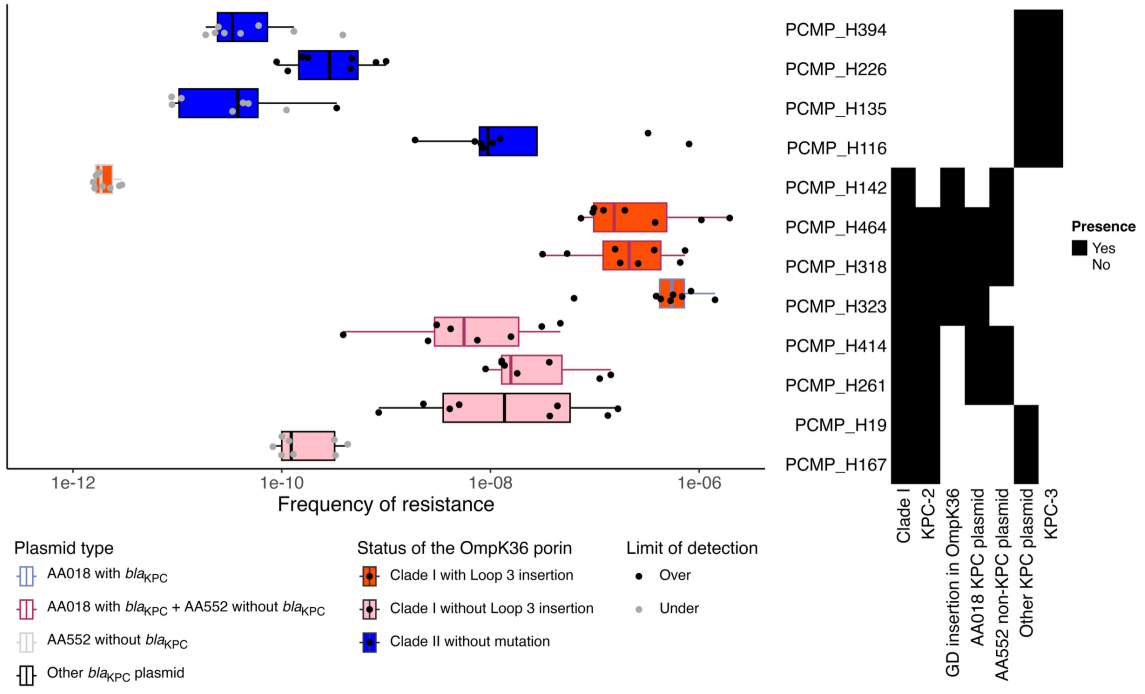


Figure 3.5 Experimental Evolution Corroborated the Influence of Genetic Background on the Evolution of Meropenem-Vaborbactam Resistance

Selection for meropenem-vaborbactam was performed on 12 representative isolates. The frequency of resistance was determined by dividing the number of colonies obtained from plating on meropenem-vaborbactam-containing plates by the number of colonies plated on Mueller-Hinton agar. Meropenem-vaborbactam-impregnated Mueller-Hinton agar plates contained 8 µg/mL of meropenem and 4 µg/mL of vaborbactam. On the left, the distribution of resistance frequencies is shown as a boxplot. Boxplot fill indicates their clade and the presence of loop 3 insertions in OmpK36. The boxplot border color represents the combination of resistance-associated plasmids. Each replicate is provided on the plot, with point color detailing whether resistance was detected. For isolates with no detectable growth, the limit of detection was displayed. Isolates are grouped by clade and the presence of loop 3 insertions in the OmpK36 porin, as detailed in the heatmap. Abbreviations: GD, Glycine-Aspartate.

To determine the genomic determinants underlying MVB resistance, we performed whole-genome sequencing of a representative resistant colony (**Table 3.2)**. Both sequenced clade II isolates harbored mutations in *ompK36*: one with a 15-amino-acid deletion and the other with ISKpn26 integration. The three sequenced clade I isolates without a loop 3 insertion acquired porin mutations. Specifically, one had *ISKpn26* inserted near the *ompK36* promoter, one had a seven-nucleotide duplication in *ompK36*, and another had both a deletion in *ompK36* and an ISKpn14 inserted near the *ompK36* promoter. Conversely, the three sequenced clade I isolates with loop 3 insertions showed a marked increase in bla_{KPC} copy number. Two isolates had a two-fold increase in bla_{KPC} copy number on the parental AA018 plasmid background. Corroborating phylogenetic evidence from our clinical collection, we also detected evidence of *tn4401* transposition from the parental AA018 plasmid to its AA552 plasmid background, which coincided with a seven-fold increase in bla_{KPC} copy number and a four-fold increase in bla_{KPC} plasmid copy number. Collectively, this experiment corroborated clinical observations of clade-specific differences in the evolution rate of resistance and marked differences in accessible genomic pathways, supporting the hypothesis that loop 3 insertions in OmpK36 shape the accessibility of resistance via bla_{KPC} copy number variation.

Parent	Clade	Parental status of the OmpK36 porin	Evolved canonical mutation (gene [mutation]; position)	bla_{KPC}	Parental bla_{KPC} plasmid	Parental bla_{KPC} CN	Parental bla_{KPC} plasmid CN	Evolved bla_{KPC} plasmid	Evolved bla_{KPC} CN	Evolved bla_{KPC} plasmid CN	bla_{KPC} CN fold change	bla_{KPC} plasmid CN fold change	Plasmid size change (bp)
PCMP_H116	II	Wild type	*ompK36* [ISKpn26]; 3714050	bla_{KPC-3}	AA357	0.66	0.69	AA357	0.85	0.86	1.29	1.25	0
PCMP_H226	II	Wild type	*ompK36* (p.Val323_Asp338del); 3713046	bla_{KPC-3}	AA274	1.47	0.97	AA274	1.67	1.97	1.13	2.03	-1132
PCMP_H19	I	Wild type	upstream *ompK36* [ISKpn26]; 3714091	bla_{KPC-2}	AA275	0.74	0.74	AA275	1.08	1.48	1.47	2.01	-1418
PCMP_H261	I	25 C>T transition	*ompK36* (c.190_196dupGAAACCC); 3713864	bla_{KPC-2}	AA018	0.61	0.56	AA018	0.78	1.03	1.29	1.83	728
PCMP_H414	I	25 C>T transition	*ompK36* (p.Gly136_Asp137del); 3713649 & upstream *ompK36* [ISKpn14]; 3714068	bla_{KPC-2}	AA018	0.49	0.42	AA018	0.68	0.91	1.39	2.15	0
PCMP_H318	I	25 C>T transition & GD insertion in loop 3		bla_{KPC-2}	AA018	0.65	0.79	AA552	4.55	3.49	7.04	4.43	NA
PCMP_H323	I	25 C>T transition & GD insertion in loop 3		bla_{KPC-2}	AA018	0.93	0.96	AA018	2.18	2.84	2.34	2.96	-247
PCMP_H464	I	25 C>T transition & GD insertion in loop 3		bla_{KPC-2}	AA018	0.42	0.53	AA018	1.07	1.1	2.58	2.08	-11368

Table 3.2 Genetic Changes Associated With the Evolution of Resistance to Meropenem-Vaborbactam

Abbreviations: CN, copy number; GD, Glycine-Aspartate.

3.3.7 Resistance-Associated Genotypes Showed Different Propensities for Spread

After identifying putative genetic determinants of BL/BLI resistance, we next evaluated the extent to which genotypes were prone to spread among patients (**Appendix B Figure 11)**. Resistance that did not spread among patients was often mediated by convergent mutations at a small number of loci. While PBP-2 modification and putative function-altering variants in

ompK36 were observed in one small clade II cluster, these mutations were more common in resistant singletons (10/30 [33.3%]) than cluster isolates (2/28 [7.1%]) (Fisher's exact test p-value = 0.022). Conversely, virtually all clade I resistance cluster isolates (21/24 [87.5%]) harbored the *bla*$_{KPC}$-containing AA552 plasmid. The AA552 plasmid was distributed across the 12 California LTACHs in this study (**Appendix B Figure 12**). All resistance clusters containing *bla*$_{KPC}$-containing AA552 plasmids had geographic and facility overlap, with several clusters distributed across multiple facilities. As several *bla*$_{KPC}$-containing AA552 plasmids containing resistance clusters occupied the same facility, we cannot rule out resistance spread due to the transfer of AA552 among strains. Collectively, analysis of resistance-associated genotypes and BL/BLI resistance revealed that the spread of resistance was predominantly mediated by a specific *bla*$_{KPC}$-containing plasmid, amidst a background of mutations in genes implicated in the entry, efflux, and target of β-lactam antibiotics that did not spread amongst patients.

3.3.8 Patient Characteristics and Antibiotic Exposures Distinguish Episodes of Non-Carbapenemase Resistance from Plasmid-Based Resistance

Lastly, we sought to understand the role of patient characteristics and antibiotic exposures in driving the emergence and spread of resistance. Given the contribution of the *bla*$_{KPC}$-containing AA552 plasmid to the spread of resistance, we hypothesized that distinct selective pressures influenced the acquisition of resistance via carbapenemase or non-carbapenemase mechanisms. In turn, we built exploratory logistic regression models to identify risk factors for these genotypic groupings and compared them with risk factors for harboring a resistant isolate, without accounting for resistance genotype.

First, without accounting for resistance mechanism, we found that harboring a resistant isolate was positively associated with prior exposure to cephalosporin antibiotics (adjusted odds ratio [aOR], 2.09; 95% confidence interval [CI], 1.08-3.99; p-value = 0.027), prior exposure to carbapenem antibiotics (aOR, 1.96; 95% CI, 1.04-3.68; p-value = 0.037), and increased age (adjusted odds ratio [aOR], 1.04 per 1 year unit; 95% confidence interval [95% CI], 1.02-1.07; p-value = 0.0015). A negative association with being underweight and/or malnourished was also detected (aOR, 0.42; 95% CI, 0.16-0.94; p-value = 0.047).

Next, partitioning isolates by resistance genotype and performing regression modeling revealed distinct sets of clinical associations for the acquisition of resistance via carbapenemase and non-carbapenemase mechanisms. Harboring a resistant isolate with non-carbapenemase

mechanisms was positively associated with prior exposure to carbapenem antibiotics (aOR, 3.43; 95% CI, 1.53-7.81; p-value = 0.003), prior exposure to cephalosporin antibiotics (aOR, 2.60; 95% CI, 1.09-6.19; p-value = 0.03), and increased age (aOR, 1.04 per 1 year unit; 95% CI, 1.01-1.07; p-value = 0.011). Conversely, harboring a resistant isolate with the *bla*$_{KPC}$-containing AA552 plasmid was positively associated with having chronic bronchitis and/or chronic obstructive pulmonary disease (aOR, 3.23; 95% CI, 1.11-8.94; p-value = 0.025). The unadjusted and adjusted results for all outcomes are found in **Appendix B Tables 3-5**.

Collectively, these results suggest that the contribution of antibiotic exposures to BL/BLI resistance, detected as a risk factor for harboring a resistant isolate, was mediated by the subset of patients whose isolates had non-carbapenemase mechanisms of carbapenem resistance that tended not to spread among patients. In contrast, restricting risk-factor analysis to transmissible resistance associated with the *bla*$_{KPC}$-containing AA552 plasmid uncovered a potential role for patients with respiratory comorbidities in the spread of BL/BLI resistance, which was not detected when grouping all resistant isolates together.

3.4 Discussion

Amidst the growing threat of antibiotic resistance, it is critical to understand the drivers of resistance to last-line therapeutics. To this end, we leveraged bacterial whole-genome sequencing and clinical metadata from a comprehensive isolate collection to characterize the dynamics of BL/BLI resistance across a network of long-term acute care hospitals. Our analysis revealed evidence for the repeated emergence and cross-transmission of resistant strains, prior to clinical introduction of the cognate antibiotics. Placing resistance determinants in a phylogenetic context uncovered a role of genetic background in shaping the available evolutionary trajectories to resistance and identified genotypes with an elevated propensity to spread among patients, which was subsequently validated by experimental evolution of resistance. Genotypically informed risk-factor analysis implicated distinct patient characteristics and antibiotic exposures in promoting resistance via carbapenemase and non-carbapenemase mechanisms. Together, these findings have immediate implications for genomic surveillance and antibiotic stewardship efforts, as well as for identifying and tracking the bacterial genotypes and clinical practices that promote resistance.

The discovery of pre-existing resistant strains before the clinical deployment of BL/BLI combinations is concerning. These findings align with earlier *in vitro* reports of resistance in

carbapenem-resistant Enterobacterales, both before and after the availability of these therapeutics[7-13]. Further support for the generalizability of our findings comes from studies reporting similar resistance-associated genotypes when experimentally evolving carbapenem and BL/BLI resistance, even among *bla*$_{KPC}$-containing CRKP strains[15,37,38], as well as from clinical case reports of BL/BLI resistance[39-41]. Our detected association between prior carbapenem and cephalosporin exposure and the emergence of BL/BLI resistance via non-carbapenemase mechanisms indicates that mono-therapy with carbapenems and cephalosporins can exert selective pressure for BL/BLI resistance among *bla*$_{KPC}$-harboring strains[42]. Moreover, our findings also indicate that *bla*$_{KPC}$-containing plasmids, at sufficient copy number to confer resistance in permissible genetic backgrounds, can emerge and spread in the absence of sustained antibiotic pressure. Collectively, our study suggests that early surveillance for nascent resistance, even before the clinical deployment of a drug, can reveal pre-existing resistance and identify bacterial genotypes, antibiotic use patterns, and patient populations at greatest risk of promoting resistance following introduction. This approach is particularly useful for modified versions of existing antibiotics, such as BL/BLI combinations, in which hypotheses generated from prior experimental and clinical studies can be leveraged to inform surveillance strategies, guide genomic investigations, and predict resistance. We further postulate that pre-introduction surveillance, when coupled with clinical risk factor modeling and molecular investigations, will be invaluable to assessing the burden, illuminating evolutionary pathways, and identifying high-risk populations, thereby guiding interventions to reduce the burden of antibiotic resistance and preserve long-term efficacy of both existing and novel antibiotic classes.

Phylogenetic analysis and experimental evolution revealed the importance of chromosome and plasmid genetic background in shaping the emergence and spread of BL/BLI resistance. In clade II, resistance predominantly emerged through chromosomal, non-carbapenemase mechanisms. In contrast, loop 3 insertions in the OmpK36 porin shaped the availability of evolutionary trajectories to resistance in clade I. By reducing antibiotic diffusion into the cell[35,36], this widespread insertion can synergistically interact with carbapenemases to increase resistance to carbapenems[35,43]. Consistent with this, we observed that clade I isolates with loop 3 insertions had elevated baseline MICs, with transitions above the clinical breakpoint associated with transposition of the *tn4401* transposon to a plasmid context with higher copy number, a pathway previously reported to lead to resistance[44-47]. Clade I isolates with this

alternative plasmid context exhibited clinical resistance that spread among patients and across numerous facilities. Collectively, these findings support the hypothesis that chromosomal and plasmid genetic backgrounds influence the availability of resistance and potential for the clonal expansion of resistant lineages[26,30,35,48-52], highlighting the importance of considering the influence of genetic background and circulating plasmids on the propensity for resistance to emerge and spread upon a therapeutic's clinical deployment.

Genotypically informed risk-factor modeling sharpened our understanding of the patient factors that drive the emergence and spread of resistance. Evaluating risk factors for non-carbapenemase mechanisms of resistance, a common source of mutational resistance, revealed a role for antibiotic exposures in the emergence and spread of BL/BLI resistance. Specifically, prior exposure to carbapenem and cephalosporin antibiotics was positively associated with mutational pathways to BL/BLI resistance. Modeling risk factors for the acquisition of resistant strains harboring *bla*$_{KPC}$-containing AA552 plasmids revealed a positive association with the presence of chronic respiratory comorbidities (e.g., chronic bronchitis or chronic obstructive pulmonary disease). The failure to detect antibiotics as a risk factor for carrying the *bla*$_{KPC}$-containing AA552 plasmid highlights a potential for spread among patients without sustained antibiotic exposures. Collectively, our findings suggest that interactions between bacterial genetic background, patient comorbidities, and antibiotic exposures shape the dynamics of BL/BLI resistance.

This study has several limitations. First, the generalizability of these findings to other populations or carbapenem-resistant Enterobacterales lineages may be limited. Our analysis was restricted to *bla*$_{KPC}$-containing ST258, the dominant sequence type in our US LTACH isolate collection[20]. Regardless, the observed chromosomal mutations and mobile genetic elements have been demonstrated to be associated with BL/BLI resistance in other strains of *K. pneumoniae*[14,15,45,53-55], and more broadly, in other species of Enterobacterales, supporting the generalizability of these mechanisms[55,56]. Second, we did not evaluate whether facility-level characteristics drive the emergence or spread of resistance. Future work should evaluate the contribution of colonization burden, antibiotic stewardship, and infection prevention practices on the development and spread of resistance to BL/BLI combinations. Third, no experimental evaluation of the necessity or sufficiency of the novel resistance-associated genotypes, non-carbapenemase mechanisms of resistance, or *bla*$_{KPC}$ copy number elevation causing resistance

changes was performed, although most predicted genotypes have been demonstrated to be associated with carbapenem resistance in other contexts, were supported by our phylogenetic investigations, or detected during our experimental evolution of MVB resistance. Finally, the small sample size may have hindered our ability to differentiate risk factors for the emergence and spread of BL/BLI resistance. The application of phylogenetic contextualization to larger, densely sampled isolate collections would improve our ability to detect the patient characteristics and medical practices that distinguish these distinct situations.

In summary, we leveraged patient metadata, bacterial whole-genome sequencing, and experimental evolution to evaluate the dynamics of resistance to two promising β-lactam/β-lactamase inhibitor therapy agents before their market introduction in CRKP collected from LTACHs. Our findings demonstrate the utility of pre-introduction surveillance to illuminate the potential drivers of resistance emergence and spread within high-risk patient populations and inform future surveillance, infection prevention, and antibiotic stewardship efforts.

3.5 Methods

3.5.1 Carbapenem-Resistant Klebsiella pneumoniae Isolate Collection

Clinical isolates were collected from August 1, 2014, to July 25, 2015, at 21 United States LTACHs belonging to a national healthcare network: 12 southern California LTACHs (11 Los Angeles & 1 San Diego), six Texas LTACHs, two Florida LTACHs, and one Kentucky LTACH[20]. Bacteria from blood, respiratory, urine, and wound cultures identified as *K. pneumoniae* were tested for carbapenem resistance according to the 2015 Centers for Disease Control and Prevention criteria, as described elsewhere[20].

3.5.2 Patient Clinical Metadata

Clinical metadata were extracted from electronic medical records, as described elsewhere[20,51]. Extracted metadata included patient demographics, medical comorbidities, and the presence of indwelling devices at the time of specimen collection. Antibiotic exposure within the 30 days preceding isolate collection was also extracted from healthcare network records.

3.5.3 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing for newer and last-line therapeutics was performed in 2021 using a custom broth microdilution panel, as described elsewhere (SENSITITRE, Thermo

Scientific)[6]. Resistance was defined as a MIC within the intermediate or resistant categories. Resistance to BL/BLI combinations was defined as having resistance to at least one of the following antibiotics: IR and MVB.

3.5.4 Whole-Genome Sequencing and Genomic Data Curation

Whole-genome sequencing was performed on an Illumina HiSeq 2500 instrument, as described elsewhere[20]. Short-read genome assemblies were generated using an in-house pipeline (https://github.com/Snitkin-Lab-Umich/QCD), as described elsewhere[51].

Reference-based variant calling was performed using an in-house pipeline (https://github.com/Snitkin-Lab-Umich/snpkit). Briefly, filtered reads were mapped to the *K. pneumoniae* KPNIH1 ST258 genome (NZ_CP008827.1) using a Burrows-Wheeler aligner v0.7.17[57]. Polymerase chain reaction duplicates were removed using PicARD v2.27.4[58]. Single-nucleotide polymorphisms (SNPs) and small insertions and deletions (INDELs) were called using SAMtools v1.2 and bcftools v1.2[57,59,60].Variants were filtered using the Genome Analysis Toolkit v4.1.4.0's VariantFiltration tool[58].

The maximum-likelihood phylogenetic tree was constructed from Gubbins-recombination-filtered SNPs using a custom, in-house Snakemake pipeline (https://github.com/Snitkin-Lab-Umich/phylokit), as described elsewhere[51,61,62]. Briefly, the GTR+F+ASC+R2 substitution model was used alongside default IQ-Tree2 parameters, except that 5,000 bootstraps and 500 unsuccessful iterations were required to halt the reconstruction. To reflect the bifurcating, major clades of ST258, the phylogeny was midpoint-rooted using the *phytools* R package[29,30,63].

Insertion sequences were identified using panISA v0.1.7[64]. The impact of SNPs was predicted by sequence homology using Sorting Intolerant from Tolerant (SIFT)[65,66].

Kleborate v3.1.3 screened genome assemblies for multilocus-sequence type and antibiotic resistance determinants[67]. As over 90% of isolates belonged to the epidemic lineage ST258, our analysis focused on those isolates.

3.5.5 Characterization of Phylogenetic Emergence and Spread of Bacterial Phenotypes and Genotypes

The phylogenetics of BL/BLI resistance and bacterial genotypes were characterized using the R package *phyloAMR (https://github.com/kylegontjes/phyloAMR)*[51].

PhyloAMR's core function, *asr*, inferred episodes of trait gain, loss, and continuation across the phylogeny using *corHMM*'s joint ancestral state reconstruction algorithm[68]. Although the all rates different model (ARD) was the best-fitting model in our original analysis of California-based isolates[51] and this extended dataset, the equal rates (ER) model was leveraged based on its alignment with the genotypic data. In this context, the ER model better captures the observed distribution and transitions of putative resistance-associated genotypes, making it a more biologically appropriate choice for addressing the aims of this study.

The evolutionary history of a trait-containing isolate was inferred using *phyloAMR's asr_cluster_detection* function. The phylogeny was traversed from tip to root to infer whether an isolate with the trait was a phylogenetic singleton (i.e., evidence of *de novo* evolution of the trait) or a member of a phylogenetic cluster (i.e., evidence that the trait was inherited from a circulating trait-containing lineage). Next, the synchronous transitions of BL/BLI resistance and genotypes were inferred using *phyloAMR's synchronous_detection* function. Nodes with transitions in trait status were compared to identify instances where synchronous transitions of both traits were detected.

3.5.6 Testing for Different Rates of Resistance Evolution Across ST258 Clades

To characterize the evolution of BL/BLI resistance across ST258 clades, we leveraged *phytools*' function, *fitmultiMK* function. This function implements a modified Markov model that permits heterogeneous rates across user-specified regions of the phylogeny[63,69]. To test the hypothesis that the evolution of BL/BLI resistance was different across clades, an ER model where rate heterogeneity was permitted on each clade was compared to an ER model with uniform rates. The fit of the single- and two-regime models was compared using the likelihood ratio test.

3.5.7 Evaluating the Contribution of Mutations in Chromosomal Carbapenem Resistance-Associated Genes to BL/BLI Resistance

The fraction of BL/BLI resistance explained by mutations in chromosomal carbapenem resistance-associated genes was defined as the number of resistant isolates with the genotype divided by the total number of resistant isolates. The following genotypes were considered: *ompK35* and *ompK36* porin mutations, efflux pump upregulation via mutation in regulatory genes (*ramA* and *ramR*), and modification of penicillin-binding proteins[32-34]. As the impact of

resistance-associated genotypes can differ across genetic backgrounds[52], we only considered genotypes with a median $\log_2$ fold change >1 using our nearest-neighbor comparison algorithm, as described below.

3.5.8 Nearest-Neighbor Comparisons to Characterize a Genotype's Influence on Resistance

The influence of genotypes on BL/BLI resistance was characterized using *phyloAMR's nearest_neighbor_algorithm* function. For each genotype-containing isolate, the nearest phylogenetic neighbor without the genotype was identified using pairwise phylogenetic distance, as generated by ape's *cophenetic.phylo* function[70]. When an isolate had more than one nearest neighbor with the same pairwise phylogenetic distance metric, the isolate with the lowest pairwise SNP distance was determined using ape's *dist.dna* function[70]. The MIC and BL/BLI resistance status of these pairs was compared. Summary statistics were generated to determine the magnitude and consistency of MIC changes and transitions in resistance status.

Nearest-neighbor comparisons were performed to minimize potential false-positive genotypes generated by GWAS and curate non-carbapenemase mechanisms of carbapenem resistance for inclusion in an exploratory panel of BL/BLI resistance-associated genotypes.

3.5.9 Identification of Klebsiella pneumoniae Carbapenemase-Containing Plasmids

MOB-suite v.3.1.9 reconstructed plasmid content from our short-read genome assemblies[71]. The *Klebsiella pneumoniae* carbapenemase, bla_{KPC}, was identified using blastn v.2.15.0[72] and a database of bla_{KPC} alleles found in the Comprehensive Antibiotic Resistance Database v.4.0.0[73]. The bla_{KPC}-containing contigs were mapped to their inferred location (i.e., chromosome or plasmid) using MOB-recon's contig reports.

3.5.10 Genome-Wide Association Studies to Identify Genomic Features Driving Resistance

An unbiased convergence-based genome-wide association study on $\log_2$ MIC and BL/BLI resistance was performed using hogwash v.1.2.6[74]. Three genotype matrices were analyzed: (1) mutations in the *ompk36* porin and the presence of β-lactamase genes inferred from Kleborate, (2) bla_{KPC}-containing plasmid clusters inferred from Mob-suite, and (3) grouped-by-gene core genome variants.

As most variants were rare (4309 variants [75.2%] had a frequency < 1%), grouped-by-gene core genome matrices were curated (**Supplementary Figure 13**). The presence of at least one variant in a genetic region (e.g., coding or non-coding region) of the reference genome was

used. The following grouped-by-gene schemas were used: (1) all core genome variants identified, (2) non-synonymous coding variants (i.e., non-synonymous SNPs, coding INDELs, and coding IS), and (3) putative function-altering variants (deleterious SNPs predicted by SIFT, stop/start-modifying SNPs, frameshift INDELs, disruptive INDELs, and coding IS). A complementary analysis was performed by removing IS from our matrices. Only genotypes with a median $\log_2$ MIC fold change > |1| were retained for downstream analyses.

3.5.11 Long-Read Sequencing to Evaluate Plasmid Sharing in Clinical Clade I Isolates

Representative clinical clade I isolates underwent long-read sequencing by Plasmidsaurus using Oxford Nanopore Technology to evaluate differences between isolates with *bla*$_{KPC}$-containing AA552 plasmids and phylogenetically related isolates with other *bla*$_{KPC}$-containing plasmids. Hybrid assemblies were generated using an in-house assembly pipeline (https://github.com/Snitkin-Lab-Umich/Nanosake). Briefly, filtlong v0.2.1 cleaned Nanopore reads were assembled using Flye v.2.9.5[75,76]. The assembly is polished with long reads using Medaka v1.2.0, followed by polishing with clean, trimmed Illumina short-read sequences using Polypolish v.0.6.0[77].

Whole-genome alignments of representative genomes were generated using Mummer4[78]. Dot plots graphically represented this alignment, with Blastn v2.15 used to infer the location of the *tn4401* transposon[72].

3.5.12 Copy Number Estimation of the Klebsiella pneumoniae Carbapenemase Gene Using Coverage Depth

The copy number of *bla*$_{KPC}$ was inferred using coverage statistics via an in-house Snakemake pipeline (https://github.com/kylegontjes/CoverageStats). Filtered reads were mapped to the KPNIH1 chromosome (NZ_CP008827.1) and *bla*$_{KPC}$-containing plasmid (NZ_CP008830.1) using the Burrows-Wheeler short-read aligner v0.7.17[57]. Polymerase chain reaction duplicates were removed from sorted BAM files using SAMtools v1.19 and PicARD v.3.4.0[58-60]. The Genome Analysis Toolkit's DepthOfCoverage v4.6.2.0 calculated coverage depth[58]. The median non-zero coverage depth was calculated for the *bla*$_{KPC-3}$ gene (KPNIH1_RS28775) and normalized to the KPNIH1 chromosome's median non-zero coverage depth. The copy number of resistance plasmids was inferred by performing read mapping to the isolate's MOB-Suite-identified mash nearest neighbor.

Regression modeling was performed to evaluate the association between *bla*$_{KPC}$ copy number and BL/BLI resistance. First, simple linear regression models were constructed between $\log_2$-normalized *bla*$_{KPC}$ copy number and $\log_2$ MIC for MVB and IR. Next, multiple linear regression models were constructed to account for the contribution of mutations in *ompK36* to resistance. Mutations in *ompK36* were constructed as a categorical variable indicating the presence of putative-function-altering variants, loop 3 insertions, or neither of these mutational classes in an isolate. Finally, interaction modeling was undertaken to evaluate the relationship between *bla*$_{KPC}$ copy number and mutations in *ompK36.*

3.5.13 Experimental Evolution of Meropenem-Vaborbactam Resistance

To test the hypothesis that genetic background influenced the accessibility and genomic pathways to BL/BLI resistance, we performed experimental evolution to select for resistance to MVB.

Representative susceptible clinical isolates were chosen to test this hypothesis. Eight clade I isolates were obtained, with representative isolates chosen based on combinations of loop 3 insertions in OmpK36 and the presence of the AA018 and AA552 resistance-associated plasmids. One chosen clade I isolate harbored a loop 3 insertion in OmpK36 but lacked a carbapenemase. Four representative clade II isolates lacking mutations in *ompK36* were selected, with one obtained from each subclade. Isolate characteristics are described in **Figure 5**.

Normalized cultures of representative isolates grown overnight in Luria-Bertani (LB) broth were plated onto standard and MVB-impregnated Mueller-Hinton agar plates. MVB-impregnated plates were made using 8 μg / mL of meropenem and 4 μg / mL of relebactam. Under these conditions, parental isolates were inhibited and evolved isolates were optimally recovered, despite the reduced inhibitor concentration. The frequency of resistance was calculated by dividing the normalized colony count on MVB plates by the normalized colony count on MHA, representing the input.

Whole-genome sequencing was performed to identify genetic pathways to resistance. One representative colony was subjected to short- and long-read sequencing using Illumina and Nanopore technology by Plasmidsaurus. To generate hybrid assemblies for parental and evolved strains, parental susceptible isolates were also subjected to long-read sequencing using Nanopore technology by Plasmidsaurus. Hybrid assemblies were generated using Nanosake. Variant calling and IS element detection were performed by SNPKIT and panISA, respectively. MOB-

Suite was performed to identify changes to plasmid content. The location of *bla*$_{KPC}$ and the presence of loop 3 insertions in OmpK36 were detected using AMRFinderPlus v.4.0.23. Finally, changes in the copy number of *bla*$_{KPC}$ and the *bla*$_{KPC}$-harboring plasmids were inferred using CoverageStats.

3.5.14 Regression Modeling to Identify Patient Features Associated With the Emergence and Spread of Resistance

Logistic regression was employed to identify patient features associated with resistance to BL/BLI combinations and two genotype-informed resistance classifications: resistant isolates with non-carbapenemase mechanisms of resistance and resistant isolates with *bla*$_{KPC}$-containing AA552 plasmids. For patients with multiple isolates, their first isolate was selected unless they contributed a resistant strain, in which case the first resistant isolate was chosen. For all three outcomes, susceptible isolates served as the reference population. Patient demographics, clinical comorbidities, and antibiotic exposures were eligible for regression modeling.

The purposeful selection algorithm, with limited alterations, was employed to perform a data-driven multivariable logistic regression[79]. First, univariable logistic regression was performed on all variables. Variables with an unadjusted p-value < 0.20 were retained as candidate variables. Next, all candidate variables were included in a logistic regression model. An iterative process was used to remove variables with p-values > 0.10. After compiling this preliminary model, all initially ineligible variables were iteratively included in the pre-specified model and retained if their value was < 0.10.

3.5.15 Data Analysis and Visualization

Analyses were performed, unless otherwise stated, using R version 4.5.0[80]. The *tidyverse* v2.0.0, *ggplot2* v3.5.2, and *ggnewscale* v0.5.1 packages were used to manipulate data, generate figures, and create scales[81]. Phylogenetic analysis and visualization were performed using *ape* v5.8-1, *phytools* v2.4-4, *ggtree* v3.16.0, *corHMM* v2.10, and *phyloAMR* v0.1.0[63,68,70,82]. Heatmaps were generated using *ComplexHeatmap* v2.24.0[83]. Descriptive statistics were generated using the *tableone* v0.13.2 package[84]. Multi-panel figures were generated using *cowplot* v1.1.3[85]. Code for this project can be found at https://github.com/kylegontjes/pre-intro-blbli-resistance-ms.

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Chapter 4 Changing Molecular Epidemiology and Resistance Patterns of Carbapenem-Resistant *Klebsiella pneumoniae* in California Long-Term Acute Care Hospitals

4.1 Preamble

This chapter compares the molecular epidemiology and resistance patterns in two period prevalence studies collected across the healthcare network and separated by nearly a decade. In this chapter, I performed all data analyses and generated all data tables and figures. Other co-authors performed isolate collection, clinical metadata extraction, antibiotic susceptibility testing, and contributed their expertise to the formulation of this work. This work has been submitted to a peer-reviewed journal.

4.2 Introduction

The emergence and worldwide dissemination of multidrug-resistant organisms, such as carbapenem-resistant *Klebsiella pneumoniae* (CRKP), is a significant public health threat[1]. Infections caused by CRKP, the most prevalent carbapenem-resistant Enterobacterales (CRE) in the United States, represent a major challenge due to their clinical complexity and high mortality rates[2]. These challenges are heightened in high-acuity populations with extensive healthcare exposure and frequent antibiotic utilization, such as residents of long-term acute care hospitals (LTACHs)[3,4].

The ability to treat infections caused by CRE has been enhanced by the introduction of several antibiotics, including novel β-lactam/β-lactamase inhibitor (BL/BLI) combinations and cefiderocol[5,6]. The availability of such agents has been a major contributor to global changes in antibiotic consumption patterns and the treatment of CRE infections over the past decade[7,8].

Genomic investigations have shown that the diversity of a region's circulating CRE can change due to strain importation, clonal expansion, and selective pressures[9-11]. Prior research has indicated that genetic backgrounds can have differential propensities for resistance to emerge and spread, suggesting that certain lineages may be more primed to respond to shifting antibiotic formularies[12-15]. To preserve the long-term efficacy of promising novel antibiotics, investigations

into the prevalence and dynamics of resistance, especially in high-risk populations, are of utmost importance.

Here, we compare two period prevalence studies, both of which performed comprehensive clinical isolate collection across a large LTACH network in California, separated by nearly a decade and the availability of several novel antibiotics. Whole-genome sequencing and antibiotic susceptibility testing enabled the assessment of how the molecular epidemiology and resistance patterns of CRKP changed in a large geographic catchment during a period of shifts in antibiotic consumption.

4.3 Methods

4.3.1 Study Population

We performed two period prevalence studies. As part of a prior study, clinical CRKP isolates were obtained from August 1, 2014, to July 25, 2015 (period 1), at 12 southern California LTACHs[16,17]. All patients with a clinical CRKP culture were included. Patients could contribute multiple isolates, but only the first per calendar quarter was retained for analysis. We subsequently performed a comprehensive clinical isolate collection from the same LTACH network from July 7, 2021, to December 30, 2023 (period 2) using the same specifications. In period 2, another LTACH was added to the network.

4.3.2 Microbiological Methods

In both periods, all samples identified as CRKP were shipped from the regional microbiology laboratory to the study laboratory at the University of Pennsylvania. Confirmatory identification was conducted using VITEK-MS (bioMérieux, Durham, NC). Antibiotic susceptibility testing was performed using a custom broth microdilution panel (SENSITITRE, Thermo Scientific, Waltham, MA)[12]. The following agents were tested: meropenem, imipenem, carbapenemase-inhibiting BL/BLI antibiotics (ceftazidime-avibactam, imipenem-relebactam, and meropenem-vaborbactam), a cephalosporinase-inhibiting BL/BLI antibiotic (ceftazolone-tazobactam), recently approved tetracyclines (eravacycline and omadacycline), delafloxacin, cefiderocol, fosfomycin, and colistin. Antibiotic resistance was defined as a minimum inhibitory concentration in intermediate or resistant categories, according to the 2025 Clinical and Laboratory Standards Institute criteria (35th edition, M100). CRKP were defined as laboratory-confirmed *Klebsiella pneumoniae* demonstrating resistance to ertapenem, imipenem, and/or

meropenem. Resistance to carbapenemase-inhibiting BL/BLI agents was defined as resistance to ceftazidime-avibactam, imipenem-relebactam, and/or meropenem-vaborbactam.

4.3.3 Whole-Genome Sequencing

Isolates underwent whole-genome sequencing on an Illumina Novaseq X instrument at the University of Michigan's Advanced Genomics Core. Raw sequencing data underwent quality control, genome assembly, and taxonomical classification using an in-house pipeline (https://github.com/Snitkin-Lab-Umich/QCD)[18-20].

Species and sequence type (ST) were identified using SKANI v.0.2.1 and Kleborate v.3.1.3, respectively[21,22]. Antibiotic resistance genes and mutations in major porins, *ompK35* and *ompK36*, were detected using AMRFinderPlus v.4.0.23[23], while truncations were assessed using Kleborate. Carbapenemase-containing plasmids were reconstructed from short-read assemblies using MOB-Suite v.3.1.9[24].

Phylogenetic reconstruction was performed on the 2021-23 collection. Cognac identified core genes, defined as genes present in at least 95% genomes[25]. IQ-Tree2 reconstructed a maximum-likelihood phylogeny using extracted single-nucleotide polymorphisms (SNPs)[26-28]. Default parameters were used, except that 5000 bootstraps were performed, and 200 unsuccessful iterations were required to halt the reconstruction. The phylogeny was rooted using the non-ST258 isolate with the largest median SNP distance from our 2021-23 ST258 isolates.

Phylogeographic analyses contextualized the regional epidemiology of each of the following abundant epidemic lineages: ST14, ST17, ST258, and ST307. *K. pneumoniae* genomes were downloaded from the NCBI Pathogen database on December 10, 2025. Genome quality was evaluated using an in-house pipeline (https://github.com/Snitkin-Lab-Umich/pubQCD). QC-passing genomes with spatiotemporal data were used for phylogenetic reconstruction alongside our study isolates. To characterize the number and magnitude of regional importations, phylogenetic clustering of California isolates was performed using *phyloAMR's asr_cluster_detection* and *asr_cluster_analysis* functions[14]. Oversampling was mitigated by retaining representative isolates with distinct collection-decade and continent combinations in subtrees that did not contain California isolates.

4.3.4 Clinical Metadata Collection

Clinical metadata were extracted from the healthcare network's electronic medical records, as described previously[16,17]. The approach to clinical data ascertainment was identical across study periods.

4.3.5 Data Analysis and Visualization

First, we compared the demographics and length of stay for subjects from period 1 versus period 2. Next, we compared the distribution of STs, carbapenemases, and carbapenemase-containing plasmids across study periods. Finally, we compared the proportion of susceptible isolates for the tested antibiotics across study periods. In these comparisons, continuous variables were compared using the Student's *t*-test or the Wilcoxon rank-sum test. Categorical variables were compared using the χ^2 or Fisher's exact test.

All analyses, unless otherwise noted, were performed using R version 4.5.2 (R Core Team, 2025). The *tidyverse* packages and *ggnewscale* v.0.5.1.9 were used to manipulate and visualize data[29,30]. Descriptive statistics were calculated using *tableone* v.0.13.2[31]. Heatmaps were curated using *ComplexHeatmap* v. 2.24.1[32]. Alluvial plots were created using *ggalluvial* v.0.12.5[33]. Phylogenetic visualizations were performed using *ape* v.5.8-1, *phytools* v.2.5-2, and *ggtree* v.3.17.0[34-36]. *Cowplot* v.1.2.0 generated multi-panel figures[37]. Manuscript code can be found at https://github.com/kylegontjes/INTEGRATE-resistance-ms.

4.4 Results

4.4.1 Surveillance of Carbapenem-Resistant Klebsiella pneumoniae in California Long-Term Acute Care Hospitals

As part of a prior study, 374 clinical CRKP isolates were obtained from 329 patients from August 1, 2014, to July 24, 2015 (period 1), across 12 southern California LTACHs [17]. We subsequently performed a new collection of clinical CRKP isolates in the same LTACH network from July 7, 2021, to December 30, 2023 (period 2) using the same study methodology as in period 1**.** During the second collection, 394 patients contributed 507 CRKP isolates.

Baseline characteristics of patients were not substantially different across study periods. The proportion of male patients was similar across study periods (period 1, 51.7%; period 2, 52.0%; X^2 test p-value = 0.99). Although not collected in period 1, 140 (36.9%) patients were non-Hispanic White in period 2. The median age at first isolate collection was 73.0 years (interquartile range [IQR], 18.0) in period 1 and 69.0 years (IQR, 16.0) in period 2, a statistically

significant difference (Wilcoxon rank-sum test p-value < 0.001). Patients had shorter lengths of stay before their initial specimen collection in period 1 (median [IQR], 20.0 days [38.0]) than in period 2 (median [IQR], 32.0 days [60.0]; Wilcoxon rank-sum test p < 0.001).

The anatomical distribution of isolates differed between study periods (Fisher's exact test p-value < 0.001). The relative contribution of wound (6 [1.6%] vs. 77 [15.2%]) and blood (30 [8.0%] vs. 65 [12.8%]) isolates increased in study period 2, whereas the contribution of respiratory (217 [58.0%] vs. 242 [47.7%]) and urine (121 [32.4%] vs. 113 [22.3%]) isolates decreased. Ten additional isolates in period 2 were isolated from other anatomical sites. Finally, the distribution of isolates across the 12 facilities in both study periods did not differ statistically (X^2 test p-value > 0.05).

4.4.2 Increasing Diversity of Carbapenem-Resistant Klebsiella pneumoniae

Multi-locus sequence typing was performed to characterize the population structure of CRKP across study periods. Study period 1 had low ST diversity, with only ten STs detected (**Figure 4.1A**). ST258 (352 [94.1%]) dominated, with only four other non-singleton STs being identified: ST15 (11 [2.9%]), single-locus variants of ST15 (2 [0.5%]), ST17 (2 [0.5%]), and ST36 (2 [0.5%]). Conversely, a much more diverse population was identified in the 2021-23 study. Although 36 STs were identified, twenty-three were found in only one isolate. Most isolates belonged to epidemic lineages, notably ST258 (211 [41.6%]), ST14 (139 [27.4%]), ST307 (35 [6.9%]), ST17 (33 [6.5%]), ST35 (13 [2.6%]), ST25 (13 [2.6%]), and ST11 (9 [1.8%]).

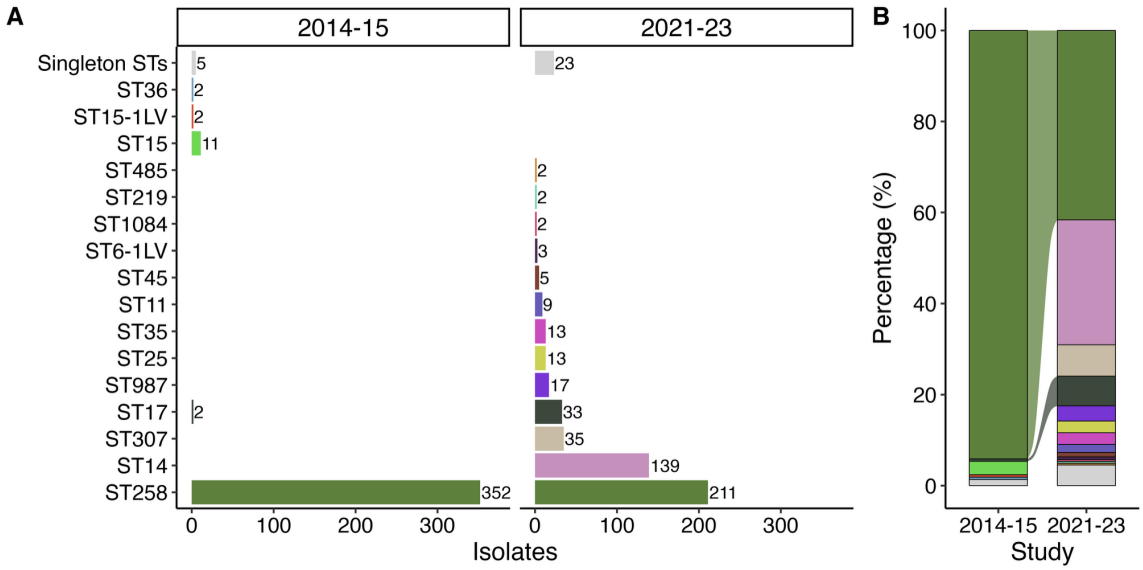


Figure 4.1 Increased Diversity of Carbapenem-Resistant *Klebsiella pneumoniae* in California Long-Term Acute Care Hospitals

(A) The frequency of *Klebsiella pneumoniae* sequence types (STs) detected across study periods. (B) Comparison of the distribution of sequence types across study periods. Sequence types that occurred only once were grouped as "Singleton STs" to aid visualization. Isolates with one allelic difference from a known sequence type were denoted with "-1LV."

Only two STs were identified across both study periods (**Figure 4.1B**). While ST258 remained the dominant lineage during the 2021-23 study period, its prevalence decreased markedly (352 [94.1%] vs. 211 [41.6%]; X^2 test, p-value < 0.001). The prevalence of epidemic lineage ST17 increased significantly (2 [0.5%] vs. 33 [6.5%]; Fisher's exact test, p-value < 0.001). Phylogeographic analysis of epidemic lineages ST14, ST17, ST258, and ST307 uncovered numerous independent introductions into the region of varying magnitude, alongside the clonal expansion of several pre-existing ST258 and ST17 lineages from the 2014-15 study period (**Appendix C Figures 1-2).**

4.4.3 Diversification of Carbapenem Resistance-Associated Genotypes

As the fitness cost of resistance genotypes can vary across genetic backgrounds [38], we hypothesized that changes in ST diversity would alter the prevalence of carbapenem resistance-associated genotypes.

Differences in the prevalence of carbapenemase families were detected across study periods (**Figure 4.2A-B).** Although the *Klebsiella pneumoniae* carbapenemase (bla_{KPC}) remained the dominant carbapenemase in study period 2, its prevalence decreased (364 [97.3%] vs. 425 [83.8%]; X^2 test p-value < 0.001). Congruent with this decrease was the detection of additional carbapenemase families at low frequencies: bla_{NDM} (11 [2.2%]) and bla_{OXA} (2 [0.4%]). The frequency of isolates without carbapenemases also increased in study period 2 (10 [2.7%] vs. 70 [13.8%]; X^2 p-value <0.001). Changes in the distribution of bla_{KPC} alleles were also detected. Specifically, the prevalence of bla_{KPC-3} (247 [66.0%] vs. 386 [76.1%]) increased in study period 2, alongside a marked decrease in the prevalence of bla_{KPC-2} (114 [30.5%] vs. 32 [6.3%]).

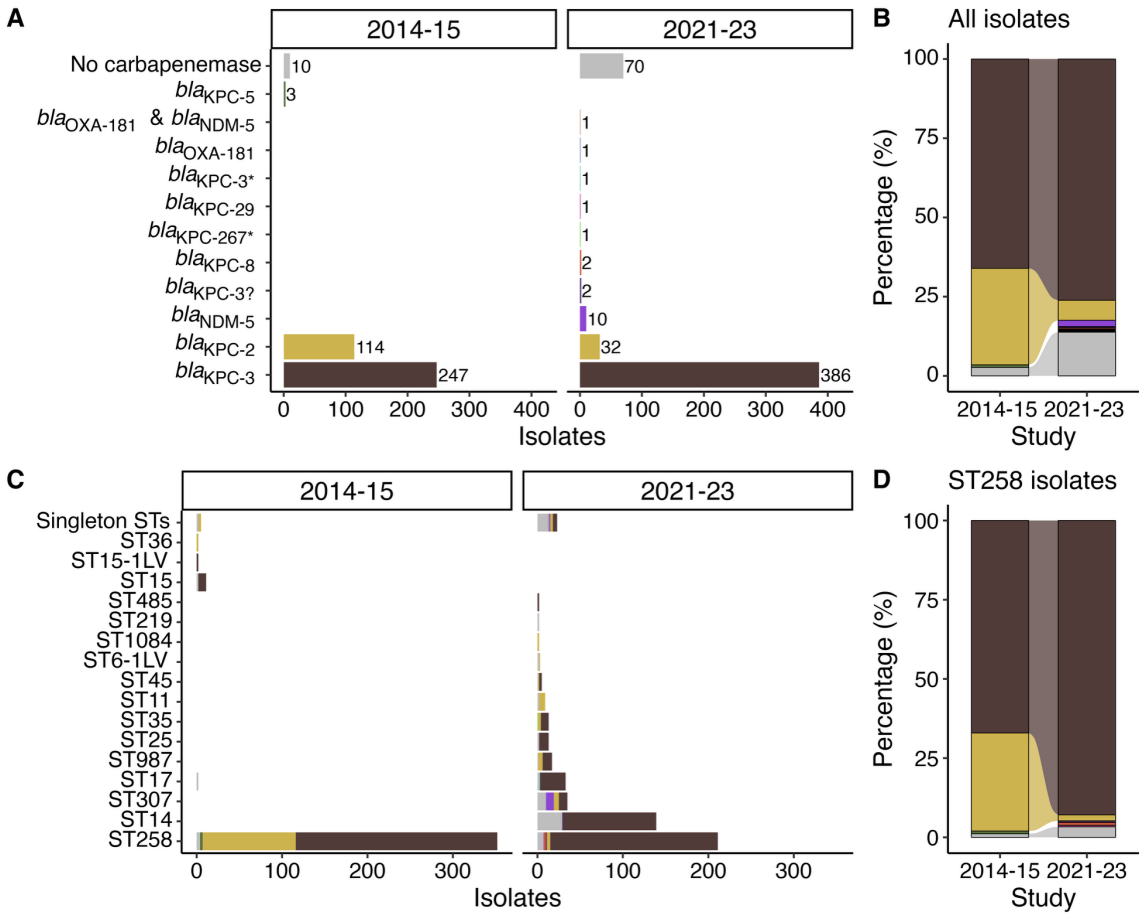


Figure 4.2 Change in Diversity of Carbapenemases Across Study Periods

(A) Carbapenemase profiles among carbapenem-resistant *Klebsiella pneumoniae* isolates across study periods. One isolate contained two carbapenemases. Genes with identity to the reference < 100% were denoted with "*", whereas genes with coverage < 100% were denoted with "?". (B) The distribution of carbapenemase genes across sequence types, stratified by study period. (C) Distribution of carbapenemase genes across sequence types (STs), stratified by study period. STs that occurred only once were grouped as "Singleton STs" to aid visualization. Isolates with one allelic difference from a known sequence type were denoted with "-1LV." (D) Distribution of carbapenemase genes among CRKP ST258 isolates, stratified by study period.

The increase in non-carbapenemase-producing isolates led us to investigate the prevalence of alternative mechanisms of carbapenem resistance, specifically mutations to major porins and the presence of other β-lactamases (**Appendix C Figure 3)**. Although mutations in the major porins, *ompK35* and *ompK36*, were common across study periods, their frequency decreased in study period 2: *ompK35* (354 [94.7%] vs. 393 [77.5%]; X^2 p-value = <0.001) and *ompK36* (156 [41.7%] vs. 110 [21.7%]); X^2 p-value < 0.001). Porin mutations were common in isolates that did not harbor carbapenemases: *ompK35* (period 1, 5/10 [50.0%]; period 2, 47/70 [67.1%]) and *ompK36* (period 1, 4/10 [40.0%]; period 2, 23/70 [32.9%]). Virtually all non-

carbapenemase-containing isolates had other β-lactamases (period 1, 10/10 [100%]; period 2, 68/70 [97.1%]). Most had at least one β-lactamase and a porin mutation (period 1, 7/10 [70.0%]; period 2, 58 [82.9%]). Across both study periods, the co-occurrence of porin mutations and β-lactamases often clustered by ST (**Figure 4.2C & Appendix C Figures 3-5).**

Given the abundance of epidemic lineage ST258 isolates in both study periods, we next compared the diversity of carbapenem resistance-associated genotypes (**Figure 4.2D**). Supporting the existence of both major circulating ST258 clades in the 2014-15 study period[14,17,39], *bla*$_{KPC-2}$-containing (109 [31.0%]) and *bla*$_{KPC-3}$-containing (236 [67.0%]) isolates were identified. Conversely, virtually all 2021-23 ST258 genomes harbored *bla*$_{KPC-3}$ (196 [92.9%]), although a more diverse repertoire of minor *bla*$_{KPC}$ alleles was detected. A statistically significant reduction in the presence of *ompK36* mutations was also observed among 2021-23 ST258 genomes (153 [43.5%] vs. 34 [16.1%]; X^2 test p-value <0.001). Inspection of the mutational profiles revealed the loss of an abundant *bla*$_{KPC-2}$-containing lineage with a Glycine-Aspartate (GD) insertion in the loop 3 of the OmpK36 porin (99 [28.1%] vs. 4 [1.9%]; Fisher's exact test p-value <0.001), coinciding with the near-total absence of major clade I of ST258 in the 2021-23 collection (**Appendix C Figures 1, 4-5)**.

4.4.4 Dynamics of Carbapenemase-Containing Plasmids Reflect Changes to Population Structure

Next, we investigated whether changes in ST diversity reflected changes to the prevalence of circulating carbapenemase-containing plasmids. Among the 801 carbapenemase-containing isolates (period 1, 364; period 2, 437), 23 carbapenemase-containing plasmid clusters were identified. MOB-Suite plasmid clusters are characterized in **Appendix C Figure 6**.

Although ten plasmids were identified in both study periods, their distribution was statistically different (Fisher's exact test p-value < 0.001) (**Figure 4.3A-B**). Several plasmids increased in prevalence in study period 2: AA356 (38 [10.4%] vs. 94 [21.5%]) and AA038 (3 [0.8] vs. 30 [6.9%]). Conversely, the prevalence of several resistance plasmids decreased in study period 2, notably AA018 (95 [26.1%] vs. 3 [0.7%]), AA552 (23 [6.3%] vs. 9 [2.1%]), AA357 (43 [11.8%] vs. 3 [0.7%]), and AA275 (21 [5.8%] vs. 10 [2.3%]). The prevalence of chromosomally encoded carbapenemase-producing isolates increased in study period 2 (16 [4.4%] vs. 130 [29.7%]).

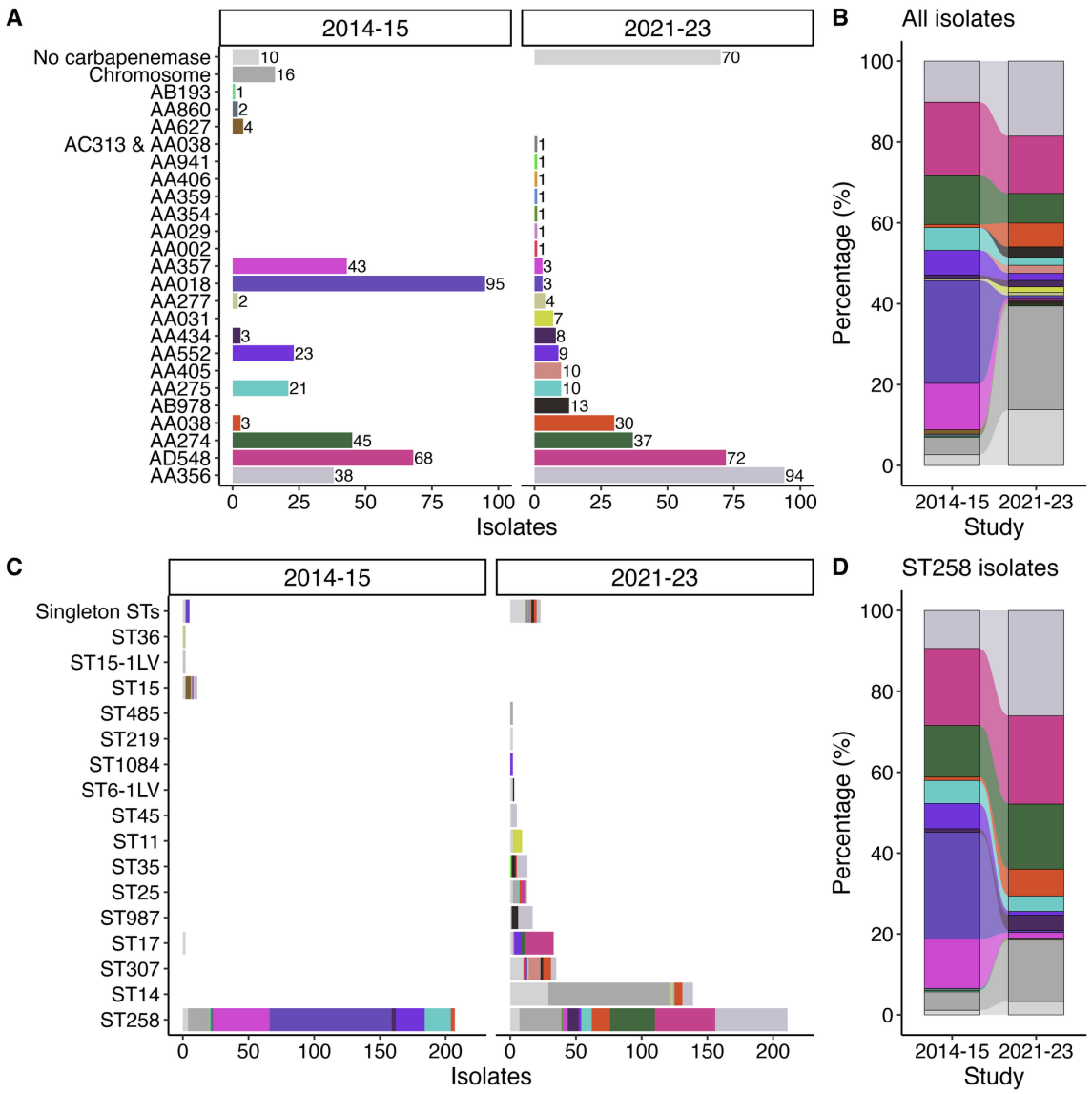


Figure 4.3 Change in Abundance and Diversity of Carbapenemase-Containing Plasmids in California

(A) The carbapenemase-containing plasmid profiles of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolates across study periods. One isolate contained two carbapenemase plasmids. (B) The distribution of carbapenemase-containing plasmids across the study period. (C) Distribution of carbapenemase-containing plasmids across sequence types (STs), stratified by study period. STs that occurred only once were grouped as "Singleton STs" to aid visualization. Isolates with one allelic difference from a known sequence type were denoted with "-1LV." (D) Distribution of carbapenemase-containing plasmids among CRKP ST258 isolates, stratified by study period.

Changes in plasmid dynamics were tightly linked to changes in population structure across study periods (**Appendix C Figure 7**). First, the increased prevalence of chromosomal carbapenemases was driven by select sequence types, notably ST14 (**Figure 4.3C**). Second, the ST258 carbapenemase-producing population (period 1, 348; period 2, 204) experienced

considerable changes to the diversity of its circulating plasmids. Although 9/12 (75%) plasmids were identified in both periods, their distribution was different (**Figure 4.3D**). Finally, considerable carbapenemase-containing plasmid sharing occurred across STs, with several abundant ST258 *bla*$_{KPC-3}$-associated plasmids, particularly AA356 and AD548, being detected in minor STs.

4.4.5 Differences in Resistance Patterns Exist Across Study Periods

To evaluate whether the introduction of new CRKP-targeting antibiotics and concurrent changes in antibiotic prescribing reflected a changing epidemiology of antibiotic resistance, we measured resistance to several key antibiotics by broth microdilution.

In the 2021-23 study, virtually all isolates were resistant to the recently approved fluoroquinolone, delafloxacin (481/507 [94.9%]). Resistance to the novel cephalosporin-inhibiting BL/BLI antibiotic, ceftazolane-tazobactam, was almost uniformly detected (486/507 [95.9%]). Resistance to recently approved tetracycline antibiotics was detected: eravacycline (112/507 [22.1%]) and omadacycline (100/507 [19.7%]). Resistance to cefiderocol was less common (29/505 [5.7%]). Distributions and correlations among minimum inhibitory concentrations are presented in **Appendix C Figures 8** and **9**, respectively. Cross-resistance among antibiotics is reported in **Appendix C Figure 10.**

Statistically significant differences were detected between study periods (**Figure 4.4A**). Although no statistical differences in resistance to carbapenemase-inhibiting BL/BLI agents were detected across study periods (50/374 [13.4%] vs. 68/507 [13.4%]; X^2 p-value = 1.0), differences were observed for specific agents. Resistance to meropenem-vaborbactam was lower in study period 2 (35/374 [9.4%] vs. 10/507 [2.0%]; X^2 p-value <0.001), while resistance to ceftazidime-avibactam increased (4/374 [1.1%] vs. 46/507 [9.1%], Fisher's exact test p-value <0.001). Although resistance to plazomicin was rare in the 2014-15 study, a moderate increase in resistance was detected in the contemporary collection (2/374 [0.5%] vs. 29/507 [5.7%]; Fisher's exact test p-value <0.001). Finally, decreases in resistance to colistin (131/374 [35.0%] vs. 102/506 [20.2%]; X^2 p-value < 0.001) and fosfomycin (101/374 [27.0%] to 68/507 [13.4%]; X^2 p-value <0.001) were also detected in study period 2.

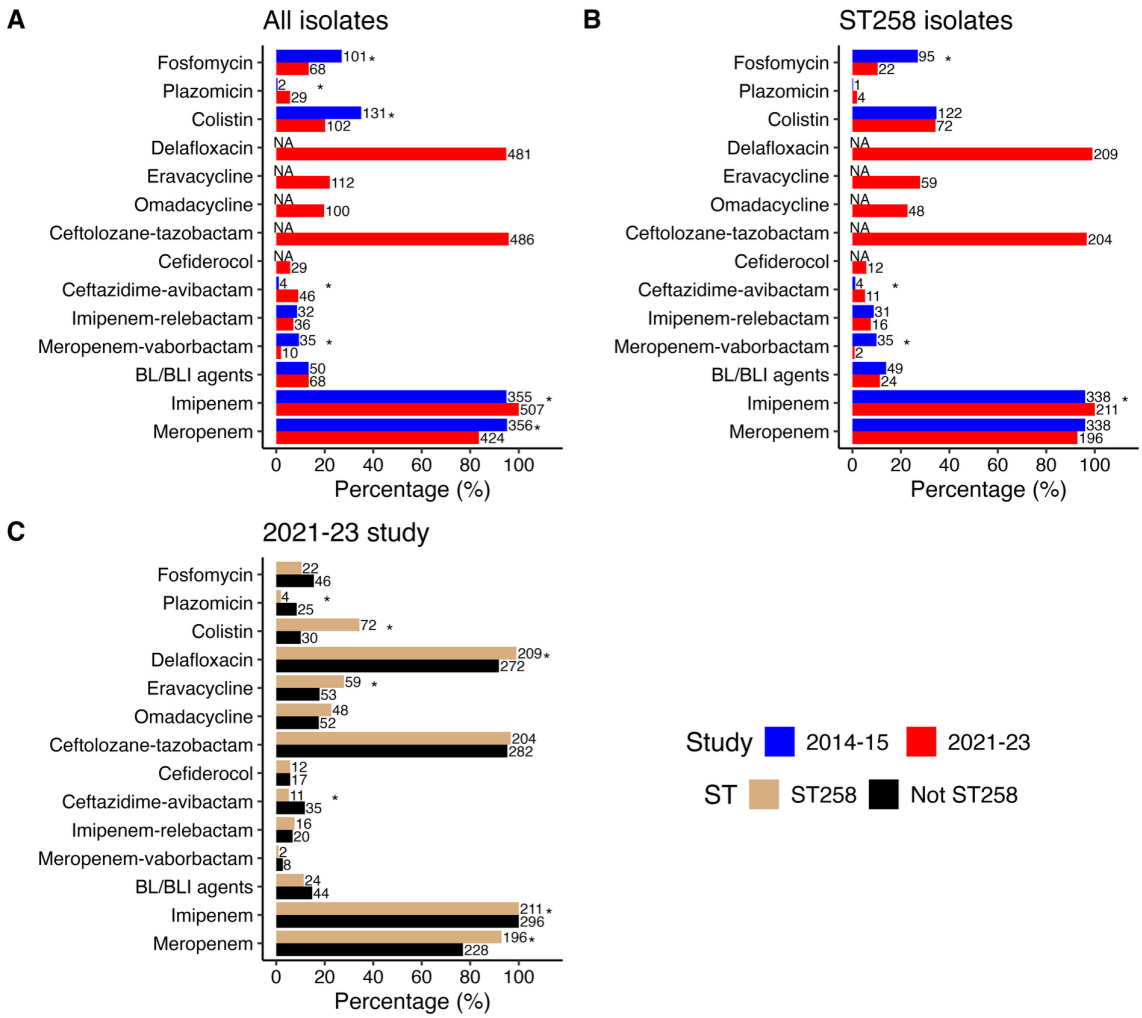


Figure 4.4 Differences in Resistance to Last-Resort and Recently Approved Antibiotics Across Study Periods

(A) The percentage of isolates resistant to tested antibiotics across both study periods. (B) The percentage of *Klebsiella pneumoniae* sequence type 258 (ST258) isolates resistant to tested antibiotics across both study periods. (C) The percentage of resistant isolates among *Klebsiella pneumoniae* ST258 and non-ST258 isolates in the 2021-23 study. The absolute number of isolates resistant to the antibiotic is shown in the figure. Resistance to ß-lactam/ß-lactamase inhibitor (BL/BLI) agents is a composite outcome that indicates resistance to at least one of the following antibiotics: ceftazidime-avibactam, imipenem-relebactam, or meropenem-vaborbactam. Abbreviations: *, statistically significant p-value <0.05; NA, not tested in 2014-15 study.

4.4.6 Differences in Resistance Were Driven by Subpopulations of Carbapenem-Resistant Klebsiella pneumoniae

We hypothesized that the changes in antibiotic resistance correlated with differences in ST. As ST258 dominated both study periods, we first compared the resistance landscape within this population (**Figure 4.4B**). Although no difference in resistance to carbapenemase-inhibiting BL/BLI agents was detected across study periods (49/352 [13.9%] vs. 24/211 [11.4%], X^2 p-value = 0.46), differences in specific agents were detected: meropenem-vaborbactam decreased

in study period 2 (35/352 [9.9%] vs. 2/211 [0.9%], Fisher's exact test p-value <0.001), while resistance to ceftazidime-avibactam increased (4/352 [1.1%] vs. 11/211 [5.2%], Fisher's exact test p-value = 0.006). A statistically significant decrease in fosfomycin resistance was also observed in study period 2 (95/352 [27.0%] vs. 22/211[10.4%], X^2 p-value <0.001). Contrary to the overall population-level decrease in colistin resistance across study periods, no statistically significant decrease was detected among ST258 isolates (122/352 [34.7%] vs. 72/210 [34.3%], X^2 p-value = 1.0).

These findings led us to hypothesize that the emergent, non-ST258 lineages contributed to the differences in antibiotic resistance observed between the two study periods. To test this hypothesis, we compared the frequency of resistance to several novel antibiotics between ST258 and non-ST258 genomes collected from the 2021-23 study (**Figure 4.4C**). ST258 isolates had elevated frequencies of resistance to delafloxacin (209/211 [99.1%] vs. 272/296 [91.8%]; Fisher's exact test p-value <0.001) and colistin (72/210 [34.3%] vs. 30/296 [10.1%]; X^2 test p-value <0.001) than non-ST258 genomes. Conversely, non-ST258 genomes had a higher frequency of plazomicin resistance than ST258 genomes (4/211 [1.9%] vs. 25/296 [8.4%]; Fisher's exact test p-value = 0.002).

Inspection of resistance patterns across STs in study period 2 revealed tight associations between resistance and select circulating lineages (**Appendix C Figures 11-12**). First, ST258 isolates accounted for most of those exhibiting resistance to colistin (72/102 [70.6%]) and to novel tetracyclines: eravacycline (59/112 [53.7%]) and omadacycline (48/100 [48%]). Second, ST17 accounted for a large proportion of isolates exhibiting resistance to cefiderocol (13/29 [44.8%]), fosfomycin (28/68 [41.2%]), and BL/BLI agents (18/68 [26.5%]). Third, the abundant epidemic lineage, ST14, had relatively low rates of resistance to the tested antibiotics. Finally, numerous minor STs had elevated resistance to select antibiotics, highlighting the potential for resistance to emerge across diverse genetic backgrounds. Phylogenetic investigation confirmed that resistance profiles were largely shaped by STs displaying differential propensities of resistance emergence and spread (**Appendix C Figure 13)**.

4.5 Discussion

Amidst the increasing burden of antibiotic resistance, it is critical to understand the evolving epidemiology of multidrug-resistant organisms. Our comparative analyses revealed an increased diversity of CRKP, driven by numerous regional introductions and clonal expansions

of epidemic lineages. These changes coincided with different patterns of resistance genotypes and phenotypic resistance. Collectively, this work highlights the ability of repeated, comprehensive surveillance to inform regional public health initiatives by improving understanding of the shifting molecular epidemiology of multidrug-resistant organisms.

Considerable changes in the molecular epidemiology of CRKP were detected across study periods. Previously dominated by epidemic lineage ST258, ST diversity increased in the 2021-23 study period. These changes reflected the introduction of other epidemic multidrug-resistant nosocomial lineages (i.e., ST14 and ST307) and clonal expansion of the then less-abundant lineage ST17. Similar to our findings, a recent study reports a shifting landscape of CRKP diversity among publicly available genomes, with the once dominant, globally disseminated lineage ST258 being replaced by other STs[40]. We show that this trend also exists in LTACHs, a critical reservoir for CRKP and other multidrug-resistant organisms[3].

Inspection of ST258 genomes revealed considerable shifts in its population structure. Virtually all 2021-23 study isolates belonged to *bla*$_{\text{KPC-3}}$-containing clade II lineages, which maintained an elevated frequency of colistin resistance. Coinciding with these clonal expansions was the loss of a *bla*$_{\text{KPC-2}}$-harboring clade I lineage with a GD insertion in the loop 3 of the OmpK36 porin. Prior investigation in this population revealed considerable clade-specific differences in anatomical site distribution and the propensity for resistance evolution and spread[13,14,41]. Longitudinal studies have also supported the dynamic nature of ST258, with adaptive mutations, changes in accessory genome content, the introduction of novel lineages, and the stability of resistance plasmids influencing the dynamics of circulating lineages[42-44]. Future work should determine the contribution of strain replacement, clonal expansion, and resistance evolution in shaping the evolutionary trajectory of ST258 in this region. Additionally, future work should investigate how facility-wide antibiotic usage, infection prevention practices, and changes in patient population characteristics influence the epidemiology and evolution of CRKP lineages.

The shifting characteristics of CRKP represent a pivotal nexus in understanding and predicting antibiotic resistance. Despite their introduction over the past 10 years, our data demonstrate reasonable retention of susceptibility for recent novel antibiotics (i.e., BL/BLI agents, recently approved tetracyclines, and cefiderocol). This indicates that, at least to date, the introductions of these drugs have not led to appreciable emergence of resistance to these agents.

Our study also identified reductions in resistance to several older antibiotics (i.e., colistin, fosfomycin, and plazomicin). This is presumed to be due to reduced selective pressure from antibiotic stewardship programming and decreased usage of these agents following the introduction of newer agents. These hypotheses should be tested in larger studies examining the correlation between facility-level antibiotic usage and resistance patterns.

Inspection of resistance patterns across STs revealed the potential for distinct lineages to drive the emergence and spread of resistance. Early recognition of epidemic STs with potential for cross-transmission could aid in forecasting regional- or facility-level trends and directing appropriate interventions. Integration of intensive, regional molecular surveillance with commonly used antibiotic stewardship strategies, such as regional antibiograms[45], will enable clinicians and public health practitioners to design and implement effective, tailored interventions to slow the development of resistance.

Our study has several limitations. First, longitudinal surveillance was not performed during 2015-2021, the period during which several promising antibiotics were introduced. Second, restriction to the patient's first clinical isolate per quarter minimized the contribution of subsequent infection episodes to our analyses. This was chosen to minimize bias from differences in culturing rates. Third, we only evaluated changes in clinical CRKP isolates. As gastrointestinal colonization often precedes infection[46], colonized patients can be an integral contributor to the regional burden of CRKP.

In conclusion, molecular surveillance across two time periods illuminated the changing epidemiology of CRKP in California LTACHs. Our regional surveillance study highlights the contribution of shifting CRKP diversity to the shaping of circulating resistance genotypes and to the emergence and spread of antibiotic resistance. Future studies should investigate the influence of changing antibiotic formularies on the evolution and epidemiology of multidrug-resistant organisms across diverse geographic and healthcare contexts.

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Chapter 5 Discussion

5.1 Overview

The clinical deployment of antibiotics revolutionized our ability to treat bacterial infections[1]. The emergence and dissemination of multidrug-resistant bacteria, such as carbapenem-resistant *Klebsiella pneumoniae* (CRKP), has complicated this achievement[2,3]. CRKP-targeting antibiotics have been introduced in recent years, including β-lactam/β-lactamase inhibitor (BL/BLI) combinations[4]. Reports of nascent resistant subpopulations exemplify the importance of studying the dynamics of last-resort antibiotics, especially in high-risk patient populations[5]. The generation of such data is integral to developing innovative strategies to slow the evolution and dissemination of bacterial lineages with resistance to last-resort antibiotics.

This dissertation investigates the genomic epidemiology of resistance to last-resort antibiotics in CRKP isolates obtained from a long-term acute care hospital (LTACH) network. Chapter 2 established a phylogenetic framework to characterize the emergence and spread of resistance, revealing varying contributions of *de novo* evolution and cross-transmission to five last-resort antibiotics among CRKP isolates belonging to sequence type 258 (ST258). Phylogenetically informed risk-factor modeling generated novel insights into how bacterial genetic background, host characteristics, and medical practices drive the emergence and spread of resistance. Chapter 3 extended this framework to study the drivers of resistance to BL/BLI combinations in epidemic lineage CRKP ST258. Investigations revealed considerable clade-specific differences in resistance evolution, cross-transmission, and genetic pathways to resistance, highlighting the role of genetic background in shaping resistance dynamics. Chapter 4 examines isolates obtained nearly a decade later to assess the changing epidemiology of CRKP in this LTACH network. Genomic analyses revealed a substantial increase in sequence type (ST) diversity, which coincided with shifts in resistance phenotypes and genotypes. Phylogenetic analysis illuminated that lineage importation, the loss of abundant pre-existing ST258 lineages, and the clonal expansion of pre-existing lineages shaped this region's molecular epidemiology and resistance patterns. Collectively, these findings demonstrate how genomic epidemiology

advances our understanding of resistance dynamics and bacterial evolution, generates testable hypotheses about resistance dynamics, and provides actionable insights for designing interventions to reduce the global burden of antibiotic resistance and infectious diseases.

5.2 Phylogenetics Improves Understanding of Antibiotic Resistance

Discerning the distribution and determinants of antibiotic resistance is critical to slowing its development and dissemination. Although traditional studies have improved our understanding of the epidemiology of resistance, the increasing availability of sequencing data provides a unique opportunity to deepen our knowledge. To leverage such data, researchers need innovative and accessible methodologies to study the evolution and epidemiology of resistance.

To this end, I developed *phyloAMR*, an open-source R package that performs phylogenetic analysis of genome-influenced traits, such as virulence and resistance. This package leverages ancestral state reconstruction to infer trait gain, loss, and persistence across a phylogeny, enabling the characterization of a trait's evolutionary history and investigation of genotype-phenotype associations[6]. Alongside the application to resistance described in this dissertation, *phyloAMR* has been used to study the epidemiology of an *ermC*-containing plasmid and describe the value of uncertainty measurements in ancestral state reconstruction[7,8].

This dissertation's phylogenetic analyses improved our understanding of the epidemiology and evolutionary dynamics of antibiotic resistance. Specifically, these analyses clarified the contributions of *de novo* evolution and cross-transmission to resistance dynamics, strengthened risk-factor modeling, and sharpened genotype-phenotype association testing.

5.2.1 Dynamics of Resistance Emergence and Spread Vary Across Antibiotics

This dissertation's phylogenetic analysis of resistance to five last-resort antibiotics in clinical CRKP isolates reshaped our understanding of how resistance emerges and spreads across a healthcare network. Ancestral state reconstruction revealed considerable differences in the evolutionary rate of resistance across tested antibiotics. Evaluation of shared evolutionary history revealed differential contributions of *de novo* evolution and cross-transmission to resistance dynamics, even among antibiotics with comparable frequencies. Enumeration of phylogenetic patterns identified substantial differences in the frequency of resistance emergence, the magnitude of cross-transmission among patients and across healthcare facilities, and the propensity for reversion to susceptibility.

Differences in evolutionary dynamics across clinically important and last-resort antibiotics underscore the growing appreciation for how sequencing can deepen our understanding of resistance emergence and spread. Epidemiological investigations have reported considerable differences in the prevalence and phylogenetic distribution of resistance across bacterial pathogens, including *K. pneumoniae*[5,9]. Experimental studies have observed nonuniformity in resistance evolution rates across antibiotics, including within the same class[10-12]. Differences in the relative contributions and fitness costs of mutational resistance and mobile genetic elements have also been documented across antibiotics[13,14]. These observations may reflect differences in the magnitude of selection, mutational accessibility, fitness costs, or the contribution of host dynamics to the emergence and spread of antibiotic resistance[15].

To design interventions that slow the development and spread of antibiotic resistance, the dynamics of resistance evolution and cross-transmission must be considered. Interventions incorporating host-, pathogen-, and syndrome-specific information have successfully driven clinical decision-making, especially antibiotic treatment decisions[16,17]. Although simulation studies have modeled impacts of interventions on resistance evolution and cross-transmission, their integration into clinical or public health practice has been limited[18,19]. Future research is needed to improve the utility of phylogenetic context in shaping clinical and public health decision-making. Given this dissertation's focus on CRKP ST258, future work should assess the consistency of these findings across other epidemic lineages in the high-risk LTACH population. Additionally, investigating the epidemiology and evolution of last-resort resistance across diverse microbial species and healthcare settings is necessary to determine the feasibility of designing evolutionarily aware interventions to slow the emergence and spread of resistance.

5.2.2 Phylogenetically Informed Risk Factor Modeling Sharpens Appraisal of Resistance Dynamics

Traditional epidemiological studies use antibiotic susceptibility testing and pharmacokinetic-pharmacodynamic data to infer clinically significant levels of phenotypic resistance[20]. Abundant epidemiological studies have leveraged this methodology to identify many convincing risk factors for harboring resistant bacteria, including the presence of indwelling devices, functional dependence, and antibiotic exposure[21,22].

This dissertation's phylogenetic analysis of antibiotic resistance suggests that modeling resistance as a binary trait (i.e., susceptible or non-susceptible) oversimplifies and masks the

complexity of resistance epidemiology and evolution. Phylogenetically informed risk factor modeling suggests that risk factors for phenotypic resistance often segregate into models of resistance emergence or spread. This analysis supports the growing appreciation that host characteristics and exposures may exert distinct selective pressures on the evolution and cross-transmission of resistant lineages[19]. Our regression modeling also revealed that antibiotics can have distinct roles in the emergence and spread of resistance, with markedly different effect sizes. These findings are supported by evidence that antibiotics differentially influence host susceptibility to strain acquisition and exert varying magnitudes of selection for resistance[23,24]. Finally, while models for resistance spread included prior antibiotic exposure, proxies for complexity of care, such as patient comorbidities and indwelling medical devices, were often uniquely associated with the acquisition of circulating resistant lineages. High-acuity patients often require frequent healthcare contact, resulting in a high burden of multidrug-resistant bacteria and an important role in the transmission of resistance[25-27]. Collectively, these findings support the value of integrating bacterial genomic data and patient features to identify the host, pathogen, and environmental factors associated with the emergence and spread of resistance.

Future studies should investigate whether differential risk factors for resistance emergence and spread exist across microbial and patient populations. *In vitro* and *in vivo* experimentation could test hypotheses that host features and medical exposures uniquely contribute to resistance dynamics. Finally, epidemiological studies should evaluate the roles of non-antibiotic exposures and polypharmacy, a common phenomenon among older adults[28], in the emergence and spread of antibiotic resistance.

5.2.3 Phylogenetic Contextualization Improves Genotype-Phenotype Association Testing

Whole-genome sequencing has improved our ability to identify the determinants of complex traits, such as antibiotic resistance and virulence[29]. The identification of genetic determinants has been hindered by population stratification, epistasis, recombination, and varying phenotypic penetrance across bacterial populations[30]. Advances in modeling convergent evolution and kinship have improved our ability to understand the epidemiology and evolution of complex traits[31].

This dissertation's genomic investigations exemplify the value of phylogenetic context in genotype-phenotype association testing. First, phylogenetic prioritization can filter spurious associations and reduce false positives. Supporting the importance of considering lineage effects

during association testing[32], signatures of convergent evolution were evident for BL/BLI resistance, although consistency and effect size varied across genotypes. Second, concurrent analysis of candidate genotypes can illuminate the complex evolutionary history of resistance. Corroborating evidence from *in vitro* evolution studies of BL/BLI resistance[33], computational analysis of synchronous transitions of *bla*$_{KPC}$-containing plasmids revealed a correlation between plasmid modification, often through transposition of *bla*$_{KPC}$-containing, and the elevation of *bla*$_{KPC}$ copy number in a genetic background harboring a loop 3 insertion in the major porin, OmpK36. Specifically, the transposition of *bla*$_{KPC}$-containing *tn4401* transposon from an ancestral plasmid to a co-existing plasmid elevated *bla*$_{KPC}$ copy number and increased BL/BLI resistance among CRKP ST258 isolates harboring a loop 3 insertion in OmpK36. Third, integrating genotypic data into risk-factor modeling illuminated the drivers of distinct pathways to BL/BLI resistance. Supporting the role of cognate exposure in driving BL/BLI resistance[34], carbapenem exposure, alongside cephalosporin exposure, was positively associated with the acquisition of resistant isolates with non-carbapenemase mechanisms of resistance. Conversely, the presence of respiratory comorbidities was positively associated with harboring circulating resistant strains that carry a high-copy, *bla*$_{KPC}$-associated plasmid.

5.3 Genomic Epidemiology Improved Understanding of Antibiotic Resistance

Whole-genome sequencing provides invaluable insights into the epidemiology and evolution of antibiotic resistance. Genomic characterization and molecular subtyping methods, such as multilocus sequence typing, have enhanced the appraisal of bacterial genetic diversity and its connection to genome-influenced traits[35]. Investigations of bacterial population structure have strengthened the understanding of the evolution and epidemiology of antibiotic resistance.

This dissertation revealed striking intra- and inter-population differences in the rate of resistance evolution and cross-transmission. Specifically, genomic investigations in clinical CRKP isolates improved understanding of the emergence and spread of last-resort resistance across an LTACH network. Comparison of CRKP obtained across two time periods, separated by nearly a decade and the introduction of antibiotics, revealed considerable changes in population structure and resistance dynamics.

5.3.1 Genomic Epidemiology Revealed Considerable Differences in a Major Epidemic Lineage

Genomic investigation into the epidemiology of CRKP revealed that the epidemic lineage ST258 dominated the California LTACH population. Since its emergence in the 1990s and clinical detection in 2001, this *K. pneumoniae* lineage rapidly disseminated carbapenem resistance worldwide, often in conjunction with bla_{KPC}-harboring plasmids[36]. This population is broken into two clades, each with distinct associations with carbapenemase alleles and capsular serotypes, although extensive capsule locus variation has been detected within this lineage[36,37]. This lineage's emergence and clonal expansion were facilitated by remarkable genomic plasticity[38-40]. The acquisition of distinct bla_{KPC}-containing plasmids and accessory adaptive genes, alongside chromosomal mutagenesis, has been shown to be influential in the emergence, niche establishment, and long-term success of ST258 sublineages[38-40].

Genomic investigations into the population structure of ST258 have shed insights into the epidemiology and evolution of this lineage. Tight associations exist between carbapenemase content, resistance plasmids, and resistance patterns across clades of ST258. Considerable enrichment of resistance to trimethoprim-sulfamethoxazole, gentamicin, and colistin was identified, as numerous large, phylogenetic clusters were present in clade I[41]. Phylogenetic analysis has also identified a clade II subclade with a disrupted capsular locus, adaptation to the respiratory tract, and increased virulence[42]. Conversely, clade I had elevated resistance to amikacin and concerning levels of resistance to two BL/BLI agents before their clinical deployment[5]. This dissertation's phylogenetic modeling corroborated such clade-specific differences in resistance evolution, mutational availability, and transmissibility. Regression modeling also revealed variance in the magnitude of risk for resistance emergence and cross-transmission, with clade consistently being associated with the spread of resistant lineages. These findings suggest that investigations into the genetic background, especially using more precise measurements of bacterial diversity and sophisticated lineage-tracking methodologies, are necessary to more accurately discern how resistance evolves and spreads within a population. Future research should evaluate whether subclade differences in last-resort resistance exist in resistant lineages, such as ST258, collected from LTACHs and other populations.

The importance of bacterial genetic background in driving the emergence and spread of antibiotic resistance is exemplified by this dissertation's analysis of BL/BLI resistance in ST258. Phylogenetic analysis revealed substantial differences in the prevalence, emergence, and cross-transmission of BL/BLI resistance among ST258 clades. Genomic investigations revealed

minimal spread of resistance in clade II, predominantly due to mutations in *ompK36* porins and penicillin-binding proteins, which can confer high fitness costs[43-45]. The marked increase in baseline levels of resistance in clade I was predominantly due to the presence of loop 3 insertions in OmpK36. Elevation of bla_{KPC} copy number in this genetic background increased clinical resistance levels and the propensity for spread across the healthcare network.

As loop 3 insertions in OmpK36 are distributed across epidemic CRKP lineages[43,44], the interaction between porin modification and bla_{KPC} copy number with resistance reflects a concerning tale of how genetic background influences the availability and transmissibility of resistance. Future research should evaluate the uniformity of these observations across epidemic CRKP lineages collected from diverse patient populations. Whether distinct evolutionary and epidemiological trajectories are represented in contemporary collections obtained after the clinical deployment of these antibiotics remains an important, active area of investigation.

To investigate the fate of these resistant lineages, we characterized the molecular epidemiology of ST258 in a contemporary isolate collection obtained several years after the introduction and widespread use of BL/BLI combinations[46]. Phylogenetic inspection revealed marked alterations to the population structure of ST258. Nearly all isolates belonged to the bla_{KPC-3}-containing clade II lineages, which maintained a high frequency of colistin resistance. Coinciding with the clonal expansion of several bla_{KPC-3}-containing clade II lineages was the loss of the bla_{KPC-2}-harboring clade I lineage with a Glycine-Aspartate insertion in the loop 3 of OmpK36. These observations were corroborated by prior reports detailing the marked reduction in the prevalence of KL106-containing clade I ST258 isolates[47]. Reflecting this lineage loss, the prevalence and cross-transmission of last-resort resistance, including resistance to BL/BLI combinations, was markedly lower. However, the elevated frequency of resistance to colistin, aminoglycosides, and fosfomycin remained in this population.

Further investigations into the population structure of epidemic lineages will enrich our understanding of how bacterial lineages evolve and adapt to novel selective pressures. Future research should investigate the epidemiological and evolutionary reasons for this altered population structure. Analyses into the contribution of strain replacement and clonal expansion in shaping the evolutionary trajectory of ST258 clades in this LTACH network. Whether host, bacterial, and environmental changes, notably antibiotic use and infection prevention practices, influenced the epidemiology and evolution of CRKP lineage reflects an important avenue for

future research. The clonal expansion of a colistin-resistant, respiratory-associated ST258 subpopulation and the subsequent loss of the ST258 clade I remains a particularly interesting area for future investigation, as widespread changes in antibiotic prescribing occurred in recent years, with polymyxins being replaced by novel agents, such as BL/BLI combinations[46].

5.3.2 Genomic Epidemiology Revealed an Increasing Diversity of Carbapenem-Resistant Klebsiella pneumoniae Lineages

Molecular subtyping has been an invaluable strategy for characterizing the diversity of bacterial populations and identifying bacterial lineages responsible for the emergence and global dissemination of antibiotic resistance. In *K. pneumoniae*, over 9,000 allelic profiles have been identified via multilocus sequence typing, including numerous *bla*$_{KPC}$-containing lineages that have shaped the global epidemiology of carbapenem resistance[48].

Alongside changes in the population structure of ST258, the diversity of circulating CRKP STs increased in our California LTACH network. Previously dominated by ST258, population diversity increased in the contemporary collection, predominantly due to the introduction of other epidemic multidrug-resistant nosocomial lineages (i.e., ST14 and ST307) and the clonal expansion of the then less-abundant, opportunistic ST17 lineage. Recent reports have detailed similar shifts in the global epidemiology of CRKP, with the once-dominant, globally disseminated ST258 lineage being replaced by other STs[47,49].

These changes in population structure markedly influenced the dynamics of antibiotic resistance. Sharp associations between resistance genotypes were observed across lineages, with certain epidemic lineages serving as sources for carbapenemase dissemination. The increased ST diversity revealed differences in the prevalence and host range of previously-detected resistance plasmids. Alongside these alterations, the prevalence of chromosomally encoded carbapenemase-producing and non-carbapenemase-producing CRKP genomes increased in our population, reflecting a shifting ecology of resistance genotypes.

Corresponding with these changes were differential patterns of resistance to last-resort antibiotics. The abundant epidemic lineage ST258 showed a higher propensity for resistance to colistin and novel tetracyclines. Conversely, the abundant lineage ST17 showed higher resistance to cefiderocol, fosfomycin, and BL/BLI combinations. This genetically diverse epidemic lineage contributes to healthcare spread, although many susceptible lineages have been reported[50]. Conversely, the abundant lineage ST14 showed notably low resistance rates to the tested

antibiotics, suggesting a reduced propensity to develop resistance to last-resort antibiotics. Numerous minor STs showed elevated resistance rates, highlighting the potential for resistance to emerge across diverse genetic backgrounds.

The identification of associations between circulating bacterial lineages and the regional epidemiology of antibiotic resistance supports the emerging appreciation for the design of regional infection prevention[51-53]. Whole-genome sequencing can facilitate the identification of high-risk lineages with an elevated propensity to evolve and spread resistance across a healthcare network. Real-time whole-genome sequencing can enhance the detection of healthcare outbreaks of resistant bacteria[54]. Future research should investigate whether prioritizing interventions within lineages enables the design of cost-effective antibiotic stewardship and infection prevention interventions.

Future research into the epidemiology and evolution of CRKP in this population is also necessary. First, investigations into the genotypic and clinical differences between ST258 and other epidemic lineages would illuminate potential reasons for changes in population structure. Prior reports indicate considerable differences in resistance genotypes, clinical characteristics, and medical exposures among patients infected with ST258 isolates[55]. Second, the influence of changes in antibiotic prescribing patterns on ST diversity and the evolution of last-resort resistance must be tested in larger studies that correlate facility-level antibiotic use and resistance dynamics. Future research should leverage clinical metadata to investigate whether resistance trends reflect variation in experienced selective pressures or proclivities to cause clinical infection. Finally, incorporating colonization screening into the evaluation of proclivities for infection and resistance evolution will sharpen understanding of the differences in resistance patterns across these epidemic lineages.

5.4 Conclusions

Only by identifying the drivers of resistance emergence and spread will it be possible to develop innovative strategies to slow the development and transmission of, antibiotic resistance. This dissertation examined the epidemiology of resistance to last-resort antibiotics in CRKP, a multidrug-resistant pathogen with limited available therapeutics and a substantial clinical and economic burden[56]. Genomic investigations studied the epidemiology and evolution of resistance in CRKP collected across an LTACH network. Considerable differences in the evolution and cross-transmission were detected across epidemic CRKP lineages. Phylogenetic investigations

illuminated differential risk factors for the emergence and spread of last-resort resistance. The integration of genomic data into such analyses, as exemplified by studies of resistance to BL/BLI combinations, highlights the value of understanding how patient characteristics, medical practices, and bacterial genetic backgrounds shape the epidemiology and evolution of antibiotic resistance. These contributions support the growing importance of studying the genomic epidemiology of antibiotic resistance and exemplify the need to design analytical frameworks and strategies to evaluate the epidemiology and evolution of multidrug-resistant pathogens in clinically-important populations.

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Appendices

Appendix A: Chapter 2 Supplemental Material

Supplementary Methods

Whole-Genome Sequencing

Whole-genome sequencing of bacterial isolates was performed, as described previously[1]. Briefly, deoxyribonucleic acid was extracted using the DNeasy Blood & Tissue kit (QIAGEN). Short-read sequencing was performed on the Illumina HiSeq2500 instrument at the Pennsylvania Children's Hospital of Philadelphia Microbiome Center. Sequencing quality was assessed using FastQC[2]. Trimmomatic trimmed adapter sequences and low-quality bases[3]. Sequencing data are available under the BioProject PRJNA415194.

Variant calling was performed using SNPKIT v2.0 (https://github.com/Snitkin-Lab-Umich/snpkit) using the *K. pneumoniae* KPNIH1 reference genome (GenBank accession: GCF_000281535.2). The maximum-likelihood phylogenetic tree was reconstructed from Gubbins-recombination-filtered polymorphic sites using a custom, in-house Snakemake pipeline (https://github.com/Snitkin-Lab-Umich/phylokit)[4]. First, Gubbins identified recombination regions within the whole-genome alignment[5]. Phage regions identified by PHASTEST and repeat regions identified by nucmer were masked[6,7]. The initial phylogenetic tree was reconstructed using FastTree's Jukes-Cantor algorithm[8]. Subsequently, IQ-TREE was employed to determine the GTR+F+ASC model as the suitable model for use during Gubbins' final round of tree reconstruction, which was performed using RAxML [9,10]. Next, each isolate's Gubbins-identified recombination regions were masked. Following this, IQ-TREE version 2.4.0 was executed using the GTR+F+ASC+R2 substitution model, which was inferred as the best model by their ModelFinder Plus algorithm[9,11]. Default parameters were used, except that a total of

5,000 bootstraps and 500 unsuccessful iterations were required to halt the reconstruction. A single, closely related non-ST258 genome from this sample collection was used as the outgroup. Given the identification of numerous clades within our phylogeny, midpoint rooting was performed using the *phytools* R package to reflect the two bifurcating clades of ST258 accurately[12,13].

Phylogenetic Analysis of Antibiotic Resistance Using Joint Ancestral State Reconstruction

The easy-to-use, open-source R package, *phyloAMR*, was leveraged to study the emergence and spread of antibiotic resistance (https://github.com/kylegontjes/phyloAMR).

PhyloAMR's core function, *asr*, leverages *corHMM's* ancestral state reconstruction algorithm to characterize the gain, loss, and continuation of a trait across a phylogenetic tree. The equal rates and all rates different models were fit to the data. The model with the lowest sample-size corrected Akaike information criterion (AICc) was used for joint ancestral state reconstruction [14]. Leveraging inferred ancestral states and tip-based data, edges on the phylogeny were evaluated to determine episodes where the trait continued (i.e., susceptible -> susceptible or resistant -> resistant), was gained (i.e., susceptible -> resistant), or was lost (i.e., resistant -> susceptible). This edge matrix can be leveraged for numerous applications, including characterizing the frequency of trait transitions across a phylogeny and investigating genotype-phenotype associations.

We developed the phylogenetic tree traversal algorithm, *asr_cluster_detection*, to infer the evolutionary history of trait-containing isolates by tracing their ancestral states. This algorithm takes an isolate's trait data and walks upward on the tree to classify trait-containing isolates as phylogenetic singletons (i.e., evidence of *de novo* evolution of a trait) or members of a phylogenetic cluster of the trait (i.e., evidence that the trait was inherited from a common ancestor of circulating trait-containing lineage). Extending to the study of antibiotic resistance, resistant isolates with gain events inferred at the tip were classified as phylogenetic singletons. However, instances where a resistant isolate had a gain event at the tip and a reversion event at its parental node were eligible for classification as members of a phylogenetic cluster. Resistant isolates were classified as members of a phylogenetic cluster if their ancestral gain event was shared with at least one additional resistant isolate. Resistant isolates that did not share an ancestral gain event with any other resistant isolate were classified as phylogenetic singletons.

Phylogenetic clusters where all isolates belonged to one patient were not considered clusters and were reclassified as redundant phylogenetic singletons.

To describe the evolutionary history of antibiotic resistance, we characterized both the transitional data and the phylogenetic clustering of each antibiotic resistance phenotype. The frequency of trait gain, loss, and continuation events was characterized using *phyoAMR*'s *asr_transition_analysis* function. Descriptive statistics for phylogenetic clustering were generated using *phyloAMR*'s *asr_cluster_analysis* function. Specifically, the crude frequency of the trait, descriptive statistics on the number of singletons, clusters, and summary statistics for cluster size. Two measures were employed to characterize the phylogenetics of a trait: phylogenetic occurrence (**Equation 1**) and clustering (**Equation 2**).

(1) Frequency of phylogenetic occurrence =

$$\frac{No.singleton\ events + No.clusters}{No.singleton\ events + No.clusters + No.isolates\ without\ the\ trait}$$

(2) Frequency of clustering =

$$\frac{No.\ clusters}{No.singleton\ events + No.clusters}$$

Tables

Agent	Susceptible	Resistance	
		Intermediate	Resistant
Trimethoprim-sulfamethoxazole[1]	≤ 8/40 µg/mL	-	≥16/80 µg/mL
Gentamicin	≤ 4 µg/mL	8 µg/mL	≥ 16µg/mL
Amikacin	≤ 4 µg/mL	8 µg/mL	≥ 16µg/mL
Colistin	-	≤ 2µg/mL	≥ 4µg/mL
Imipenem-relebactam	≤ 1/4 µg/mL	2/4 µg/mL	≥ 4/4 µg/mL
Meropenem-vaborbactam	≤ 4/8 µg/mL	8/8 µg/mL	≥ 16/8 µg/mL

Appendix A Table 1 Minimum-Inhibitory Concentration Breakpoints Applied in This Study

[1.] Due to the regional clinical microbiology's susceptibility reporting practices (sulfamethoxazole doubling from 20 µg/mL to 320 µg/mL), we adjusted the 2024 Clinical and Laboratory Standards Institute breakpoints for trimethoprim-sulfamethoxazole accordingly. All remaining antibiotics used susceptibility testing breakpoints from the 2021 Clinical and Laboratory Standards Institute's Performance Standards for Antimicrobial Susceptibility Testing report.

Variables	Type	Description
Demographics		
Age	Continuous	Age in years
Sex	Binary	Female or male sex
Length of stay	Continuous	Days in the facility preceding isolate collection
Indwelling devices	Binary	Presence of the device on the day of isolate collection
Tracheostomy tube		
Central venous catheter		
Indwelling urinary catheter		
Gastrostomy tube		
Comorbidities	Binary	Presence of comorbidities on the day of isolate collection
Acute kidney injury		
Chronic kidney disease		
Ventilator-dependent respiratory failure		
Underweight or malnourished		Body mass index $\leq$ 18.5 kg/m^2
Congestive heart failure		
Decubitus ulcer stage IV/V		
COPD or chronic bronchitis		Chronic obstructive pulmonary disease or chronic bronchitis
Brain injury		Anoxic brain injury from a cerebral vascular accident, causing paresis
Malignancy		
Obesity		Body mass index $\geq$ 30 kg/m^2
Antibiotic exposures in the preceding 30 days	Binary	Exposure of $\geq$1 day of antibiotics modeled as a binary variable
Intravenous vancomycin		
Carbapenem		Exposure to ertapenem, imipenem, and/or meropenem
Cephalosporin		Exposures to third-, fourth-, and/or fifth-generation cephalosporins, notably ceftriaxone, ceftazidime, cefepime, or ceftaroline
Aminoglycoside		Exposures to amikacin, tobramycin, and/or gentamicin. For data-driven, multivariable regression modeling, aminoglycoside exposure was modeled separately, as gentamicin and amikacin resistance had distinct distributions across the phylogenetic tree
Metronidazole		
Fluoroquinolone		Exposure to levofloxacin and/or ciprofloxacin
Linezolid		
Tigecycline		
Piperacillin-tazobactam		
Polymyxin		Exposure to polymyxin B and/or colistin
Daptomycin		
Aztreonam		
Trimethoprim-sulfamethoxazole		

Appendix A Table 2 Variables Considered for Multivariable Logistic Regression Modeling

Characteristic[1]	Overall (n=312)
General characteristics	
Age, y, median (IQR)	72.72 (18.2)
Female sex	149 (47.8)
Length of stay before culture, median (IQR)	21 (37)
Indwelling device presence	
Device count, median (IQR)	2 (2)
Any device present	274 (87.8)
Tracheostomy tube	219 (70.2)
Central venous catheter	180 (57.7)
Indwelling urinary catheter	174 (55.8)
Gastronomy tube	117 (37.5)
Medical comorbidities	
Comorbidity count, median (IQR)	2 (3)
Presence of multiple comorbidities	201 (64.4)
Acute kidney injury	152 (48.7)
Chronic kidney disease	107 (34.3)
Ventilator-dependent respiratory failure	96 (30.8)
Underweight or malnourished	82 (26.3)
Congestive heart failure	65 (20.8)
Decubitus ulcer stage IV/V	65 (20.8)
COPD or chronic bronchitis	61 (19.6)
Brain injury	59 (18.9)
Malignancy	39 (12.5)
Obesity	17 (5.4)
Systemic antibiotic exposure in the preceding 30 days	
Any antibiotic exposure	227 (72.8)
Intravenous vancomycin	138 (44.2)
Carbapenem	99 (31.7)
Cephalosporin	79 (25.3)
Aminoglycoside	63 (20.2)
Amikacin	40 (12.8)
Tobramycin	21 (6.7)
Gentamicin	11 (3.5)
Metronidazole	60 (19.2)
Fluoroquinolone	58 (18.6)
Linezolid	46 (14.7)
Tigecycline	42 (13.5)
Piperacillin-tazobactam	42 (13.5)
Polymyxin	37 (11.9)
Daptomycin	12 (3.8)
Trimethoprim-sulfamethoxazole	11 (3.5)
Aztreonam	10 (3.2)

Appendix A Table 3 Characteristics of the Study Population at the Time of First Isolate Collection

[1.] Data are presented as No. (%) unless noted otherwise.

Phenotype	Model	No. parameters	Rate 1[1]	Rate 2	Log likelihood	AICc	Best model[2]
TMP-SMX	ER	1	12301.25	NA	-186.86	375.72	ARD
TMP-SMX	ARD	2	14266.6	9772.79	-185.73	375.49	
Gentamicin	ER	1	10271.29	NA	-162.08	326.17	ER
Gentamicin	ARD	2	10791.82	9820.55	-162.01	328.05	
AMK	ER	1	23413.38	NA	-226.98	455.96	ARD
AMK	ARD	2	24663.74	42368.73	-222.47	448.97	
Colistin	ER	1	8707.65	NA	-159.26	320.53	ER
Colistin	ARD	2	7875.24	11609.37	-158.5	321.02	
BL/BLI	ER	1	7843.71	NA	-166.53	335.06	ARD
BL/BLI	ARD	2	12924.48	76998.04	-145.38	294.79	

Appendix A Table 4 Comparison of Ancestral State Reconstruction Models Using Akaike Information Criteria

Abbreviations: AICc, sample-size corrected Akaike information criterion; ARD, all rates different; BL/BLI, β-lactam/β-lactamase inhibitor; ER, equal rates; NA, not applicable; TMP-SMX, trimethoprim-sulfamethoxazole.

[1.] For the all rates different model, rate 1 represents transitions from susceptible to resistant, while rate 2 represents transitions from resistant to susceptible.
[2.] The model with the lowest sample-size corrected Akaike information criterion value for each phenotype was chosen for downstream analyses.

Phenotype	Clade	Resistant isolates	Resistance frequency	Phylogenetic events	Singletons	Singleton isolates	Clusters	Cluster isolates	Cluster size median (range)	Phylogenetic frequency	Clustering frequency
TMP-SMX	I	40	29.85%	19	14	18	5	22	4 (2-8)	16.81%	26.32%
TMP-SMX	II	167	66.27%	18	14	14	4	153	2.5 (2-146)	9.33%	22.22%
Gentamicin	I	36	26.87%	18	13	17	5	19	3 (2-7)	11.84%	27.78%
Gentamicin	II	163	64.68%	9	6	6	3	157	8 (2-147)	6.29%	33.33%
Amikacin	I	79	58.96%	21	13	14	8	65	7 (2-25)	7.34%	38.10%
Amikacin	II	53	21.03%	26	21	24	5	29	4 (2-15)	8.58%	19.23%
Colistin	I	19	14.18%	14	13	13	1	6	6 (6-6)	8.92%	7.14%
Colistin	II	110	43.65%	14	7	7	7	103	4 (2-71)	5.83%	50.00%
BL/BLI	I	37	27.61%	21	16	17	5	20	2 (2-10)	15.11%	23.81%
BL/BLI	II	19	7.54%	17	16	17	1	2	2 (2-2)	6.80%	5.88%

Appendix A Table 5 Differences in Phylogenetic Clustering of Antibiotic Resistance Across Clades of *Klebsiella pneumoniae* Sequence Type 258

Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; TMP-SMX, trimethoprim-sulfamethoxazole.

[1.] Phylogenetic frequency accounts for the number of episodes a trait occurs across the phylogenetic tree relative to the total number of possible events.
[2.] Clustering frequency characterizes the proportion of phylogenetic events that are phylogenetic clusters.

		fitmultiMK single-regime model				*fitmultiMK* two-regime model					Model comparison via the likelihood ratio test		
Phenotype	**Model**	**Rate 1**	**Rate 2**	**Log Likelihood**	**AIC**	**Clade I Rate 1**	**Clade I Rate 2**	**Clade II Rate 1**	**Clade II Rate 2**	**Log Likelihood**	**AIC**	**X^2 value**	**p-value**
TMP-SMX	ER	12300.44	NA	-186.86	375.71	12935.96	NA	12016.96	NA	-186.84	377.67	0.04	0.843
TMP-SMX	ARD	14277.61	9773.99	-185.75	375.49	13558.78	18657.04	18620.48	9281	-184.26	376.51	2.98	0.225
Gentamicin	ER	10271.16	NA	-162.08	326.16	15179.17	NA	8494.82	NA	-160.89	325.77	2.39	0.122
Gentamicin	ARD	10765.84	9852.66	-162.02	328.04	18208.26	41250.16	9512.24	8328.1	-158.65	325.29	6.75	0.034
Amikacin	ER	23429.23	NA	-226.98	455.95	398075.94	NA	12357.89	NA	-212	428	29.95	<0.001
Amikacin	ARD	24665.84	42370.76	-222.47	448.94	202426.53	140691.98	16090.46	55069.54	-202.83	413.67	39.27	<0.001
Colistin	ER	8707.39	NA	-159.26	320.52	9924.87	NA	8150.87	NA	-159.14	322.27	0.25	0.616
Colistin	ARD	7889.8	11658.15	-158.52	321.04	184614.13	1097808.65	8487.38	7764.6	-146.18	300.36	24.68	<0.001
BL/BLI	ER	7844.06	NA	-166.53	335.05	22055.86	NA	4480.41	NA	-158.75	321.51	15.54	<0.001
BL/BLI	ARD	12926.43	77010.14	-145.38	294.75	45899.85	139580.52	8497.99	82031.48	-142.39	292.77	5.98	0.05

Appendix A Table 6 Evaluation of Single-Regime and Clade-Specific Evolutionary Rate Model Using *fitmultiMK*

Abbreviations: AIC, Akaike information criterion; ARD, all rates different; BL/BLI, β-lactam/β-lactamase inhibitor; ER, equal rates; NA, not applicable; TMP-SMX, trimethoprim-sulfamethoxazole.

Phenotype	Model	Rate 1	Rate 2	Single-rate Log Likelihood	Single-rate AICc	Category I Rate 1	Category I Rate 2	Category II Rate 1	Category II Rate 2	Multi-rate Log Likelihood	Multi-rate AICc	Best model
TMP-SMX	ER	12301.83	NA	-186.86	375.72	1.44e+12	NA	8.14e-07	NA	-162.37	332.84	Two rate categories
TMP-SMX	ARD	14265.06	9773.29	-185.73	375.49	3.15e+11	3.90e+11	3251.3	1.91e-06	-160.25	332.73	Two rate categories
Gentamicin	ER	10270.57	NA	-162.08	326.17	6.30e+11	NA	0	NA	-157.21	322.52	Two rate categories
Gentamicin	ARD	10792.83	9821.52	-162.01	328.05	3.60e+11	1.18e+12	0.01	4660.81	-156.67	325.55	Two rate categories
Amikacin	ER	23430.82	NA	-226.98	455.96	5.96e+05	NA	1782.15	NA	-202.12	412.35	Two rate categories
Amikacin	ARD	24662.95	42366.02	-222.47	448.97	4.84e+05	7.47e+05	4859.19	51051.42	-197.35	406.92	Two rate categories
Colistin	ER	8707.02	NA	-159.26	320.53	8.61e+17	NA	1326.06	NA	-152.17	312.45	Two rate categories
Colistin	ARD	7876.33	11610.01	-158.5	321.02	1.71e+06	1.31e+05	43726.54	293.61	-147.27	306.76	Two rate categories
BL/BLI	ER	7844.23	NA	-166.53	335.06	1.96e+17	NA	0	NA	-134.75	277.6	Two rate categories
BL/BLI	ARD	12924.8	76995.92	-145.38	294.79	1.35e+06	4.66e+05	1.02e-09	1903.25	-129.65	271.53	Two rate categories

Appendix A Table 7 Modeling of Single- and Multi-Rate Categories Using *corHMM*

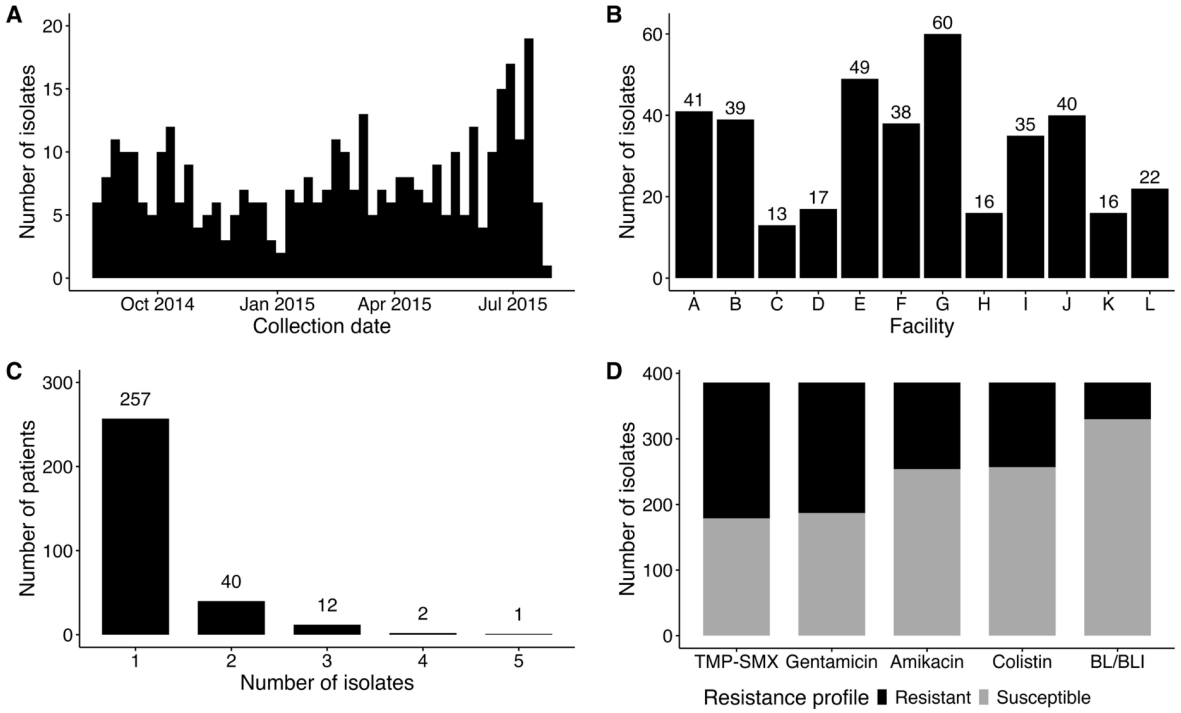
Abbreviations: AICc, sample-size corrected Akaike information criterion; ARD, all rates different; BL/BLI,β-lactam/β-lactamase inhibitor; ER, equal rates; NA, not applicable; TMP-SMX, trimethoprim-sulfamethoxazole.

Phenotype	Multivariable regression models								
TMP-SMX	**Present (n = 170)**	**OR (95% CI)**	**p-value**	**Emergence (n = 28)**	**OR (95% CI)**	**p-value**	**Spread (n = 142)**	**OR (95% CI)**	**p-value**
	Cephalosporin exposure	0.40 (0.22-0.72)	0.0022	TMP-SMX exposure	119.13 (5.95-5891.04)	0.0041	Acute kidney injury	0.41 (0.23-0.70)	0.0014
	TMP-SMX exposure	24.97 (4.02-504.50)	0.0044	Female sex	0.35 (0.13-0.86)	0.0284	Cephalosporin exposure	0.40 (0.22-0.73)	0.0031
	Acute kidney injury	0.49 (0.29-0.81)	0.006	Tigecycline exposure	3.66 (1.10-11.75)	0.0292	TMP-SMX exposure	18.93 (3.13-368.12)	0.0077
	Gastrostomy tube	1.86 (1.11-3.18)	0.0203	Piperacillin-tazobactam exposure	0.09 (0.00-0.67)	0.0709	Fluoroquinolone exposure	0.42 (0.20-0.82)	0.0132
	Decubitus ulcer stage IV/V	2.08 (1.12-3.96)	0.0227	Polymyxin exposure	0.10 (0.00-0.80)	0.0892	Decubitus ulcer stage IV/V	2.10 (1.11-4.06)	0.0247
	Tigecycline exposure	2.34 (1.12-5.11)	0.0278				Gastrostomy tube	1.89 (1.09-3.33)	0.0256
	Gentamicin exposure	0.17 (0.02-0.76)	0.0346				Gentamicin exposure	0.09 (0.00-0.54)	0.0286
	Amikacin exposure	0.46 (0.21-0.98)	0.0465				Tracheostomy tube	1.67 (0.94-3.01)	0.0831
	Fluoroquinolone exposure	0.53 (0.28-1.01)	0.0541						
	Malignancy	0.52 (0.24-1.08)	0.0803						
Gentamicin	**Present (n = 171)**	**OR (95% CI)**	**p-value**	**Emergence (n =19)**	**OR (95% CI)**	**p-value**	**Spread (n = 152)**	**OR (95% CI)**	**p-value**
	Decubitus ulcer stage IV/V	2.23 (1.22-4.20)	0.0105	Congestive heart failure	3.61 (1.21-10.75)	0.0199	Decubitus ulcer stage IV/V	2.57 (1.37-4.96)	0.0038
	Cephalosporin exposure	0.49 (0.28-0.84)	0.0108	Cephalosporin exposure	0.13 (0.01-0.67)	0.0513	Chronic kidney disease	0.47 (0.27-0.82)	0.0082
	Chronic kidney disease	0.52 (0.31-0.88)	0.0152	Carbapenem exposure	0.27 (0.06-0.95)	0.0626	TMP-SMX exposure	10.85 (1.96-203.63)	0.0259
	TMP-SMX exposure	12.95 (2.22-249.77)	0.0193				Cephalosporin exposure	0.56 (0.32-0.98)	0.0438
	Malignancy	0.48 (0.22-1.00)	0.0525				Acute kidney injury	0.60 (0.36-1.00)	0.0501
	Acute kidney injury	0.65 (0.39-1.06)	0.0858				Gentamicin exposure	0.23 (0.03-1.01)	0.0773
Amikacin	**Present (n = 119)**	**OR (95% CI)**	**p-value**	**Emergence (n = 33)**	**OR (95% CI)**	**p-value**	**Spread (n = 86)**	**OR (95% CI)**	**p-value**
	Tobramycin exposure	2.82 (1.14-7.40)	0.0279	Tobramycin exposure	3.79 (1.03-12.65)	0.0334	Cephalosporin exposure	1.88 (1.06-3.33)	0.0297
	Gentamicin exposure	4.44 (1.24-20.80)	0.0318	Gentamicin exposure	5.61 (0.94-33.04)	0.0474	Gentamicin exposure	4.61 (1.08-23.30)	0.0424
	Malignancy	1.93 (0.96-3.88)	0.0647	Malignancy	2.55 (0.89-6.69)	0.0649	COPD or chronic bronchitis	1.80 (0.96-3.35)	0.0618
							Tobramycin exposure	2.52 (0.88-7.24)	0.0806
Colistin	**Present (n = 111)**	**OR (95% CI)**	**p-value**	**Emergence (n = 20)**	**OR (95% CI)**	**p-value**	**Spread (n = 91)**	**OR (95% CI)**	**p-value**
	Polymyxin exposure	3.85 (1.85-8.28)	4e-04	Indwelling urinary catheter	0.15 (0.04-0.49)	0.0039	Polymyxin exposure	3.19 (1.45-7.11)	0.0039
	Ventilator-dependent respiratory failure	0.54 (0.30-0.95)	0.0353	Polymyxin exposure	7.87 (1.92-34.78)	0.0042	Underweight or malnourished	0.47 (0.24-0.87)	0.0208
	Underweight or malnourished	0.53 (0.29-0.95)	0.0361	COPD or chronic bronchitis	4.60 (1.54-14.26)	0.0064	Length of stay, 1 day unit	0.99 (0.98-1.00)	0.0667
				Tigecycline exposure	3.95 (1.11-13.41)	0.0277			
BL/BLI	**Present (n = 50)**	**OR (95% CI)**	**p-value**	**Emergence (n = 31)**	**OR (95% CI)**	**p-value**	**Spread (n = 19)**	**OR (95% CI)**	**p-value**
	Age, 1 year unit	1.04 (1.01-1.06)	0.0036	Age, 1 year unit	1.03 (1.01-1.06)	0.025	Cephalosporin exposure	5.74 (2.03-17.14)	0.0011
	Cephalosporin exposure	2.21 (1.13-4.27)	0.0186	Carbapenem exposure	2.41 (1.08-5.37)	0.0299	COPD or chronic bronchitis	4.16 (1.38-12.54)	0.0102
	Carbapenem exposure	1.94 (1.02-3.69)	0.0424	Tigecycline exposure	2.32 (0.90-5.60)	0.0683	Age, 1 year unit	1.04 (1.00-1.09)	0.0383
	Underweight or malnourished	0.42 (0.16-0.95)	0.0498	Linezolid exposure	0.17 (0.01-0.87)	0.0894	Indwelling urinary catheter	3.10 (1.07-10.49)	0.0478
				Underweight or malnourished	0.39 (0.11-1.06)	0.093	Underweight or malnourished	0.29 (0.06-1.00)	0.0744

Appendix A Table 8 Complete Multivariable Logistic Regression Modeling Results for Risk Factors for Resistance Emergence and Spread

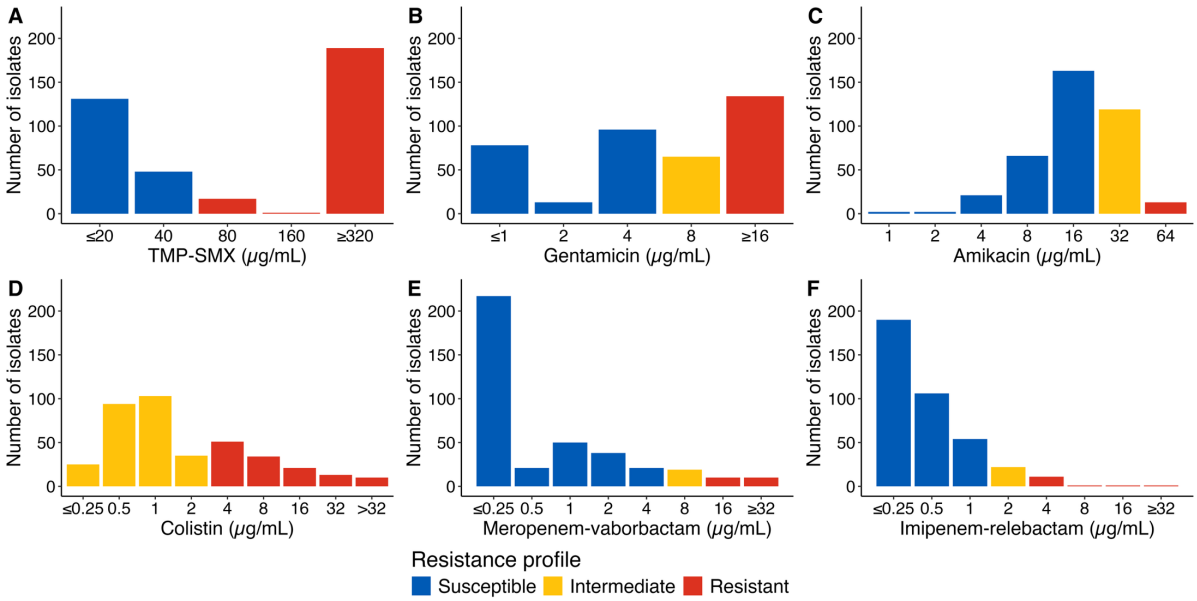
Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; CI, confidence interval; COPD, chronic obstructive pulmonary disease; OR, odds ratio; TMP-SMX, trimethoprim-sulfamethoxazole.

Figures



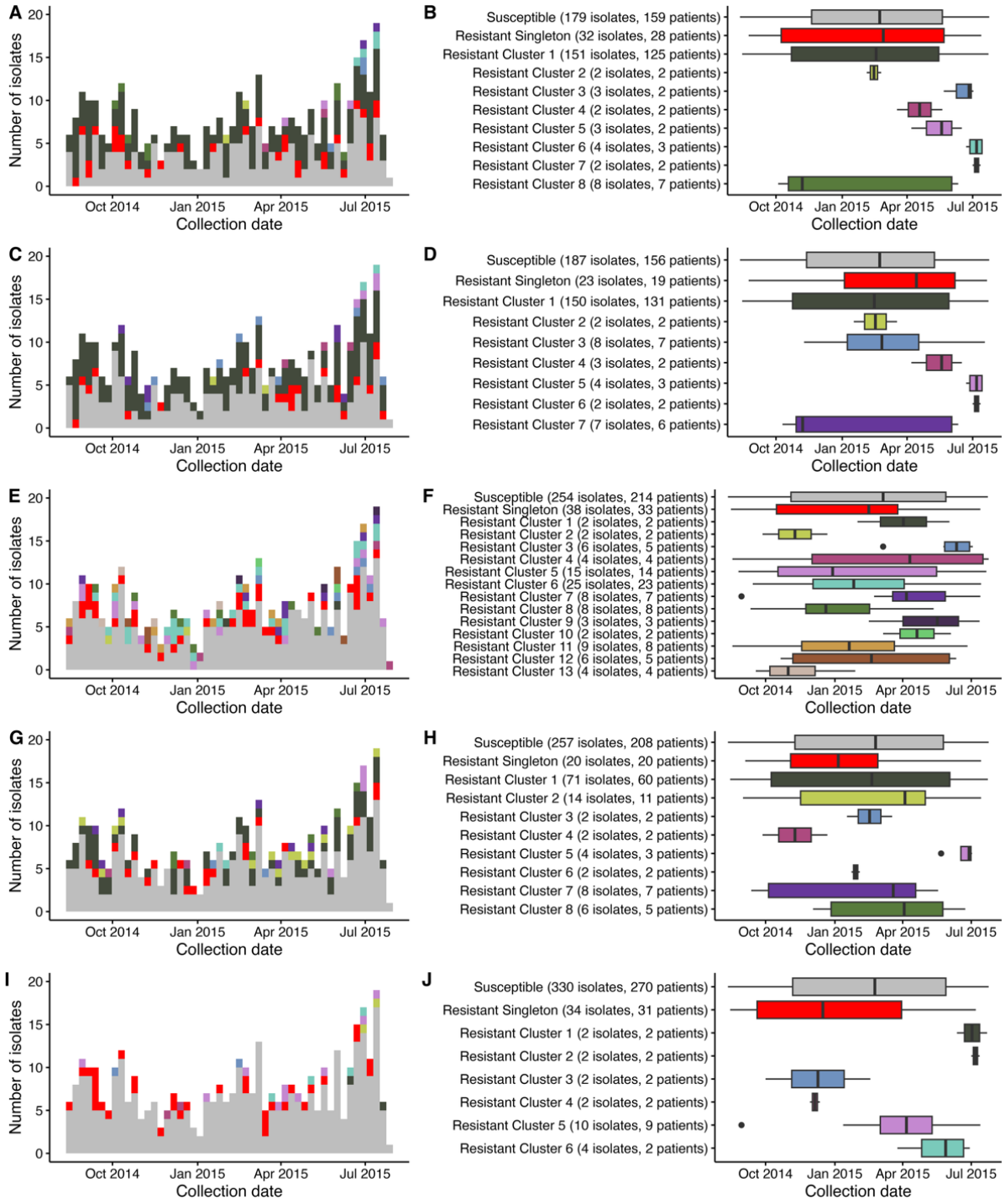
Appendix A Figure 1 Comprehensive Sampling of Carbapenem-Resistant *Klebsiella pneumoniae* That are Resistant to Last-Resort or Recently Introduced Antibiotics

(A) Number of clinical carbapenem-resistant *Klebsiella pneumoniae* sequence type 258 isolates collected from August 1, 2014, to July 25, 2015. (B) Number of specimens collected across each of the twelve California long-term acute care hospitals. (C) Number of specimens contributed by each patient. (D) Number of resistant and susceptible isolates for the five resistance phenotypes. Antibiotic resistance was defined as having a minimum-inhibitory concentration in either the resistant or intermediate category, per the 2021 Clinical and Laboratory Standards Institute criteria for all agents, except TMP-SMX, for which resistance was defined as MIC $\geq$ 16/80 μg/mL. Resistance to BL/BLI agents was determined as a composite of resistance to either imipenem-relebactam and/or meropenem-vaborbactam. Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; TMP-SMX, trimethoprim-sulfamethoxazole.



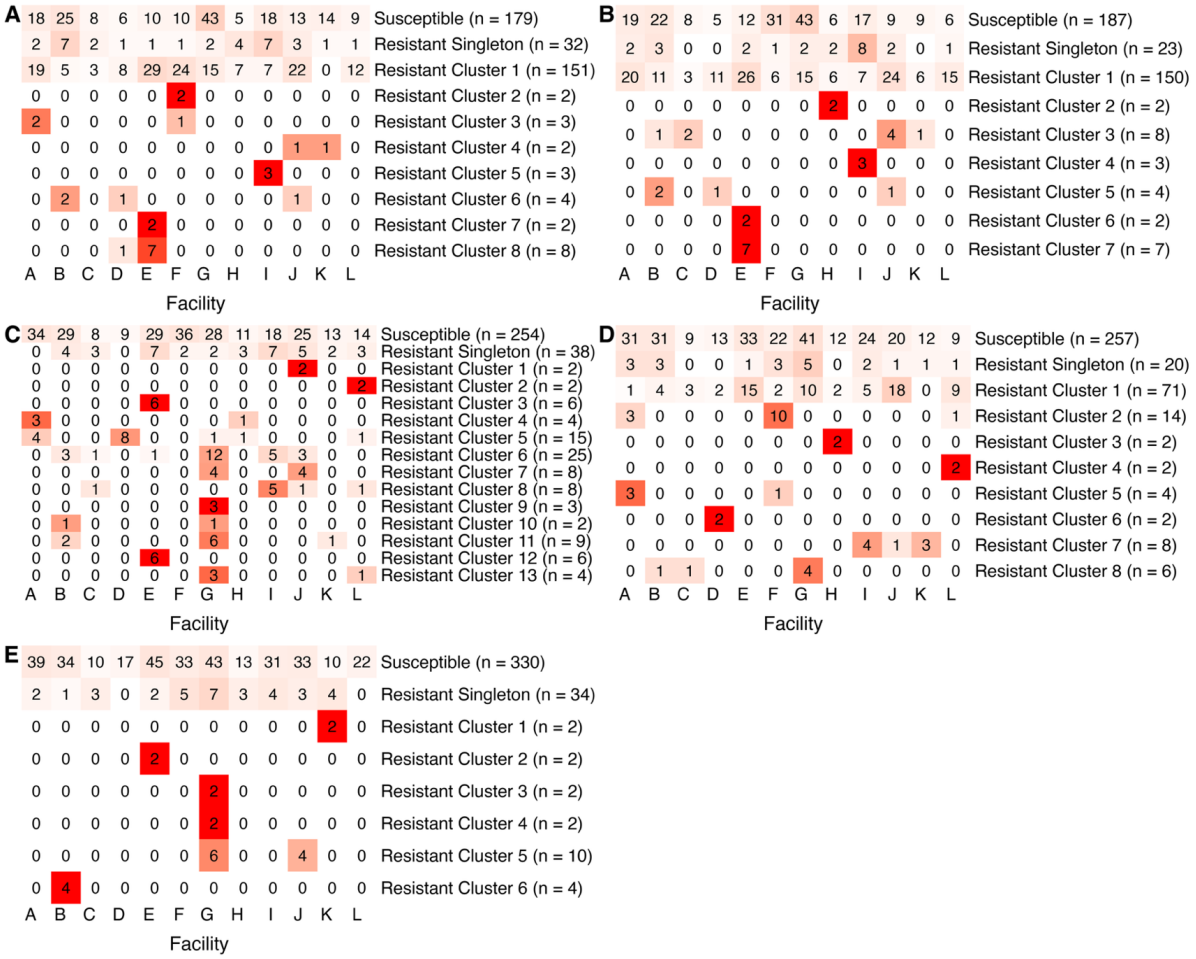
Appendix A Figure 2 Minimum-Inhibitory Concentration Histogram for Each Antibiotic of Interest

Abbreviations: TMP-SMX, trimethoprim-sulfamethoxazole.



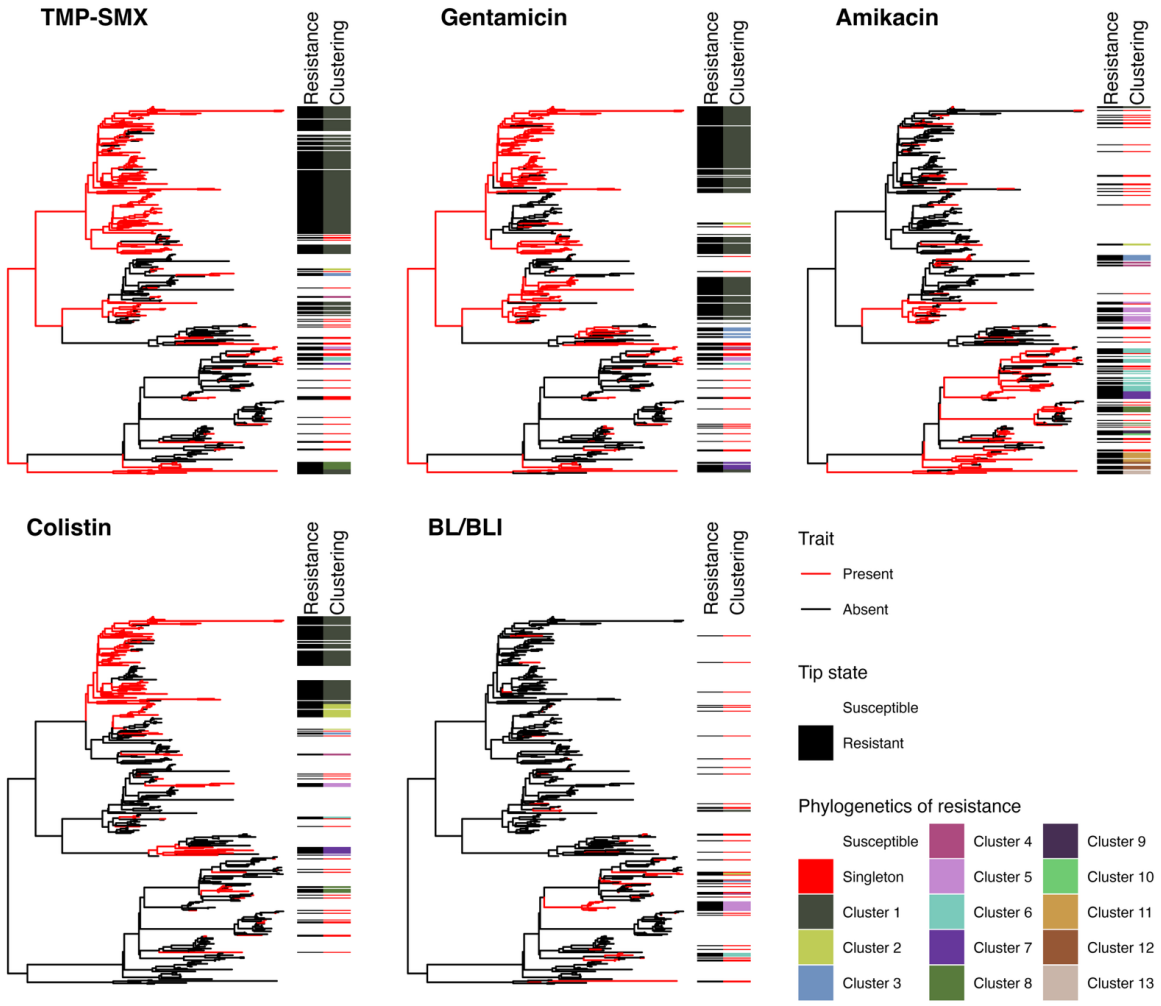
Appendix A Figure 3 Temporal Distribution of Phylogenetic Groups

The distribution of phylogenetic groups over time for (A-B) trimethoprim-sulfamethoxazole, (C-D) gentamicin, (E-F) amikacin, (G-H) colistin, (I-J) and resistance to β-lactam/β-lactamase inhibitor (BL/BLI) combinations. Resistance to BL/BLI agents was determined as a composite of resistance to either imipenem-relebactam and/or meropenem-vaborbactam.



Appendix A Figure 4 Distribution of Phylogenetic Groups Across Study Facilities

The distribution of phylogenetic groups across study facilities for (A) trimethoprim-sulfamethoxazole, (B) gentamicin, (C) amikacin, (D) colistin, and (E) resistance to β-lactam/β-lactamase inhibitor (BL/BLI) combinations. Resistance to BL/BLI agents was determined as a composite of resistance to either imipenem-relebactam and/or meropenem-vaborbactam.



Appendix A Figure 5 Ancestral State Reconstruction and Evolutionary History Inferences on the Phylogenetic Tree

Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitors; TMP-SMX, trimethoprim-sulfamethoxazole.

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Appendix B: Chapter 3 Supplemental Material

Tables

Mechanism of interest	Overall (n=412)	Clade I (n=145)	Clade II (n=267)	p-value
Carbapenem entry				
ompK35 porin truncation at 25%	412 (100.0)	145 (100.0)	267 (100.0)	NA
ompK36 porin	131 (31.8)	129 (89.0)	2 (0.7)	<0.001
Cytosine-to-thymine transition at position 25	124 (30.1)	124 (85.5)	0 (0.0)	<0.001
Loop 3 insertion	119 (28.9)	119 (82.1)	0 (0.0)	<0.001
Glycine-Aspartate (GD) insertion	119 (28.9)	119 (82.1)	0 (0.0)	<0.001
Threonine-Aspartate (TD) insertion	5 (1.2)	5 (3.4)	0 (0.0)	0.005
Non-synonymous mutation	22 (5.3)	13 (9.0)	9 (3.4)	0.029
Putative function-altering variants	20 (4.9)	13 (9.0)	7 (2.6)	0.007
Truncation	8 (1.9)	1 (0.7)	7 (2.6)	0.27
ompK36 intergenic region				
Any mutation	31 (7.5)	5 (3.4)	26 (9.7)	0.02
Insertion sequences	24 (5.8)	1 (0.7)	23 (8.6)	0.001
Carbapenem efflux				
acrAB-tolC efflux pump mutant	2 (0.5)	0 (0.0)	2 (0.7)	0.543
ramR efflux pump regulator putative function-altering variants	27 (6.6)	11 (7.6)	16 (6.0)	0.537
ramA efflux pump activator putative function-altering variants	10 (2.4)	4 (2.8)	6 (2.2)	0.746
Carbapenem target modification				
Non-synonymous mutations in penicillin-binding-proteins	9 (2.2)	3 (2.1)	6 (2.2)	1.00
Penicillin-binding protein-2	8 (1.9)	2 (1.4)	6 (2.2)	0.718
Penicillin-binding protein-4	2 (0.5)	2 (1.4)	0 (0.0)	0.123
B-lactamases				
$bla_{\text{CMY-2.v2}}$	12 (2.9)	9 (6.2)	3 (1.1)	0.005
$bla_{\text{CTX-M-14}}$	8 (1.9)	8 (5.5)	0 (0.0)	<0.001
$bla_{\text{CTX-M-15}}$	11 (2.7)	10 (6.9)	1 (0.4)	<0.001
$bla_{\text{KPC-2}}$	136 (33.0)	131 (90.3)	5 (1.9)	<0.001
$bla_{\text{KPC-3}}$	269 (65.3)	9 (6.2)	260 (97.4)	<0.001
$bla_{\text{KPC-5}}$	3 (0.7)	3 (2.1)	0 (0.0)	0.043
$bla_{\text{KPC-10}}$	1 (0.2)	1 (0.7)	0 (0.0)	0.756
$bla_{\text{LAP-2}}$	1 (0.2)	0 (0.0)	1 (0.4)	1.00
$bla_{\text{OXA-1}}$	10 (2.4)	10 (6.9)	0 (0.0)	<0.001
$bla_{\text{OXA-9.v1}}$	154 (37.4)	1 (0.7)	153 (57.3)	<0.001
$bla_{\text{SHV-11}}$	313 (76.0)	135 (93.1)	178 (66.7)	<0.001
$bla_{\text{SHV-12}}$	30 (7.3)	2 (1.4)	28 (10.5)	0.001
$bla_{\text{TEM-104?}}$	6 (1.5)	6 (4.1)	0 (0.0)	0.002
$bla_{\text{TEM-104*?}}$	7 (1.7)	6 (4.1)	1 (0.4)	0.009
$bla_{\text{TEM-150*?}}$	6 (1.5)	0 (0.0)	6 (2.2)	0.095
$bla_{\text{TEM-1D.v1}}$	177 (43.0)	51 (35.2)	126 (47.2)	0.024
***tn4401* bla_{KPC} transposon isoform**				
tn4401a	143 (35.0)	138 (95.8)	5 (1.9)	<0.001
tn4401b	39 (9.5)	5 (3.5)	34 (12.8)	0.004
tn4401d	137 (33.5)	1 (0.7)	136 (51.3)	<0.001
tn4401_del_6920-7126	50 (12.2)	0 (0.0)	50 (18.9)	<0.001
tn4401_del_1-554_7008-7075	39 (9.5)	0 (0.0)	39 (14.7)	<0.001
tn4401_del_1-3391_6920-7126	1 (0.2)	0 (0.0)	1 (0.4)	1

Appendix B Table 1 Clade-Specific Differences in the Presence of Non-Carbapenemase Mechanisms of Carbapenem Resistance

Statistical significance was tested using the Chi-squared test for most variables. Fisher's exact test was performed for the following variables due to small event size: TD insertions in *ompk36* porin, acrAB-tolC efflux pump variant, *ramA*, *ramR*, penicillin-binding protein-2, penicillin-binding protein-4, $bla_{\text{CTX-M14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{KPC-5}}$, $bla_{\text{LAP-2}}$, $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$, and *tn4401 del 1-3391 5920-7126*. For β-lactamase genes, ? indicates coverage < 100%, whereas *? indicates an incomplete match and coverage <100%.

	Log_2 meropenem-vaborbactam minimum-inhibitory concentration												Log_2 imipenem-relebactam minimum-inhibitory concentration											
Predictors	**Estimate**	**SE**	**CI**	**P-value**	**Estimate**	**SE**	**CI**	**P-value**	**Estimate**	**SE**	**CI**	**P-value**	**Estimate**	**SE**	**CI**	**P-value**	**Estimate**	**SE**	**CI**	**P-value**	**Estimate**	**SE**	**CI**	**P-value**
(Intercept)	-0.64	0.1	-0.83 – -0.45	**<0.001**	-1.68	0.09	-1.86 – -1.51	**<0.001**	-1.61	0.09	-1.78 – -1.43	**<0.001**	-1.12	0.06	-1.23 – -1.01	**<0.001**	-1.63	0.06	-1.74 – -1.52	**<0.001**	-1.61	0.06	-1.72 – -1.49	**<0.001**
Log_2 *bla*$_{KPC}$ copy number	-0.12	0.09	-0.30 – 0.05	0.169	0.30	0.07	0.16 – 0.44	**<0.001**	0.10	0.08	-0.06 – 0.27	0.205	0.02	0.05	-0.08 – 0.13	0.683	0.18	0.04	0.09 – 0.27	**<0.001**	0.12	0.05	0.01 – 0.23	**0.025**
Putative function altering variants in *ompK36*					2.40	0.31	1.79 – 3.02	**<0.001**	2.35	0.31	1.74 – 2.97	**<0.001**					2.52	0.20	2.13 – 2.92	**<0.001**	2.52	0.20	2.12 – 2.92	**<0.001**
Loop 3 insertions in OmpK36					2.96	0.17	2.63 – 3.28	**<0.001**	3.19	0.17	2.86 – 3.52	**<0.001**					1.22	0.11	1.01 – 1.43	**<0.001**	1.30	0.11	1.08 – 1.51	**<0.001**
Log_2 bla$_{KPC}$ copy number * Putative function altering variants in *ompK36*									0.11	0.23	-0.34 – 0.56	0.627									0.01	0.15	-0.29 – 0.30	0.959
Log_2 bla$_{KPC}$ copy number * Loop 3 insertions in OmpK36									0.89	0.17	0.55 – 1.23	**<0.001**									0.29	0.11	0.07 – 0.52	**0.009**
Observations	409				409				409				409				409				409			
R^2 / R^2 adjusted	0.005 / 0.002				0.461 / 0.457				0.495 / 0.489				0.000 / -0.002				0.387 / 0.383				0.398 / 0.390			
AIC	1695.218				1448.285				1425.753				1275.877				1079.84				1076.793			

Appendix B Table 2 Regression Modeling to Evaluate the Interaction Between Loop 3 Insertions in OmpK36 Porin with *bla*KPC Copy Number

The association between log_2 bla$_{KPC}$ copy number and log_2 minimum-inhibitory concentration (MIC) was assessed in bla$_{KPC}$-containing isolates. In simple linear regression, log_2 *bla*$_{KPC}$ copy number was not associated with increased log_2 MIC for MVB or IR. To account for the contribution of mutations to the *ompK36* porin, we adjusted for mutational status. While *ompK36* mutations exhibited strong independent associations with resistance in the additive model, a statistically significant association between log_2 *bla*$_{KPC}$ copy number and log_2 MIC for MVB and IR was also detected. Based on the marked increase in MIC associated with loop 3 insertions in OmpK36, and their restriction to clade I, we hypothesized that loop 3 insertions were necessary for *bla*$_{KPC}$ copy number variation to influence MIC. Interaction modeling was performed to evaluate the relationship between loop 3 insertions and *bla*$_{KPC}$ copy number's influence on MICs. This modeling identified that the effect of *bla*$_{KPC}$ copy number was specific to isolates with loop 3 insertions, as confirmed by statistically significant interaction terms.

Abbreviations: AIC, Akaike information criteria; CI, confidence interval; SE, standard error.

Variable	Overall (n=336)	Resistant (n=52)	Susceptible (n=284)	OR (95% CI)	OR p-value	aOR (95% CI)	aOR p-value
Age. 1 year unit	71.07 (14.66)	77.00 (11.72)	69.98 (14.90)	1.04 (1.02-1.07)	0.0017	1.04 (1.02-1.07)	0.0015
Female sex	40.48 (109.98)	41.81 (66.94)	40.23 (116.23)	1.00 (1.00-1.00)	0.9243		
Length of stay before culture, 1-day unit	160 (47.6)	28 (53.8)	132 (46.5)	1.34 (0.74-2.45)	0.3292		
Tracheostomy tube	227 (67.6)	33 (63.5)	194 (68.3)	0.81 (0.44-1.52)	0.4929		
Central venous catheter	187 (55.7)	25 (48.1)	162 (57.0)	0.70 (0.38-1.26)	0.2331		
Indwelling urinary catheter	183 (54.5)	29 (55.8)	154 (54.2)	1.06 (0.59-1.94)	0.8372		
Gastrostomy tube	121 (36.0)	16 (30.8)	105 (37.0)	0.76 (0.39-1.41)	0.3927		
Acute kidney injury	154 (45.8)	23 (44.2)	131 (46.1)	0.93 (0.51-1.68)	0.8009		
Chronic kidney disease	110 (32.7)	18 (34.6)	92 (32.4)	1.10 (0.58-2.04)	0.7537		
Ventilator-dependent respiratory failure	99 (29.5)	14 (26.9)	85 (29.9)	0.86 (0.43-1.64)	0.6622		
Underweight or malnutrition	84 (25.0)	7 (13.5)	77 (27.1)	0.42 (0.17-0.91)	0.0415	0.42 (0.16-0.94)	0.0471
Congestive heart failure	64 (19.0)	9 (17.3)	55 (19.4)	0.87 (0.38-1.82)	0.7283		
Decubitus ulcer stage 4+	67 (19.9)	7 (13.5)	60 (21.1)	0.58 (0.23-1.28)	0.2079		
Chronic bronchitis or chronic obstructive pulmonary disorder	60 (17.9)	13 (25.0)	47 (16.5)	1.68 (0.81-3.32)	0.1467		
Brain injury	63 (18.8)	11 (21.2)	52 (18.3)	1.20 (0.55-2.42)	0.6294		
Malignancy	41 (12.2)	6 (11.5)	35 (12.3)	0.93 (0.34-2.19)	0.8736		
Obesity	19 (5.7)	2 (3.8)	17 (6.0)	0.63 (0.10-2.28)	0.5425		
Carbapenem exposure	106 (31.5)	23 (44.2)	83 (29.2)	1.92 (1.04-3.51)	0.0342	1.96 (1.04-3.68)	0.0367
Amikacin exposure	41 (12.2)	8 (15.4)	33 (11.6)	1.38 (0.56-3.06)	0.4473		
Gentamicin exposure	12 (3.6)	2 (3.8)	10 (3.5)	1.10 (0.17-4.32)	0.9076		
Tobramycin exposure	21 (6.2)	5 (9.6)	16 (5.6)	1.78 (0.56-4.80)	0.2813		
Aztreonam exposure	10 (3.0)	0 (0.0)	10 (3.5)	0.00 (NA-NA)	0.9843		
Sulfamethoxazole-trimethoprim exposure	11 (3.3)	2 (3.8)	9 (3.2)	1.22 (0.18-4.92)	0.8011		
Cephalosporin exposure	84 (25.0)	20 (38.5)	64 (22.5)	2.15 (1.14-3.99)	0.0163	2.09 (1.08-3.99)	0.0268
Fluoroquinolone exposure	59 (17.6)	7 (13.5)	52 (18.3)	0.69 (0.27-1.54)	0.4003		
Daptomycin exposure	14 (4.2)	5 (9.6)	9 (3.2)	3.25 (0.96-9.84)	0.042		
Metronidazole exposure	63 (18.8)	10 (19.2)	53 (18.7)	1.04 (0.47-2.13)	0.923		
Linezolid exposure	48 (14.3)	6 (11.5)	42 (14.8)	0.75 (0.27-1.75)	0.5392		
Polymyxin and/or colistin exposure	38 (11.3)	6 (11.5)	32 (11.3)	1.03 (0.37-2.44)	0.9548		
Tigecycline exposure	47 (14.0)	11 (21.2)	36 (12.7)	1.85 (0.84-3.83)	0.1093		
Vancomycin exposure	144 (42.9)	21 (40.4)	123 (43.3)	0.89 (0.48-1.61)	0.6953		
Piperacillin/tazobactam exposure	45 (13.4)	5 (9.6)	40 (14.1)	0.65 (0.22-1.59)	0.3875		

Appendix B Table 3 Unadjusted and Adjusted Analysis of Risk Factors for Resistance to β-lactam/β-lactamase inhibitor Combinations

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; NA, not applicable; OR, odds ratio

Variable	Overall (n=317)	Resistant (n=34)	Susceptible (n=284)	OR (95% CI)	OR p-value	aOR (95% CI)	aOR p-value
Age. 1 year unit	70.75 (14.84)	77.20 (12.84)	69.98 (14.90)	1.04 (1.01-1.07)	0.0079	1.04 (1.01-1.07)	0.0112
Female sex	39.92 (110.41)	37.26 (35.17)	40.23 (116.23)	1.00 (0.99-1.00)	0.8825		
Length of stay before culture, 1-day unit	149 (46.9)	17 (50.0)	132 (46.5)	1.15 (0.56-2.36)	0.6976		
Tracheostomy tube	217 (68.2)	23 (67.6)	194 (68.3)	0.97 (0.46-2.15)	0.9375		
Central venous catheter	178 (56.0)	16 (47.1)	162 (57.0)	0.67 (0.32-1.37)	0.2701		
Indwelling urinary catheter	170 (53.5)	16 (47.1)	154 (54.2)	0.75 (0.36-1.53)	0.4296		
Gastrostomy tube	114 (35.8)	9 (26.5)	105 (37.0)	0.61 (0.26-1.32)	0.2311		
Acute kidney injury	143 (45.0)	12 (35.3)	131 (46.1)	0.64 (0.30-1.32)	0.2331		
Chronic kidney disease	103 (32.4)	11 (32.4)	92 (32.4)	1.00 (0.45-2.09)	0.9961		
Ventilator-dependent respiratory failure	93 (29.2)	8 (23.5)	85 (29.9)	0.72 (0.29-1.59)	0.4398		
Underweight or malnutrition	81 (25.5)	4 (11.8)	77 (27.1)	0.36 (0.10-0.95)	0.0615	0.40 (0.11-1.09)	0.1015
Congestive heart failure	59 (18.6)	4 (11.8)	55 (19.4)	0.56 (0.16-1.48)	0.2873		
Decubitus ulcer stage 4+	64 (20.1)	4 (11.8)	60 (21.1)	0.50 (0.14-1.32)	0.2061		
Chronic bronchitis or chronic obstructive pulmonary disorder	53 (16.7)	6 (17.6)	47 (16.5)	1.08 (0.39-2.60)	0.8711		
Brain injury	58 (18.2)	6 (17.6)	52 (18.3)	0.96 (0.34-2.28)	0.9247		
Malignancy	38 (11.9)	3 (8.8)	35 (12.3)	0.69 (0.16-2.06)	0.5542		
Obesity	18 (5.7)	1 (2.9)	17 (6.0)	0.48 (0.03-2.44)	0.4776		
Carbapenem exposure	100 (31.4)	17 (50.0)	83 (29.2)	2.42 (1.17-5.00)	0.0159	3.43 (1.53-7.81)	0.0029
Amikacin exposure	38 (11.9)	5 (14.7)	33 (11.6)	1.31 (0.42-3.37)	0.601		
Gentamicin exposure	12 (3.8)	2 (5.9)	10 (3.5)	1.71 (0.26-6.87)	0.4996		
Tobramycin exposure	20 (6.3)	4 (11.8)	16 (5.6)	2.23 (0.61-6.57)	0.1741		
Aztreonam exposure	10 (3.1)	0 (0.0)	10 (3.5)	0.00 (NA-NA)	0.9901		
Sulfamethoxazole-trimethoprim exposure	10 (3.1)	1 (2.9)	9 (3.2)	0.93 (0.05-5.16)	0.9427		
Cephalosporin exposure	77 (24.2)	13 (38.2)	64 (22.5)	2.13 (0.99-4.44)	0.0471	2.60 (1.09-6.19)	0.03
Fluoroquinolone exposure	57 (17.9)	5 (14.7)	52 (18.3)	0.77 (0.25-1.93)	0.6055		
Daptomycin exposure	12 (3.8)	3 (8.8)	9 (3.2)	2.96 (0.63-10.52)	0.1177		
Metronidazole exposure	58 (18.2)	5 (14.7)	53 (18.7)	0.75 (0.25-1.88)	0.5735		
Linezolid exposure	44 (13.8)	2 (5.9)	42 (14.8)	0.36 (0.06-1.25)	0.172	0.26 (0.04-0.99)	0.0879
Polymyxin and/or colistin exposure	37 (11.6)	5 (14.7)	32 (11.3)	1.36 (0.44-3.50)	0.5559		
Tigecycline exposure	45 (14.2)	9 (26.5)	36 (12.7)	2.48 (1.03-5.58)	0.0337		
Vancomycin exposure	136 (42.8)	13 (38.2)	123 (43.3)	0.81 (0.38-1.66)	0.5724	0.46 (0.18-1.09)	0.0857
Piperacillin/Tazobactam exposure	43 (13.5)	3 (8.8)	40 (14.1)	0.59 (0.14-1.76)	0.4015		

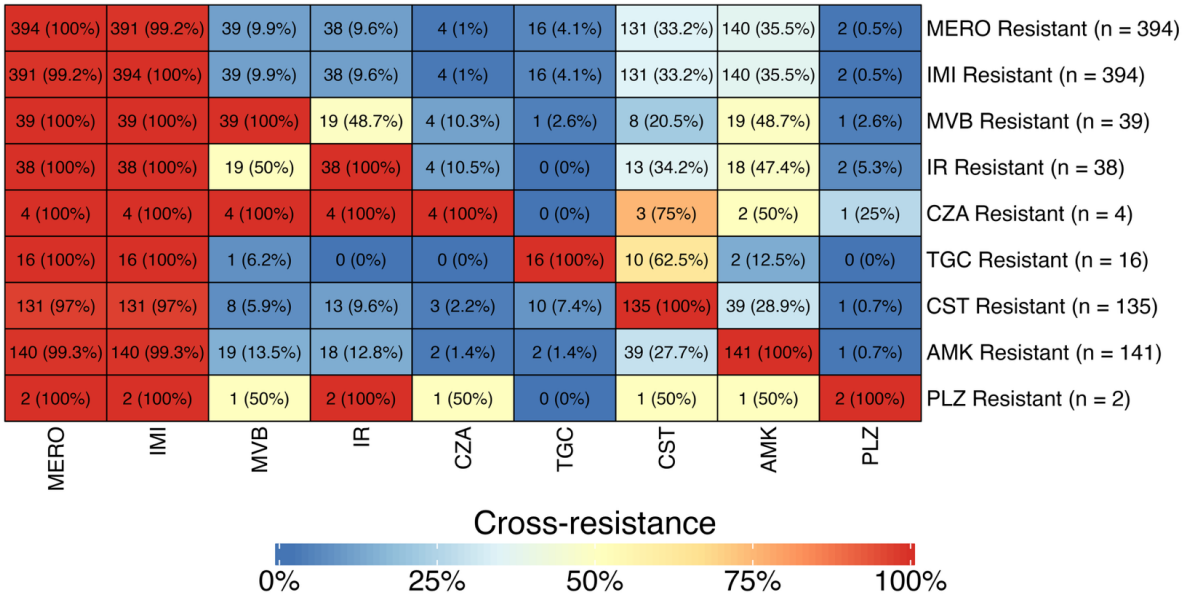
Appendix B Table 4 Unadjusted and Adjusted Analysis of Risk Factors for Non-Carbapenemase Mechanisms of Resistance to β-lactam/β-lactamase Inhibitor Combinations

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; NA, not applicable; OR, odds ratio.

Variable	Overall (n=302)	Resistant (n=18)	Susceptible (n=284)	OR (95% CI)	OR p-value	aOR (95% CI)	aOR p-value
Age. 1 year unit	70.38 (14.71)	76.63 (9.58)	69.98 (14.90)	1.04 (1.00-1.08)	0.0634	1.04 (1.00-1.08)	0.0774
Female sex	40.84 (115.43)	50.39 (104.52)	40.23 (116.23)	1.00 (0.99-1.00)	0.7227		
Length of stay before culture, 1-day unit	143 (47.4)	11 (61.1)	132 (46.5)	1.81 (0.69-5.04)	0.2336		
Tracheostomy tube	204 (67.5)	10 (55.6)	194 (68.3)	0.58 (0.22-1.57)	0.2673		
Central venous catheter	171 (56.6)	9 (50.0)	162 (57.0)	0.75 (0.29-1.98)	0.5599		
Indwelling urinary catheter	167 (55.3)	13 (72.2)	154 (54.2)	2.19 (0.80-6.99)	0.1451		
Gastrostomy tube	112 (37.1)	7 (38.9)	105 (37.0)	1.08 (0.39-2.84)	0.8703		
Acute kidney injury	142 (47.0)	11 (61.1)	131 (46.1)	1.84 (0.70-5.11)	0.2226		
Chronic kidney disease	99 (32.8)	7 (38.9)	92 (32.4)	1.33 (0.48-3.48)	0.5703		
Ventilator-dependent respiratory failure	91 (30.1)	6 (33.3)	85 (29.9)	1.17 (0.40-3.12)	0.7604		
Underweight or malnutrition	80 (26.5)	3 (16.7)	77 (27.1)	0.54 (0.12-1.68)	0.3371		
Congestive heart failure	60 (19.9)	5 (27.8)	55 (19.4)	1.60 (0.50-4.44)	0.3895		
Decubitus ulcer stage 4+	63 (20.9)	3 (16.7)	60 (21.1)	0.75 (0.17-2.35)	0.6526		
Chronic bronchitis or chronic obstructive pulmonary disorder	54 (17.9)	7 (38.9)	47 (16.5)	3.21 (1.13-8.58)	0.022	3.23 (1.11-8.94)	0.0252
Brain injury	57 (18.9)	5 (27.8)	52 (18.3)	1.72 (0.53-4.77)	0.3246		
Malignancy	38 (12.6)	3 (16.7)	35 (12.3)	1.42 (0.32-4.59)	0.5918		
Obesity	18 (6.0)	1 (5.6)	17 (6.0)	0.92 (0.05-4.94)	0.9404		
Carbapenem exposure	89 (29.5)	6 (33.3)	83 (29.2)	1.21 (0.41-3.23)	0.7112		
Amikacin exposure	36 (11.9)	3 (16.7)	33 (11.6)	1.52 (0.34-4.92)	0.5244		
Gentamicin exposure	10 (3.3)	0 (0.0)	10 (3.5)	0.00 (NA-NA)	0.9905		
Tobramycin exposure	17 (5.6)	1 (5.6)	16 (5.6)	0.99 (0.05-5.30)	0.9889		
Aztreonam exposure	10 (3.3)	0 (0.0)	10 (3.5)	0.00 (NA-NA)	0.9905		
Sulfamethoxazole-trimethoprim exposure	10 (3.3)	1 (5.6)	9 (3.2)	1.80 (0.09-10.42)	0.5883		
Cephalosporin exposure	71 (23.5)	7 (38.9)	64 (22.5)	2.19 (0.78-5.79)	0.1203	2.62 (0.91-7.25)	0.0649
Fluoroquinolone exposure	54 (17.9)	2 (11.1)	52 (18.3)	0.56 (0.09-2.04)	0.4456		
Daptomycin exposure	11 (3.6)	2 (11.1)	9 (3.2)	3.82 (0.55-16.44)	0.1034		
Metronidazole exposure	58 (19.2)	5 (27.8)	53 (18.7)	1.68 (0.52-4.66)	0.3457		
Linezolid exposure	46 (15.2)	4 (22.2)	42 (14.8)	1.65 (0.45-4.85)	0.399		
Polymyxin and/or colistin exposure	33 (10.9)	1 (5.6)	32 (11.3)	0.46 (0.03-2.38)	0.4619		
Tigecycline exposure	38 (12.6)	2 (11.1)	36 (12.7)	0.86 (0.13-3.20)	0.8462		
Vancomycin exposure	131 (43.4)	8 (44.4)	123 (43.3)	1.05 (0.39-2.73)	0.925		
Piperacillin/Tazobactam exposure	42 (13.9)	2 (11.1)	40 (14.1)	0.76 (0.12-2.82)	0.7244		

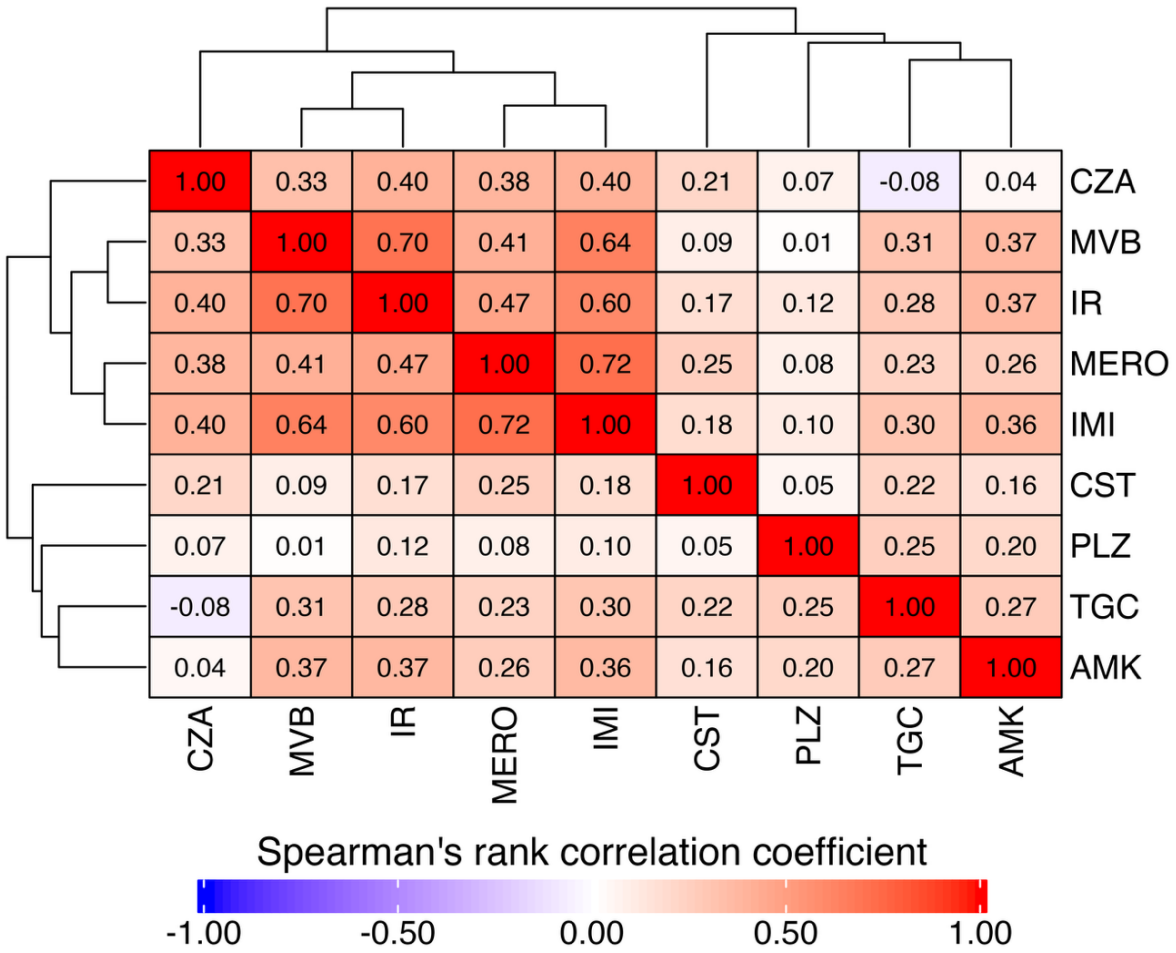
Appendix B Table 5 Unadjusted and Adjusted Analysis of Risk Factors for Plasmid-Mediated Mechanisms of Resistance to β-lactam/β-lactamase Inhibitor Combinations

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; NA, not applicable; OR, odds ratio.



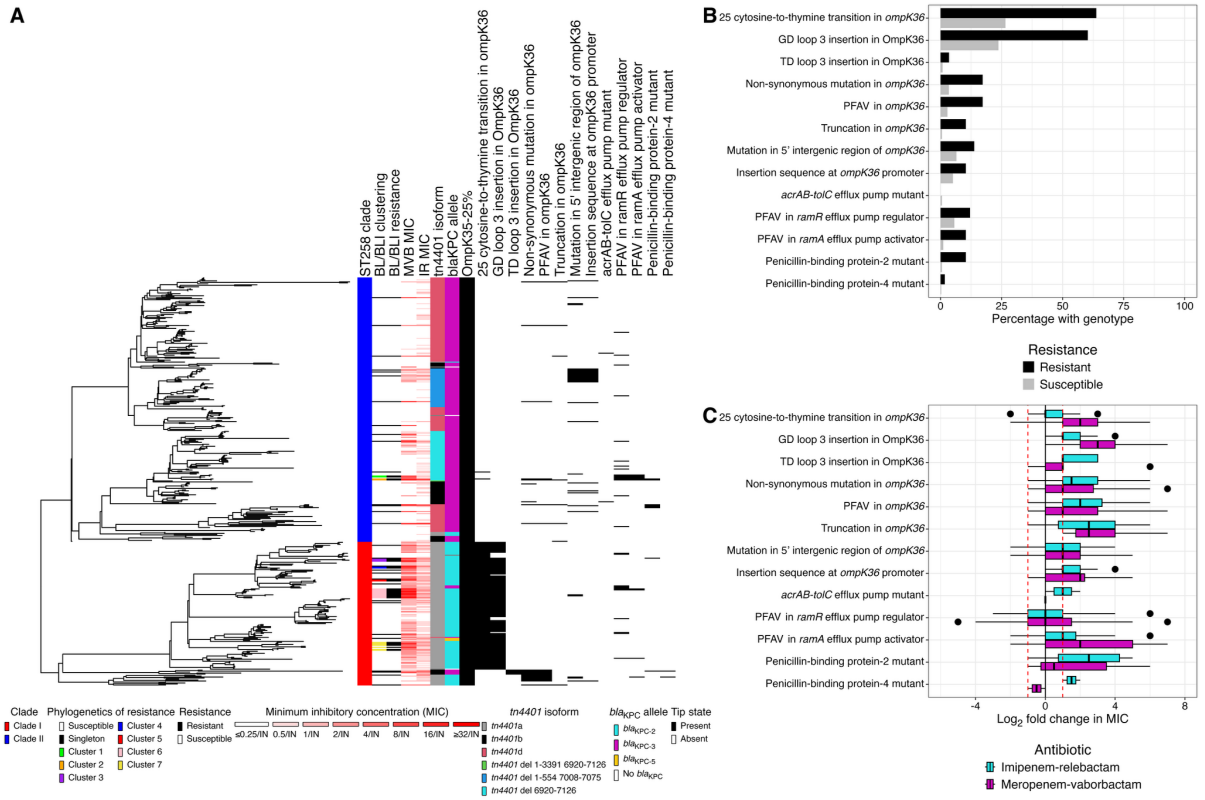
Appendix B Figure 1 Cross-Resistance Profile of Carbapenem-Resistant *Klebsiella pneumoniae* ST258 Isolates

The cross-resistance profile of 412 carbapenem-resistant *Klebsiella pneumoniae* sequence type 258 strains. This heatmap shows the number of resistant isolates (rows) that also exhibit resistance to other antibiotics (columns). Cross-resistance was evaluated for the following antibiotics: amikacin, colistin, ceftazidime-avibactam, imipenem, imipenem-relebactam, meropenem, meropenem-vaborbactam, plazomicin, and tigecycline. Resistance was defined as a minimum inhibitory concentration in the intermediate or resistant categories. Abbreviations: AMK, amikacin; CST, colistin; CZA, ceftazidime-avibactam; IMI, imipenem; IR, imipenem-relebactam; MERO, meropenem, MVB, meropenem-vaborbactam; PLZ, plazomicin; TGC, tigecycline.



Appendix B Figure 2 Spearman's Rank Correlation Coefficients Between the Minimum Inhibitory Concentration of Last-Line and Novel Agents

Spearman's rank correlation coefficient between minimum-inhibitory concentrations of last-line and novel agents for 412 carbapenem-resistant *Klebsiella pneumoniae* sequence type 258 strains. The following antibiotics were evaluated: amikacin, colistin, ceftazidime-avibactam, imipenem, imipenem-relebactam, meropenem, meropenem-vaborbactam, plazomicin, and tigecycline. Abbreviations: AMK, amikacin; CST, colistin; CZA, ceftazidime-avibactam; IMI, imipenem; IR, imipenem-relebactam; MERO, meropenem, MVB, meropenem-vaborbactam; PLZ, plazomicin; TGC, tigecycline.

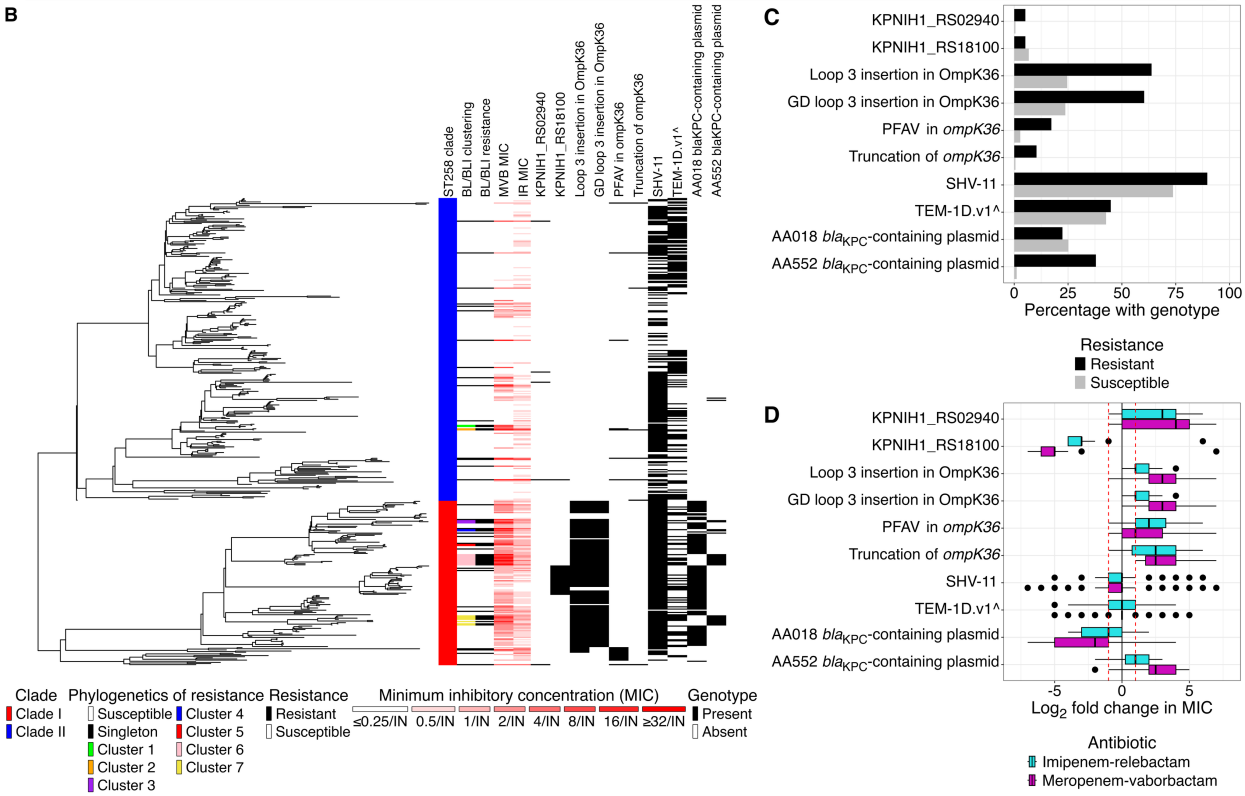


Appendix B Figure 3 Clade-Specific Differences in Non-Carbapenemase Mechanisms of Carbapenem Resistance

(A) Mechanisms of carbapenem resistance were overlaid across the sequence type 258 phylogeny. (B) The frequency of genotypes in β-lactam/β-lactamase inhibitor combination susceptible and resistant isolates. (C) The $\log_2$ fold change in imipenem-relebactam and meropenem-vaborbactam minimum inhibitory concentration for isolates with the genotype relative to their nearest phylogenetic neighbor without the genotype. Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; GD, Glycine-Aspirate; KPC, *Klebsiella pneumoniae* carbapenemase; IR, imipenem-relebactam; MVB, meropenem-vaborbactam; PFAV, putative function-altering variant.

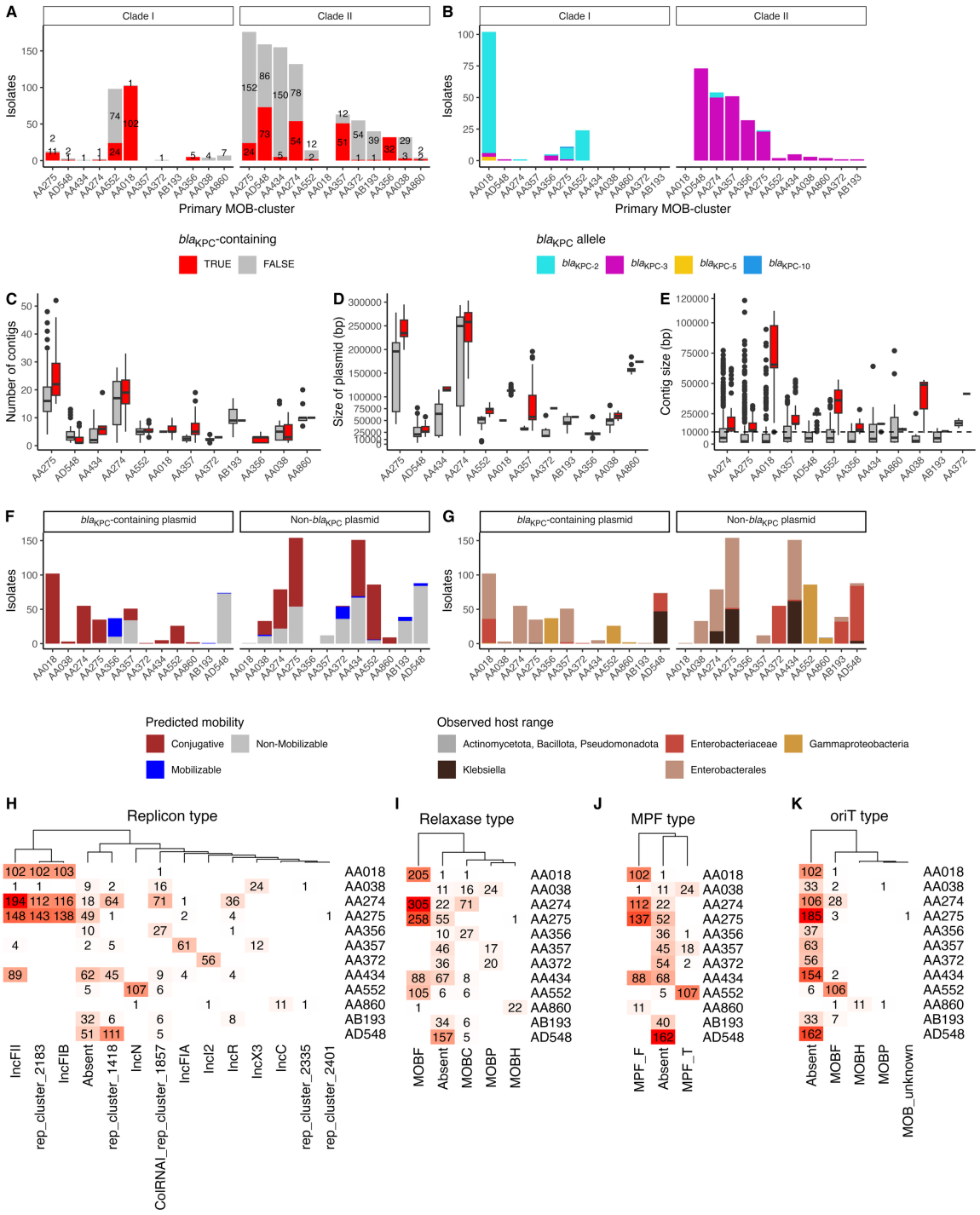
A

Significant hit	Gene	Product	Significant tests	Significant models	Phenotypes	Nearest neighbor criterion
KPNIH1_RS02940		hypothetical protein	Continuous	All variants & Non-synonymous	MVB	Pass
KPNIH1_RS18100		MdtA/MuxA family multidrug efflux RND transporter periplasmic adaptor subunit	Continuous	Putative function-altering variants	IR	Pass
Loop 3 insertion in OmpK36	*ompK36*	porin OmpC	Continuous	Kleborate	MVB	Pass
GD loop 3 insertion in OmpK36	*ompK36*	porin OmpC	Continuous	Kleborate	MVB	Pass
PFAV in *ompK36*	*ompK36*	porin OmpC	All tests	All variant models	BL/BLI & IR & MVB	Pass
Truncation of *ompK36*	*ompK36*	porin OmpC	All tests	Kleborate	BL/BLI & IR & MVB	Pass
SHV-11		Broad-spectrum β-lactamase	Synchronous	Kleborate	BL/BLI	Fail
TEM-1D.v1^		Broad-spectrum β-lactamase	Synchronous	Kleborate	BL/BLI	Fail
AA018 bla_{KPC}-containing plasmid		bla_{KPC}-containing plasmid cluster	Synchronous	bla_{KPC}-containing plasmids	BL/BLI	Pass
AA552 bla_{KPC}-containing plasmid		bla_{KPC}-containing plasmid cluster	PhyC & Synchronous	bla_{KPC}-containing plasmids	BL/BLI	Pass



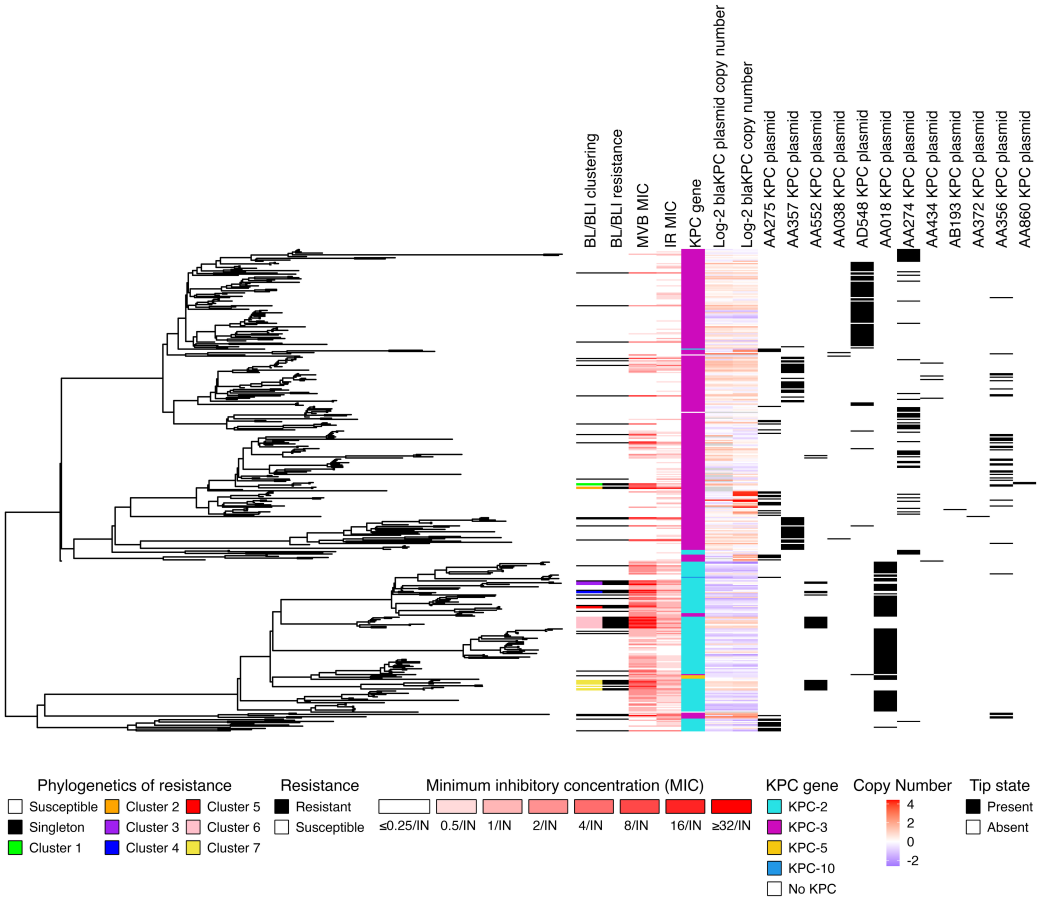
Appendix B Figure 4 All Significant Genome-Wide Association Study Hits for β-lactam/β-lactamase Inhibitor Resistance

A convergence-based genome-wide association study was performed on $\log_2$ minimum inhibitory concentration and resistance to β-lactam/β-lactamase inhibitor combinations using hogwash v.1.2.6. Hits that passed the nearest-neighbor criterion (median $\log_2$ fold change in MIC > |1|) were eligible for downstream analysis. (A) Information about each significant genome-wide association study hit. (B) The significant hits are overlaid on the phylogenetic tree. (C) The frequency of genotypes in β-lactam/β-lactamase inhibitor combination susceptible and resistant isolates. (D) The $\log_2$ fold change in imipenem-relebactam and meropenem-vaborbactam minimum inhibitory concentration for isolates with the genotype relative to their nearest phylogenetic neighbor. Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; GD, Glycine-Aspirate; IR, imipenem-relebactam; MVB, meropenem-vaborbactam; PFAV, putative function-altering variant.



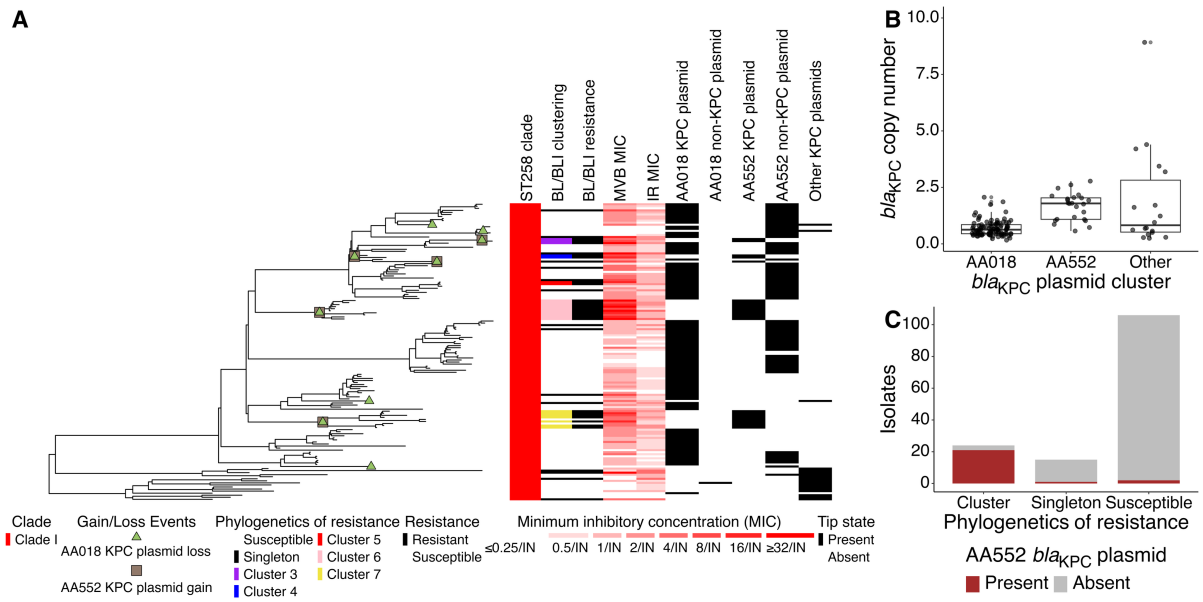
Appendix B Figure 5 Characteristics of Carbapenemase-Containing Plasmids

(A) The frequency of plasmids associated with *Klebsiella pneumoniae* carbapenemase, *bla*KPC, are displayed, with the distribution across clades and presence of the carbapenemase detailed. (B) For *bla*KPC-containing plasmids, their *Klebsiella pneumoniae* carbapenemase (KPC) allele is detailed. Distributions for the (C) number of contigs, (D**)** size of the plasmid, and (E) size of the *bla*KPC-containing contigs are detailed. The (F) predicted mobility and (G) observed host range for *bla*KPC-containing and non- *bla*KPC-containing plasmids. The (H) replicon, (I) relaxase, (J) mating pair formation (MPF) type, and (K) origin of transcription (oriT) type are detailed for all plasmids.



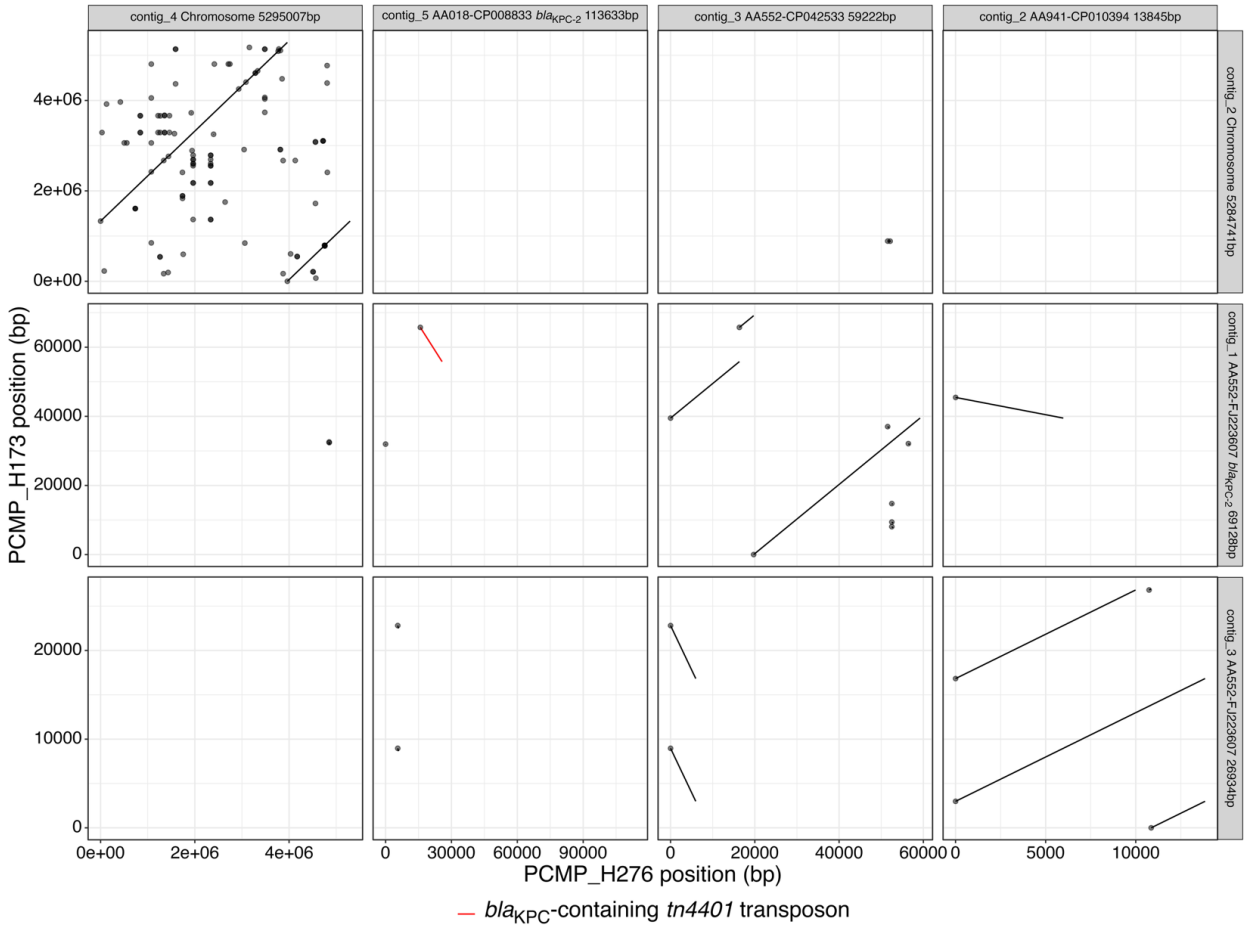
Appendix B Figure 6 Phylogenetic Distribution of Carbapenemase-Containing Plasmids

Measurements of resistance to β-lactam/β-lactamase inhibitor combinations were overlaid on the phylogeny. The *Klebsiella pneumoniae* carbapenemase (KPC) allele and the $\log_2$-transformed sequencing-inferred copy number of the carbapenemase gene, $bla_{KPC,}$ and its plasmid are represented across the phylogeny. Next, the presence of bla_{KPC}-containing plasmids is detailed on the phylogeny.



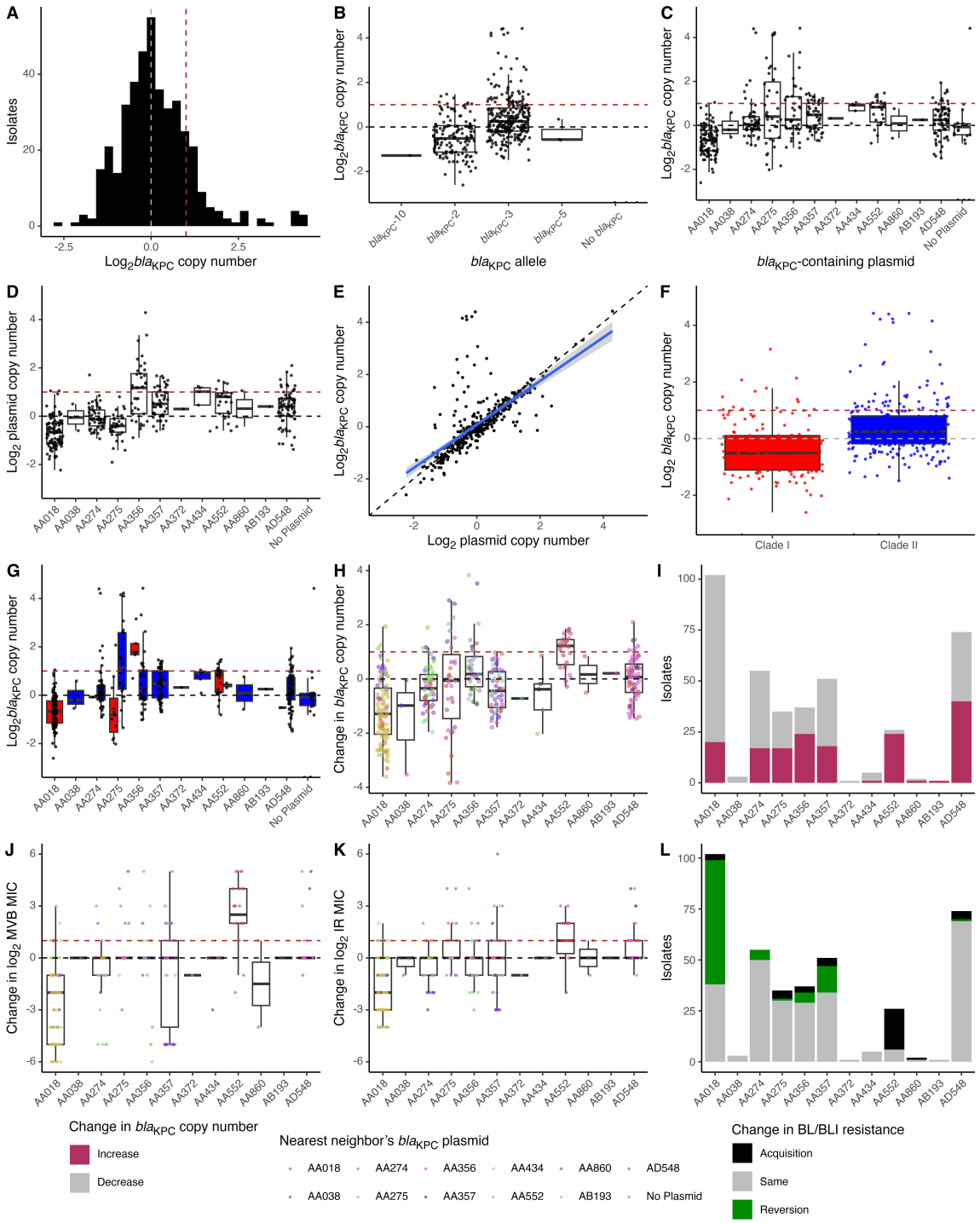
Appendix B Figure 7 Differences in *bla*$_{KPC}$ Plasmid Backbone Contribute to Elevated *bla*$_{KPC}$ Copy Number and the Spread of Resistance in Clade I

(A) The presence of dominant *Klebsiella pneumoniae* carbapenemase (*bla*$_{KPC}$)-containing plasmids across clade I. The emergence of resistance and select resistance-associated genotypes is overlaid on ancestral nodes. (B) Differences in *bla*$_{KPC}$ copy number across *bla*$_{KPC}$ plasmids. (C) The frequency of *bla*$_{KPC}$-containing AA552 plasmids in β-lactam/β-lactamase inhibitor resistant clusters, singletons, and susceptible isolates. Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; IR, imipenem-relebactam; MVB, meropenem-vaborbactam; PFAV, putative function-altering variant.



Appendix B Figure 8 Evidence of *tn4401* *bla*$_{KPC}$ Transposon Hopping from AA018 Plasmid to the AA552 Plasmid

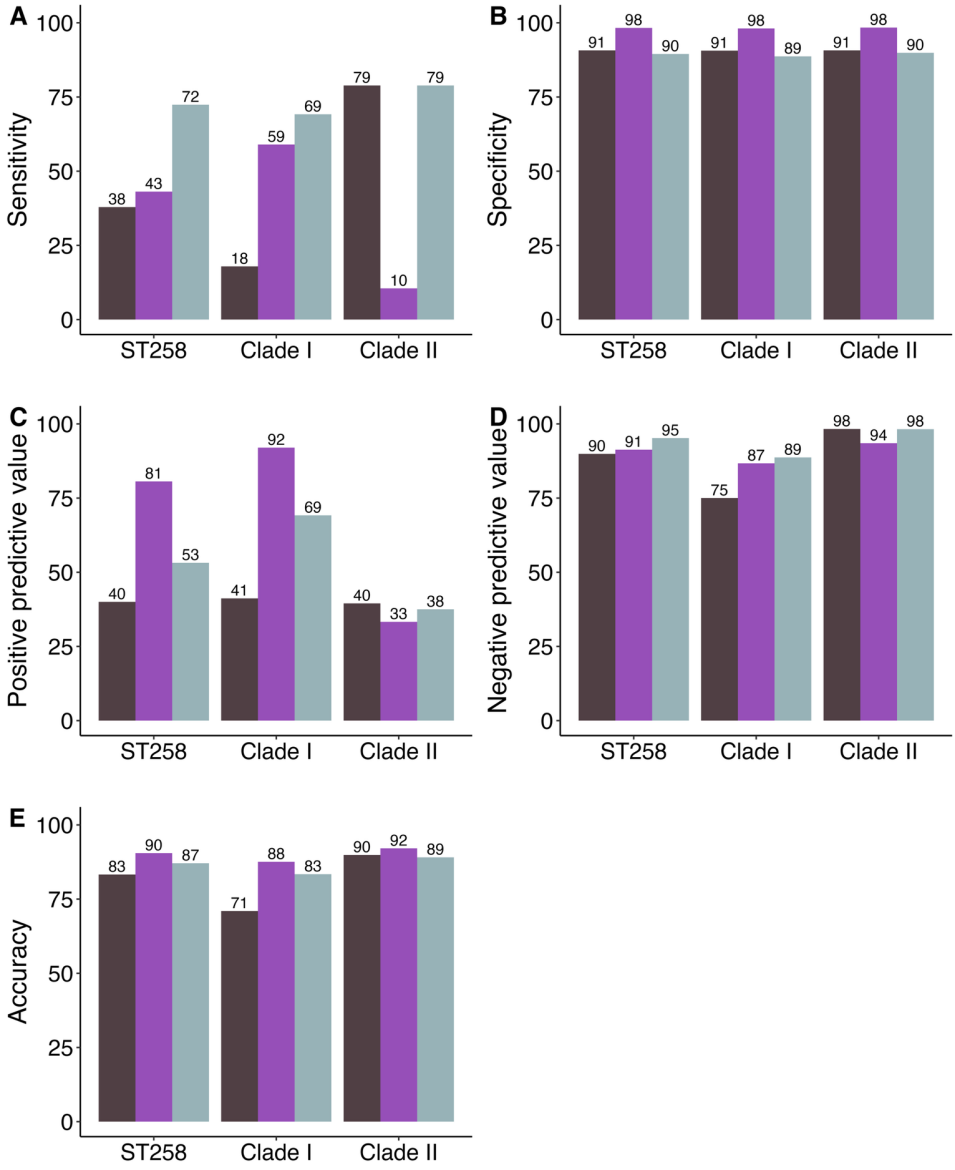
Long-read sequencing was performed on a representative clade I isolate with *Klebsiella pneumoniae* carbapenemase (*bla*$_{KPC}$)-containing AA552 plasmid and a closely related isolate with a *bla*$_{KPC}$-containing AA018 plasmid. Grids were named using the isolate's contig name, Mob-Suite plasmid calls (primary cluster – mash nearest-neighbor), the presence of the *bla*$_{KPC}$ gene, and contig size. The bla$_{KPC}$ transposon, *tn4401*, was labeled in red.



Appendix B Figure 9 Carbapenemase Copy Number and Resistance Patterns Across Carbapenemase-Containing Plasmid Clusters

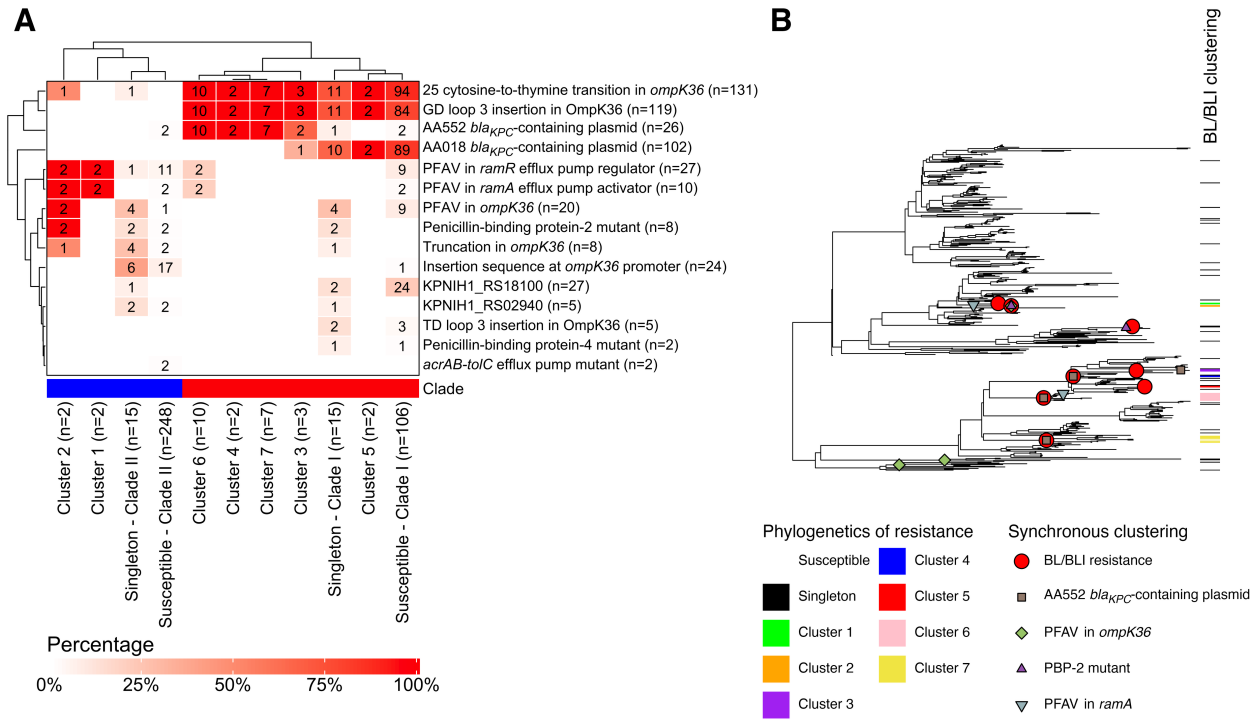
(A) The distribution of log2-transformed *bla*KPC copy number. (B) Copy number distribution across *bla*KPC alleles. (C) The *bla*KPC copy number distribution across *bla*KPC-containing plasmids. (D) Copy number of the *bla*KPC-containing plasmids. (E) Correlation between the copy number of *bla*KPC and their corresponding plasmid. (F) Differences in *bla*KPC copy number across clades. (G) Copy number of *bla*KPC-containing plasmids, stratified by clade. Next, isolates harboring a *bla*KPC-containing were compared to their phylogenetic nearest neighbor without the *bla*KPC-containing plasmid. (H) The change in *bla*KPC copy number, (I) directional change in copy number,

change in (K) meropenem-vaborbactam (MVB) and (K) imipenem-relebactam (IR) minimum-inhibitory concentration (MIC) are detailed. (I) The consistency in changes to resistance status upon phylogenetic neighbor comparison.



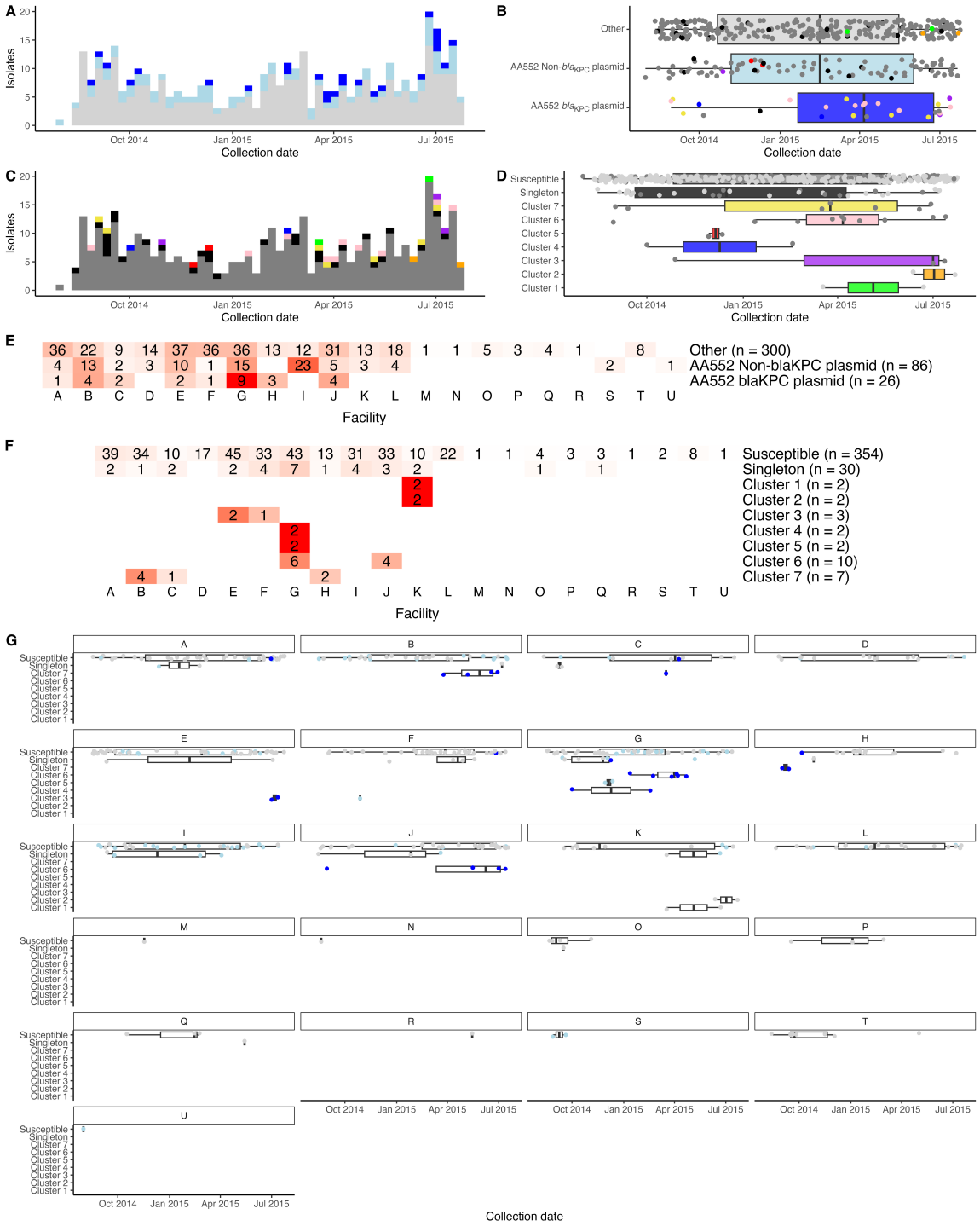
Appendix B Figure 10 Performance Statistics for Resistance and Our Genotype Panels

The sensitivity (A), specificity (B), positive predictive value (C), negative predictive value (D), and accuracy (E) of our genotype panels for resistance to β-lactam/β-lactamase inhibitor combinations. The following genotypes were considered in our known carbapenem resistance-associated panel: truncations and putative function-altering variants in *ompK36*, insertion sequences near the *ompK36* promoter, putative function-altering variants in *ramA* efflux pump activator, and non-synonymous mutations in PBPs. The following novel hits were identified in our genome-wide association study: *Klebsiella pneumoniae* carbapenemase (*bla*$_{KPC}$)-containing AA552 plasmid and the hypothetical protein, KPNIH1_RS02940. Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; IR, imipenem-relebactam; MVB, meropenem-vaborbactam; PFAV, putative function-altering variant.



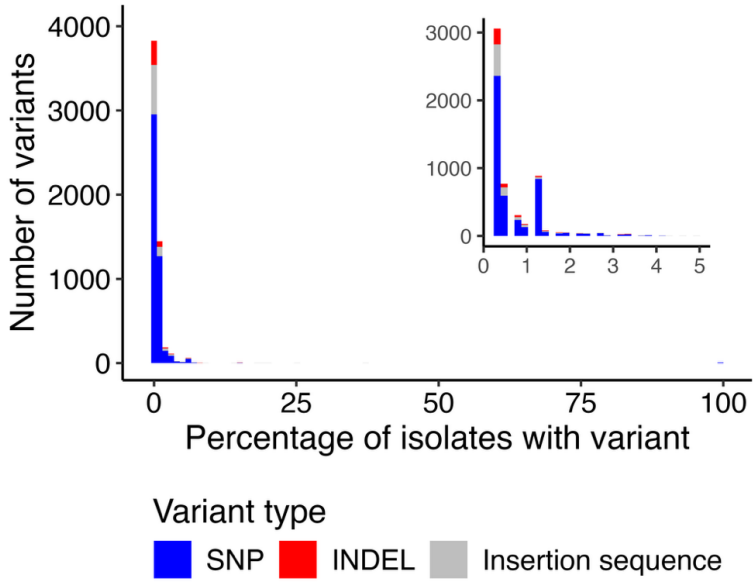
Appendix B Figure 11 Differential Potential for Emergence and Spread of β-lactam/β-lactamase Inhibitor Resistance-Associated Genotypes

(A) The frequency of genotypes in phylogenetic singletons of β-lactam/β-lactamase inhibitor (BL/BLI) resistance, clusters of BL/BLI resistance, and susceptible isolates. (B). The synchronous clustering of BL/BLI resistance and resistance genotypes is overlaid on ancestral nodes. Abbreviations: BL/BLI, β-lactam/β-lactamase inhibitor; GD, Glycine-Aspirate; PFAV, putative function-altering variants; PBP, penicillin-binding protein.



Appendix B Figure 12 Spatiotemporal Distribution of Resistant Isolates

(A) Distribution of study isolates, with color detailing the presence of AA552 plasmids. (B) Distribution of isolates across the study period, stratified by the presence of AA552 plasmids. (C) Distribution of study isolates, with color depicting the resistance status. (D) Distribution of isolates across the study period, stratified by susceptible, resistant singletons, and resistant cluster membership. (E) Distribution of AA552 plasmid-containing isolates across study facilities. (F) Distribution of resistant isolates across study facilities. (G) Spatiotemporal distribution of isolates. The y-axis indicates the resistance status of the isolate. Points detail isolate collection date, with color denoting the presence of AA552 plasmids.

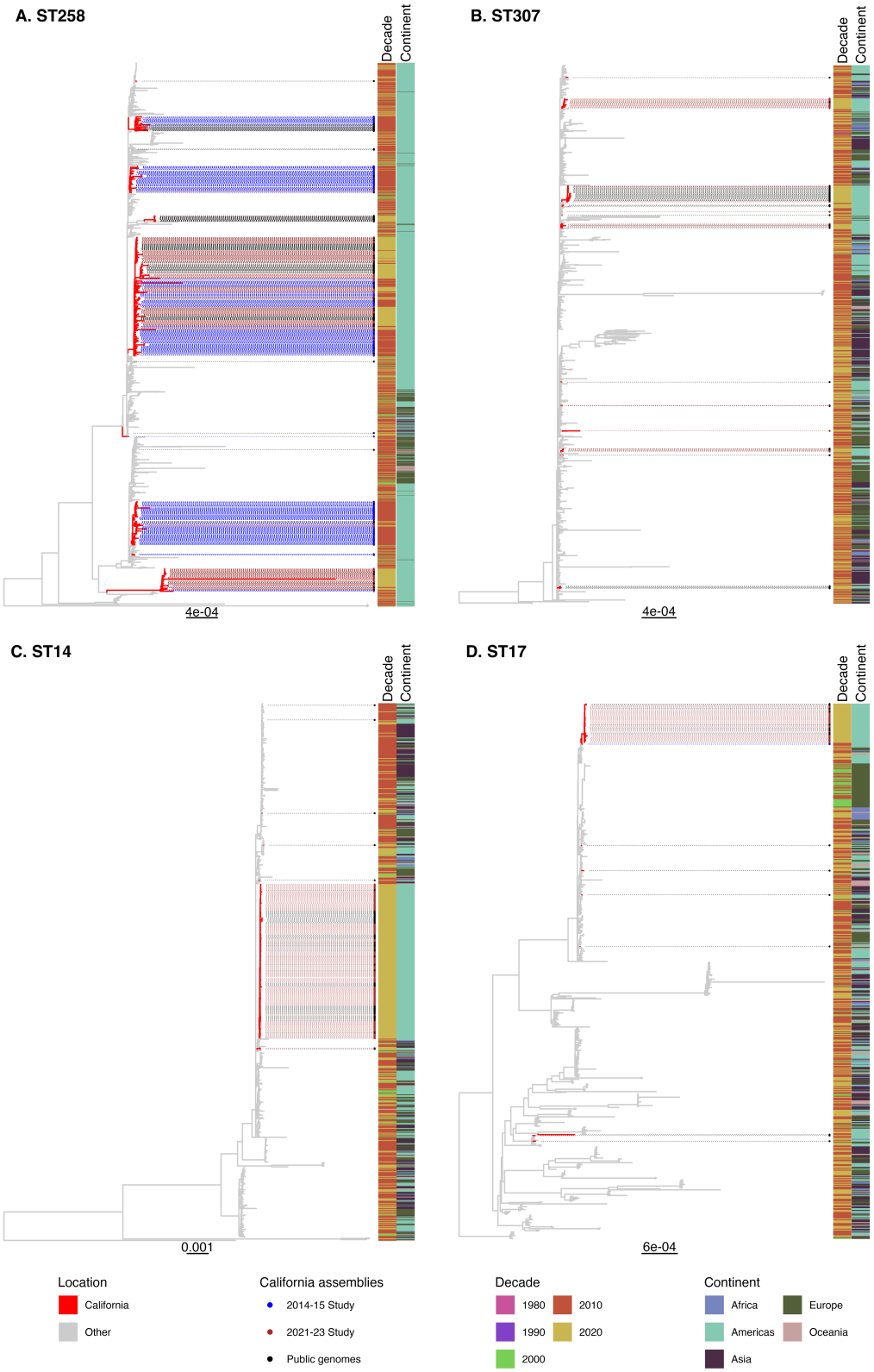


Appendix B Figure 13 Percentage of Isolates with a Core Genome Variant

The percentage of isolates with considered variants: single-nucleotide polymorphisms, insertions, deletions, and insertion sequences. The figure insert highlights the distribution of variants found in fewer than five percent of isolates. Abbreviations: INDEL, insertion or deletion; SNP, single-nucleotide polymorphism.

Appendix C: Chapter 4 Supplemental Material

Figures



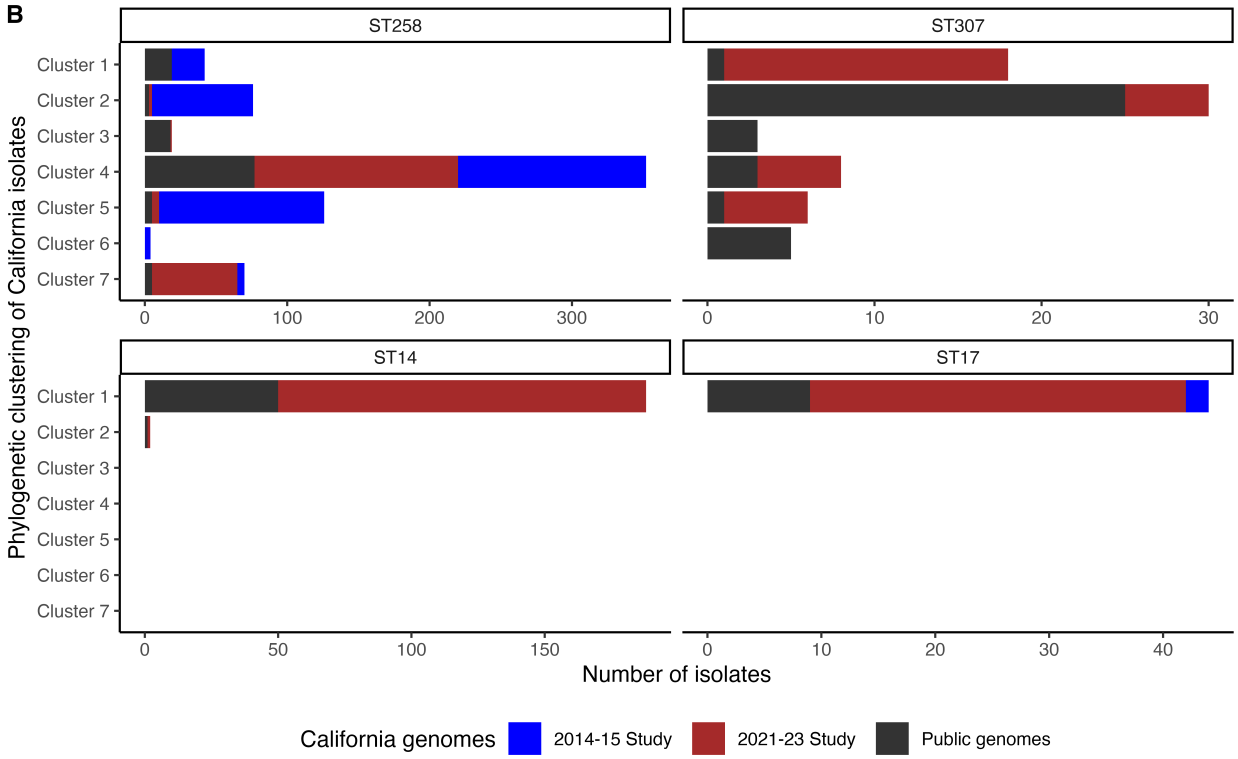
Appendix C Figure 1 Phylogeographic Analysis Reveals Numerous Introductions and Clonal Expansions of Epidemic Lineages in California

Public *K. pneumoniae* genomes were downloaded from the NCBI Pathogen database on December 10, 2025. Quality-control passing genomes with country and collection date metadata, which we included for phylogenetic reconstruction alongside our study isolates using core single-nucleotide polymorphisms. The spatiotemporal data are displayed on each maximum-likelihood phylogeny. Additionally, the tips of California isolates are colored denote whether they are a public genome or belong to either of our isolate collections. Ancestral state reconstruction was performed using *phyloAMR* to characterize the phylogenetic clustering of California isolates. Ancestral states are denoted by edge color. Ancestral state reconstruction of California. Oversampling was mitigated by retaining representative isolates with distinct collection-decade and continent combinations in subtrees that did not contain California isolates.

A

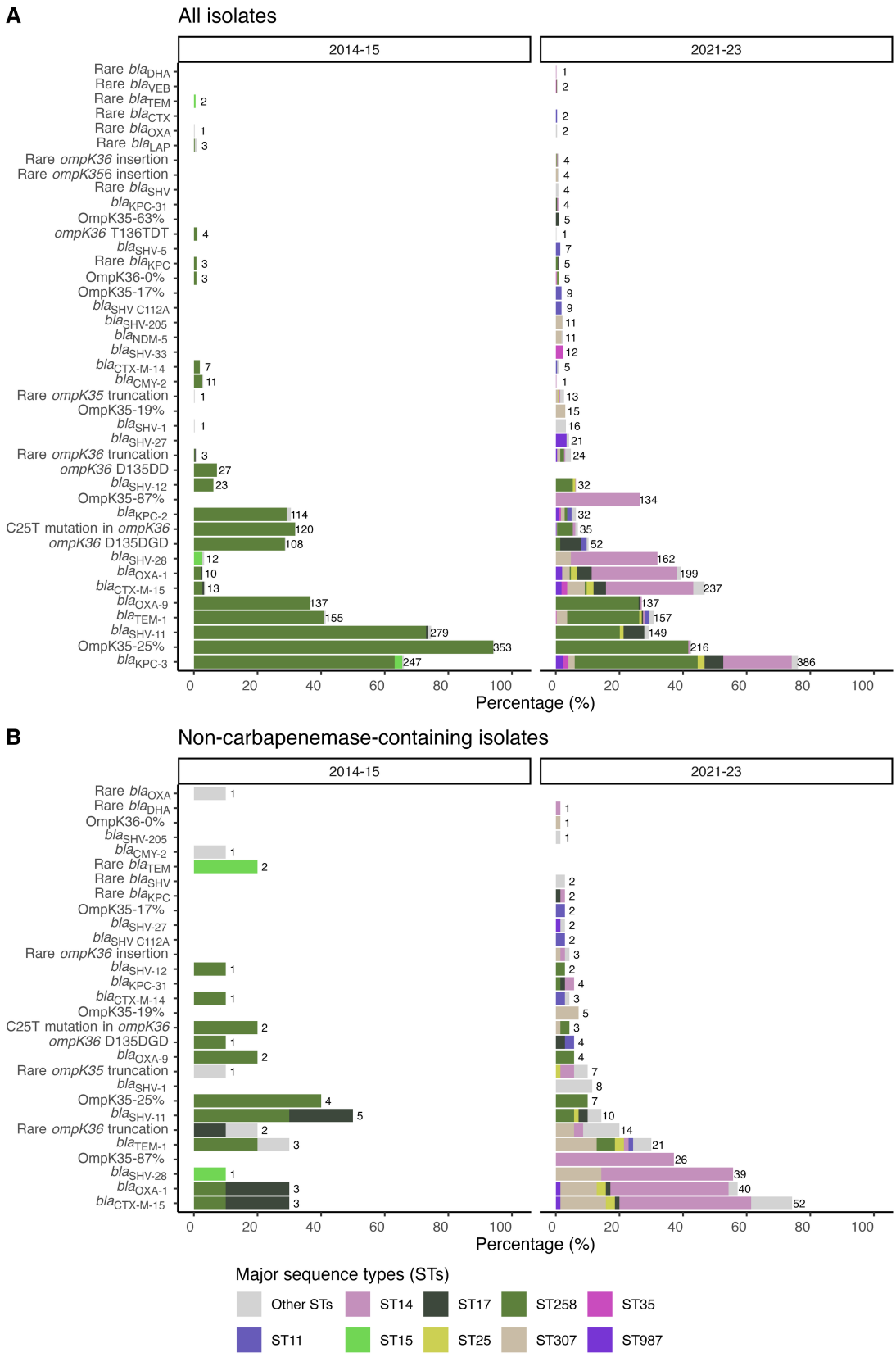
Sequence type (ST)	California isolates	Other genomes	Phylogenetic events	Singletons	Singletons (2014-15 study)	Singletons (2021-23 study)	Singletons (Public)	Clusters	Cluster isolates	Cluster size median (range)
ST258	696	1047	14	7	1	0	6	7	689	70 (4-352)
ST307	78	893	14	8	0	3	5	6	70	7 (3-30)
ST14	195	462	7	5	0	0	5	2	190	95 (2-188)
ST17	51	521	8	7	0	0	7	1	44	44 (44-44)

B



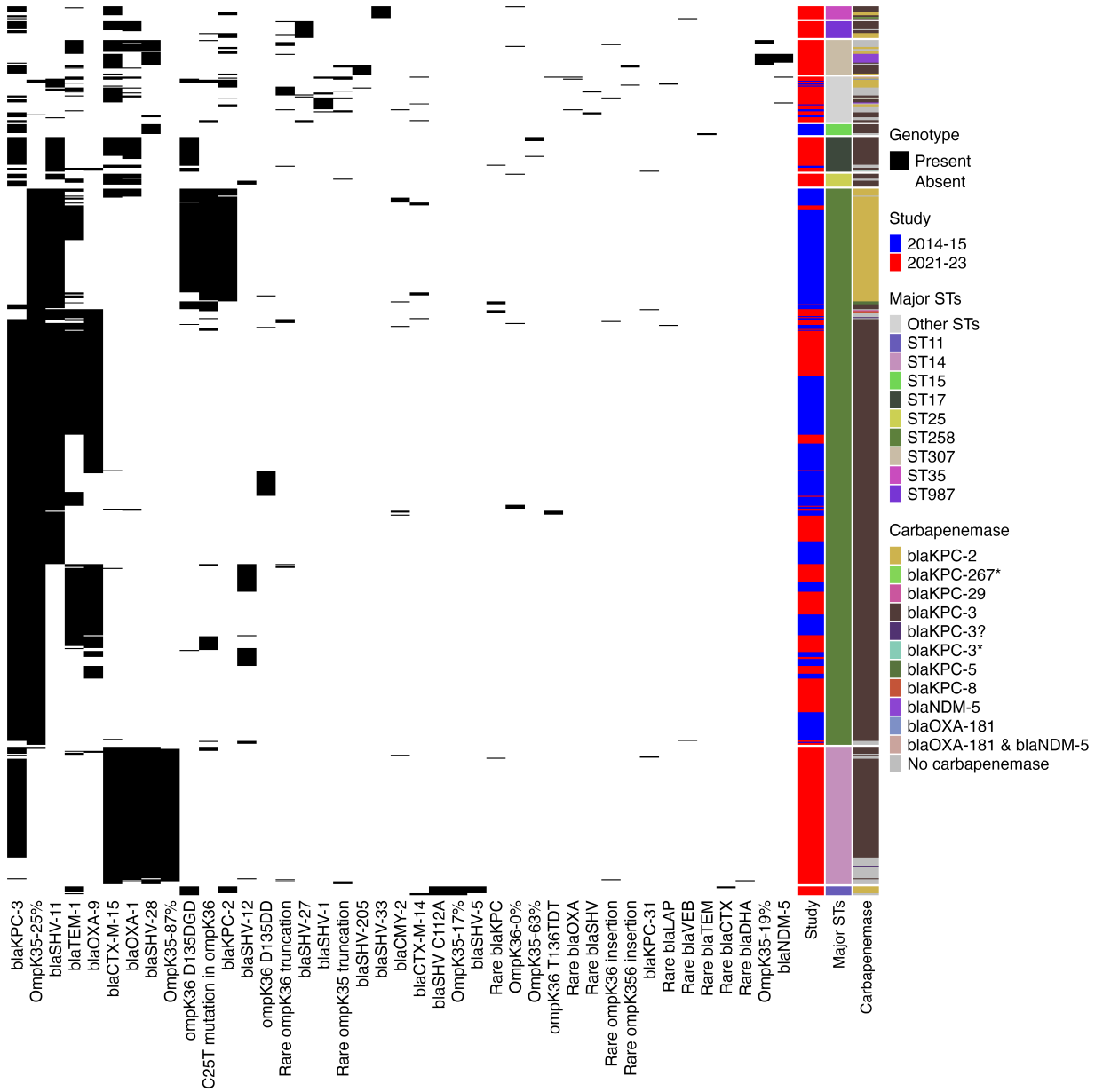
Appendix C Figure 2 Phylogenetic Clustering Patterns of California Isolates Across Major Epidemic Lineages

Public *K. pneumoniae* genomes were downloaded from the NCBI Pathogen database on December 10, 2025. Quality-control passing genomes with country and collection date metadata, which we included for phylogenetic reconstruction alongside our study isolates using core single-nucleotide polymorphisms. (A) Phylogenetic clustering of California isolates inferred from ancestral state reconstruction. (B) The size and composition of phylogenetic clusters of California isolates are reported across major sequence types.



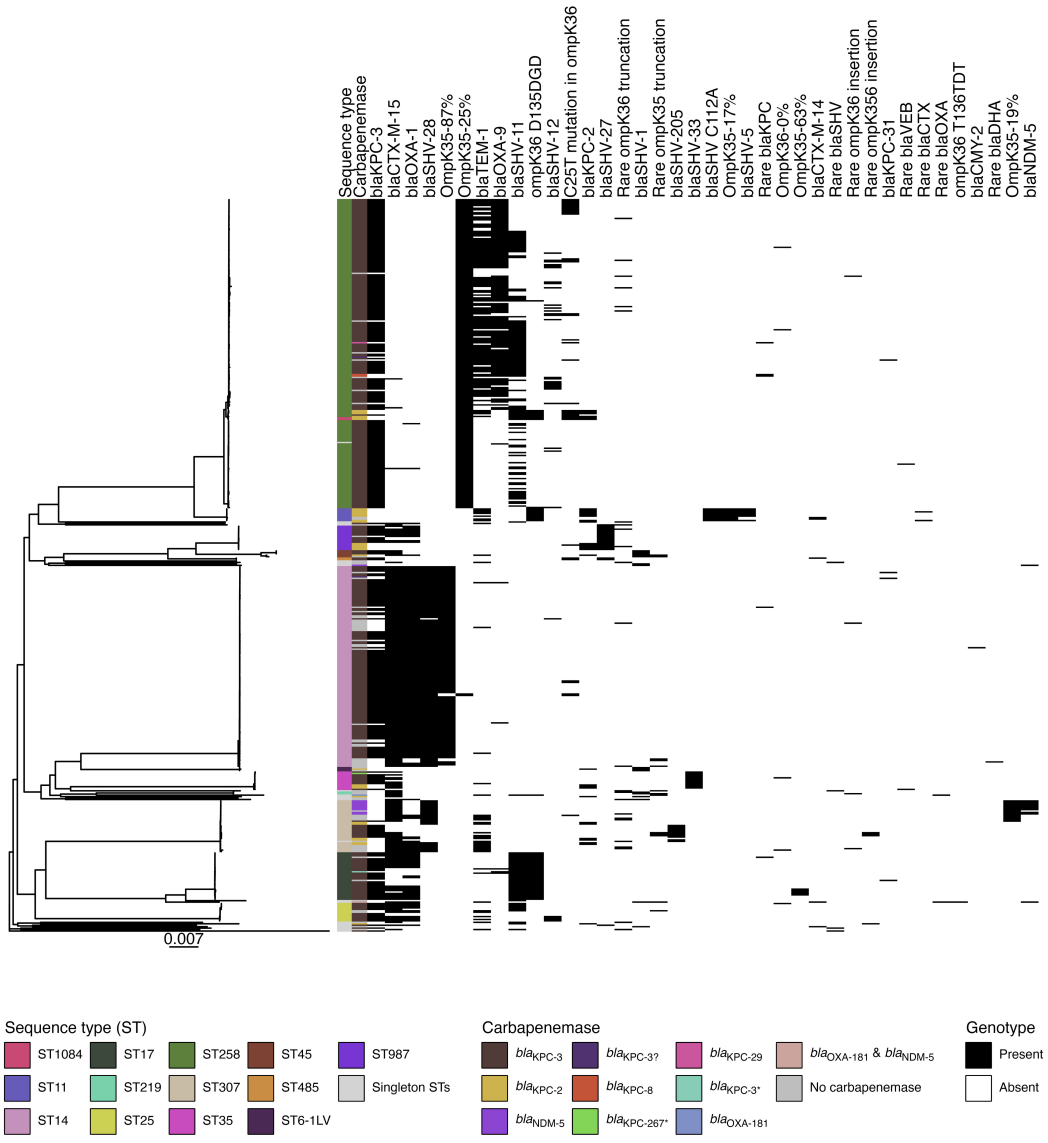
Appendix C Figure 3 Prevalence of β-lactamase and Porin Mutations Across Study Periods

Carbapenem-resistant *Klebsiella pneumoniae* isolates were screened for β-lactamases and major porin modifications using AMRFinderPlus and Kleborate. Truncations were denoted by their alignment coverage. For example, OmpK35-25% denotes a truncation at 25%. Additionally, genes with identity to the reference < 100% were denoted with "*", whereas genes with coverage < 100% were denoted with "?". (A) Frequency of carbapenem resistance-associated genotypes across study periods. (B) Frequency of carbapenem resistance-associated genotypes in non-carbapenemase-containing isolates across study periods. Rare genotypes found in less than five isolates were grouped by β-lactamase family or porin.



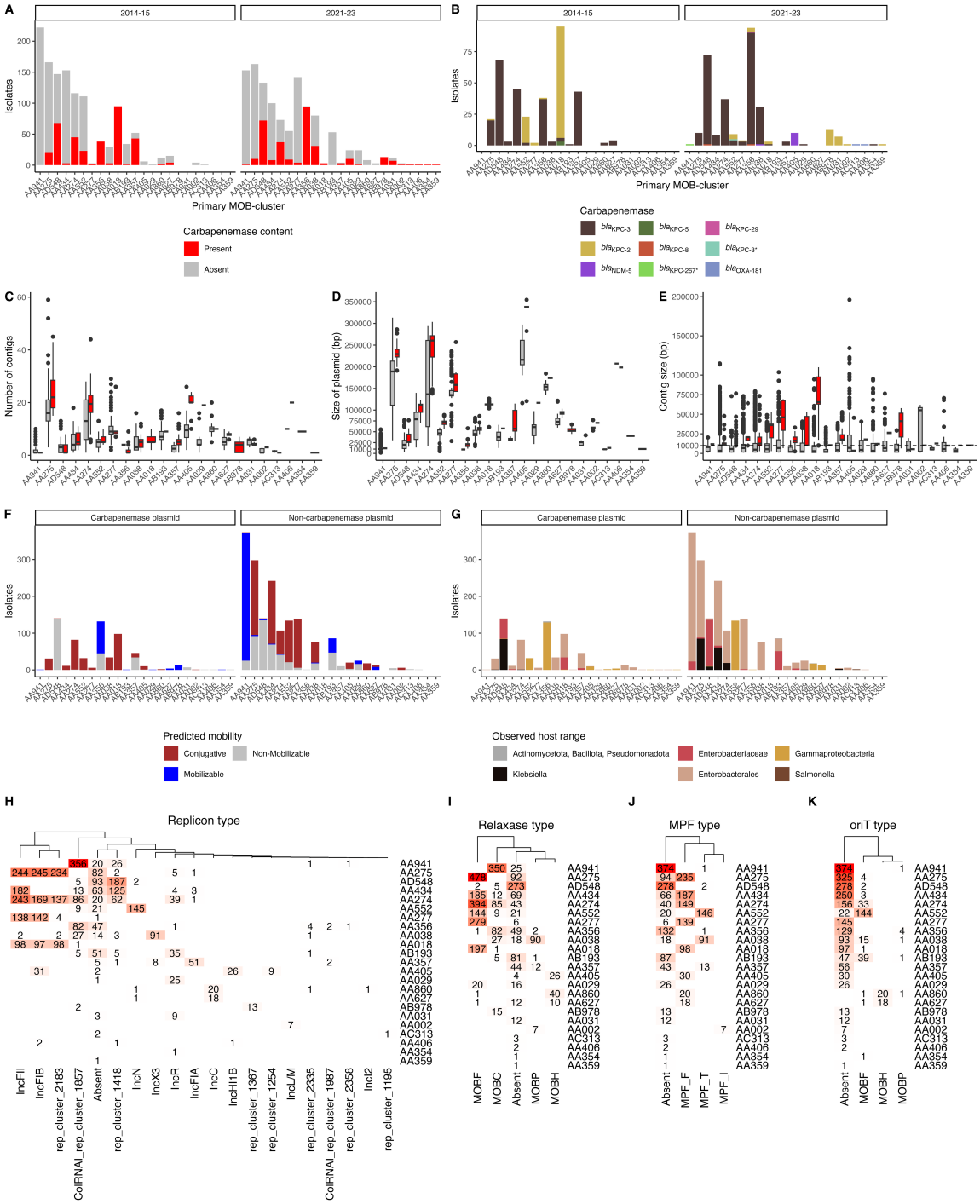
Appendix C Figure 4 Co-Occurrence of β-lactamases and Porin Modification in Carbapenem-Resistant *Klebsiella pneumoniae* Lineages Across Study Periods

Carbapenem-resistant *Klebsiella pneumoniae* isolates were screened for β-lactamases and major porin modifications using AMRFinderPlus and Kleborate. Truncations were denoted by their alignment coverage. For example, OmpK35-25% denotes a truncation at 25%. Additionally, genes with identity to the reference < 100% were denoted with "*", whereas genes with coverage < 100% were denoted with "?". Rare genotypes found in less than five isolates were grouped by β-lactamase family or porin. Isolates were partitioned into major sequence types. Intra-group clustering was performed to detail genotype clustering across study periods.



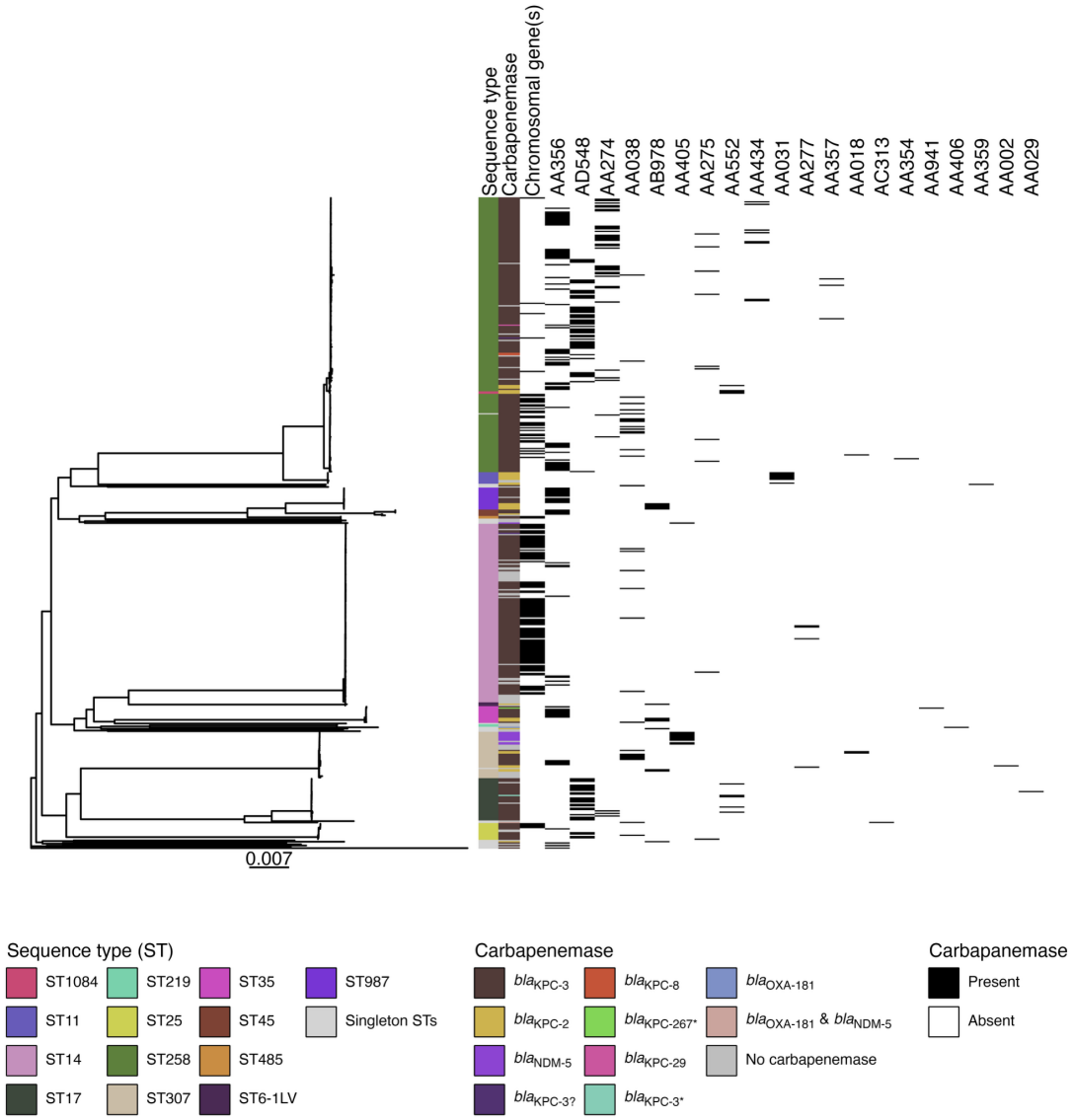
Appendix C Figure 5 Phylogenetic Distribution of β-lactamases and Porin Modification Across the 2021-23 Study

Carbapenem-resistant *Klebsiella pneumoniae* isolates were screened for β-lactamases and major porin modifications using AMRFinderPlus and Kleborate. Truncations were denoted by their alignment coverage. For example, OmpK35-25% denotes a truncation at 25%. Additionally, carbapenemases with identity to the reference < 100% were denoted with "*", whereas genes with coverage < 100% were denoted with "?". Rare genotypes found in less than five isolates were grouped by β-lactamase family or porin.



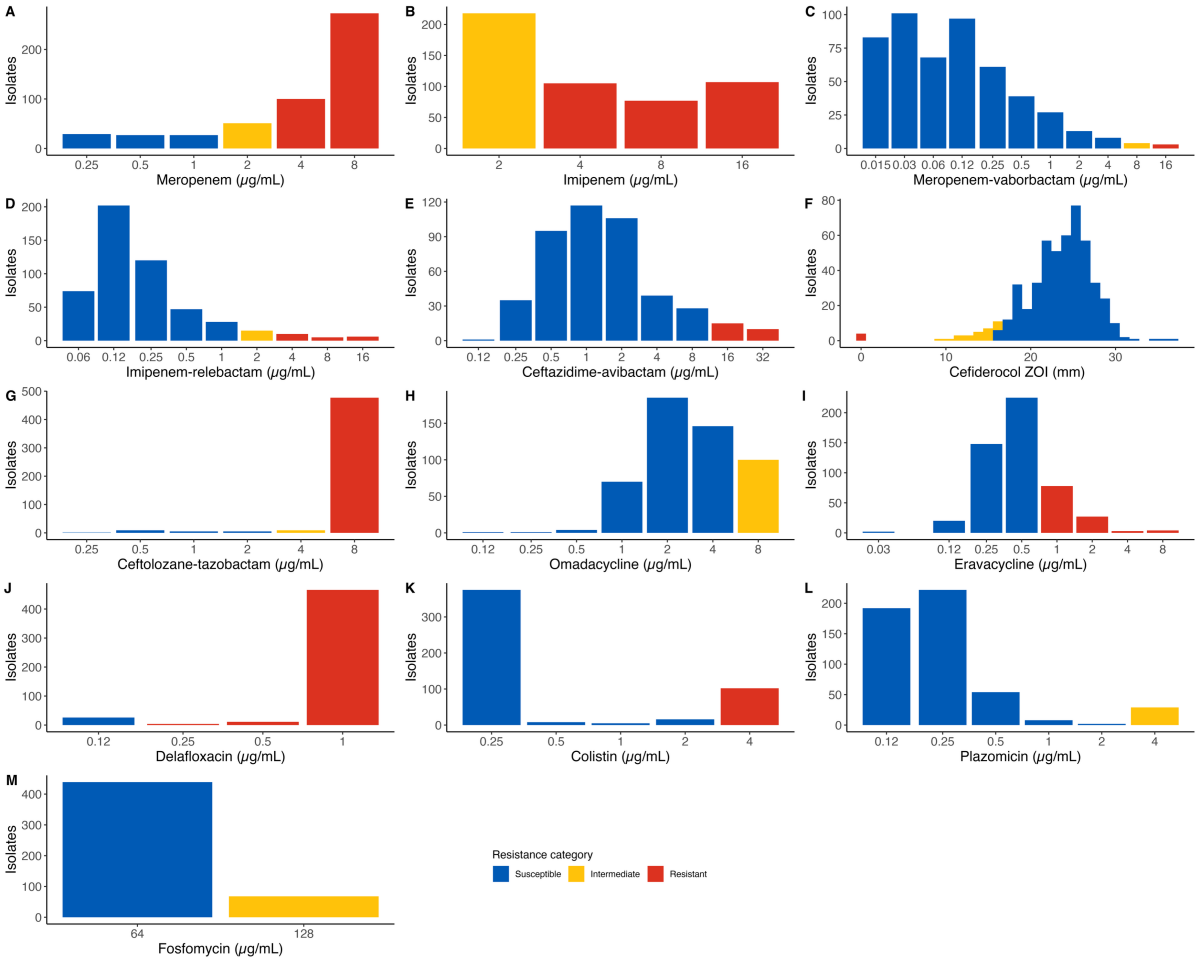
Appendix C Figure 6 Characterization of Carbapenemase-Containing Plasmid Clusters

The characteristics of each MOB-suite-identified carbapenemase-containing plasmid cluster. (A) The frequency of each plasmid cluster is denoted by carbapenemase content. (B) The specific carbapenemases identified in each plasmid cluster. (C) The number of short-read contigs for each plasmid call. (D) The size of each plasmid. (E) Contig size for each contig, stratified by the presence of carbapenemases. (F) The predicted mobility for each plasmid cluster, stratified by carbapenemase content. (G) The observed host range for each plasmid cluster, stratified by carbapenemase content. The (H) replicon type, (I) relaxase type, (J) mating pair formation (MPF) type, and (K) origin of transfer (oriT) type for each plasmid cluster.



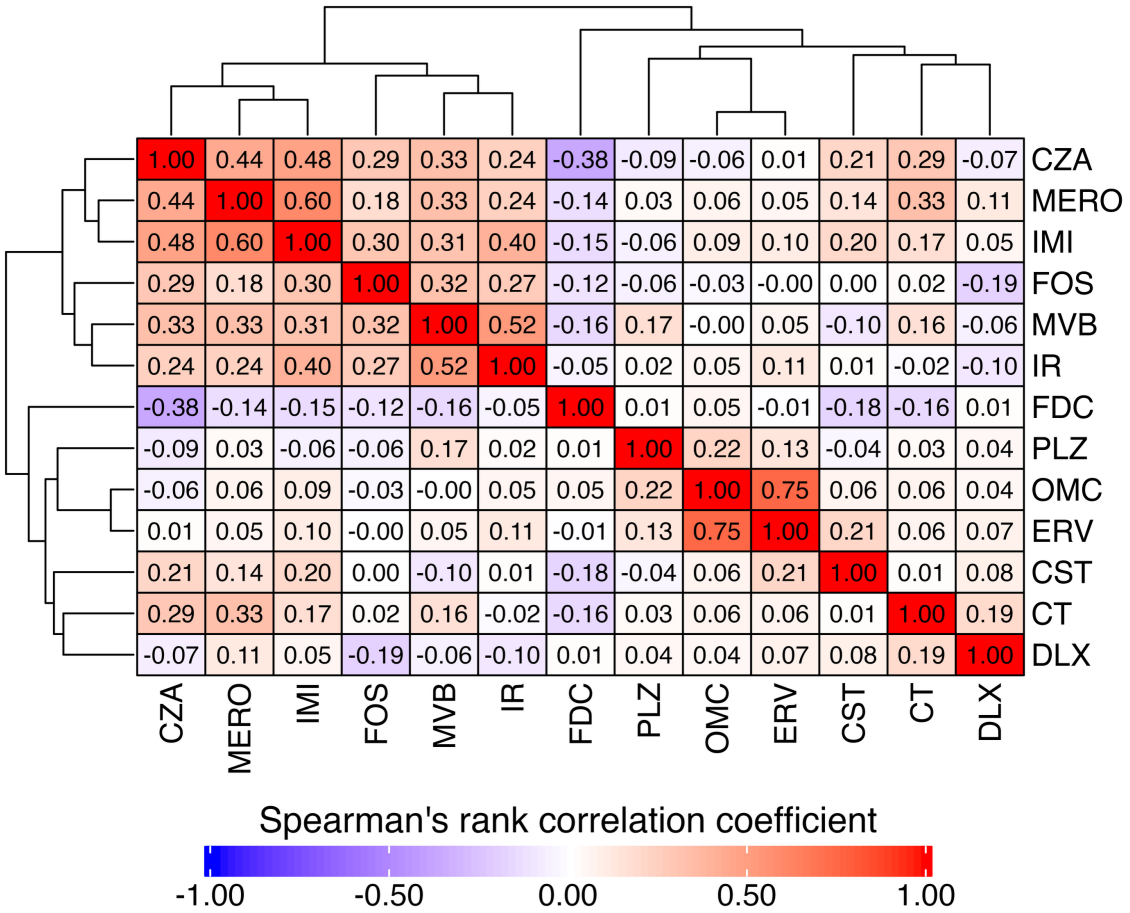
Appendix C Figure 7 Phylogenetic Distribution of Carbapenemase-Containing Plasmids Across the 2021-23 Study

Carbapenemase-containing plasmids were reconstructed from short-read assemblies using AMRFinderPlus and MOB-Suite. Carbapenemases with identity to the reference < 100% were denoted with "*", whereas genes with coverage < 100% were denoted with "?".



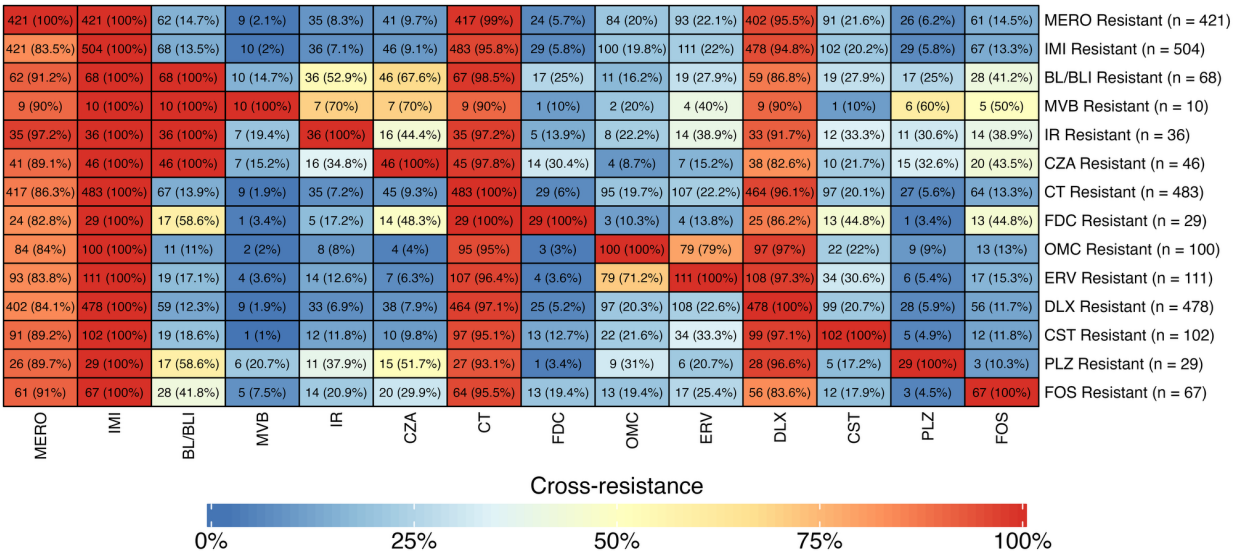
Appendix C Figure 8 Distribution of Minimum-Inhibitory Concentrations for Last-Resort and Novel Antibiotics Among 2021-23 Study Isolates

The resistance category for minimum-inhibitory concentration measurements was determined using the 2025 Clinical and Laboratory Standards Institute criteria (35th edition, M100).



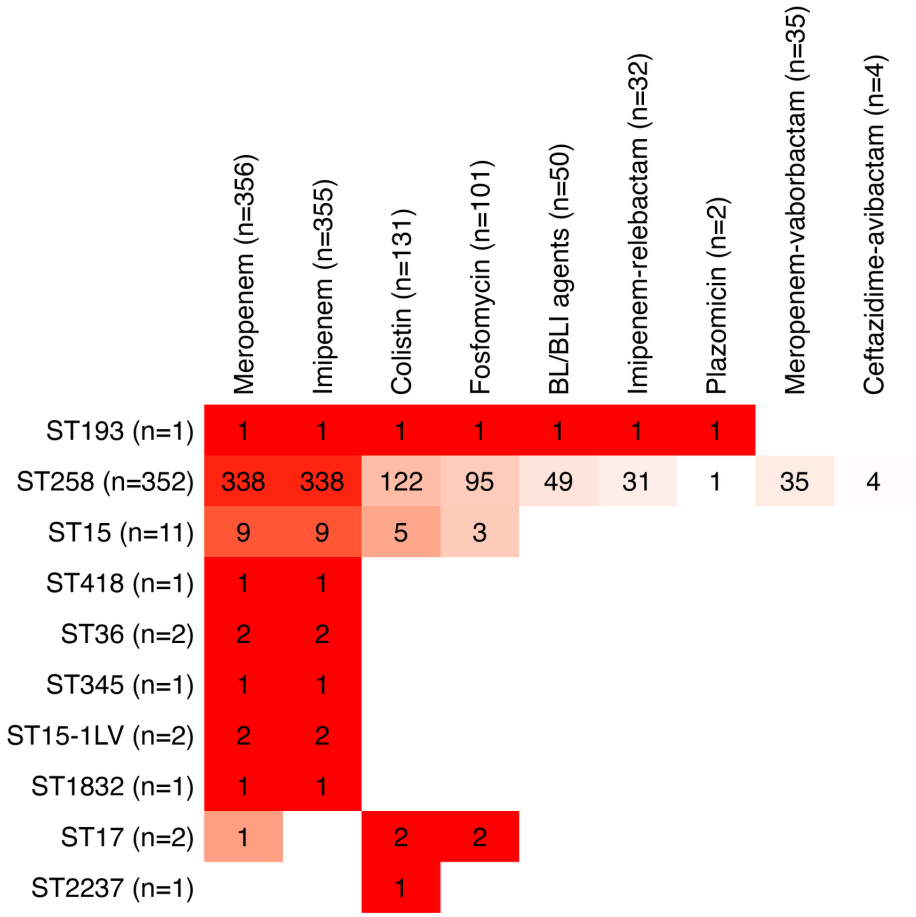
Appendix C Figure 9 Correlation Between the Log_2 Transformed Measurements of Resistance Among 2021-23 Study Isolates

Cross-resistance among carbapenem-resistant *Klebsiella pneumoniae* isolates from the 2021-2023 study, where all antibiotics were tested. Resistance was defined as an intermediate or resistant category, according to Clinical & Laboratory Standards Institute (CLSI) guidelines. Abbreviations: MERO, meropenem; IMI, imipenem; BL/BLI, β-lactam/β-lactamase inhibitor; MVB, meropenem-vaborbactam; IR, imipenem-relebactam; CZA, ceftazidime-avibactam; CT, ceftolozane-tazobactam; FDC, cefiderocol; OMC, omadacycline; ERV, ervacycline; DLX, delafloxacin; CST, colistin; PLZ, plazomicin; FOS, fosfomycin.



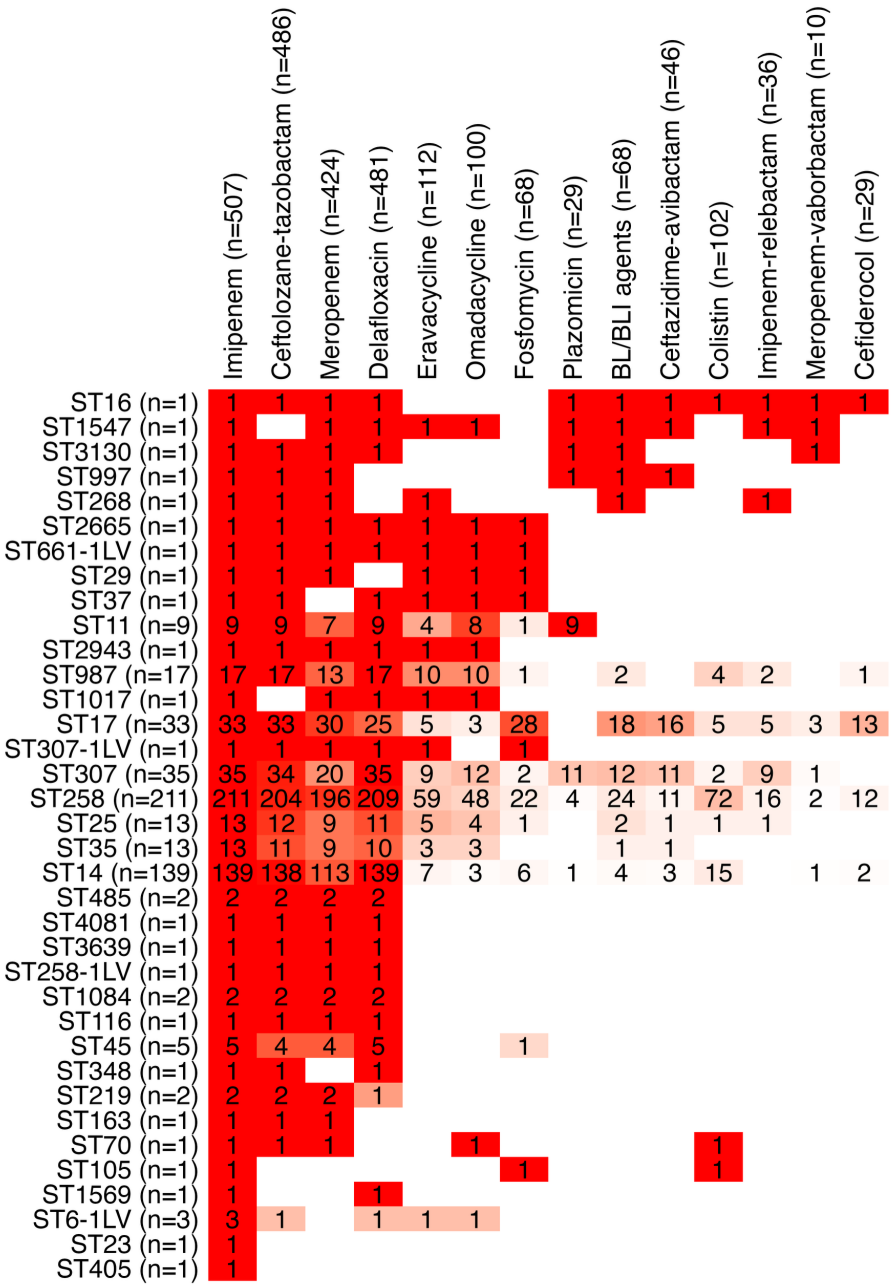
Appendix C Figure 10 Cross-Resistance Among 2021-23 Study Isolates

Cross-resistance among carbapenem-resistant *Klebsiella pneumoniae* isolates from the 2021-23 study, where all antibiotics were tested. Resistance was defined as an intermediate or resistant category, according to Clinical & Laboratory Standards Institute (CLSI) guidelines. Resistance to BL/BLI agents is a composite outcome that indicates resistance to at least one of the following antibiotics: ceftazidime-avibactam, imipenem-relebactam, or meropenem-vaborbactam. Abbreviations: MERO, meropenem; IMI, imipenem; BL/BLI, β-lactam/β-lactamase inhibitor; MVB, meropenem-vaborbactam; IR, imipenem-relebactam; CZA, ceftazidime-avibactam; CT, ceftolozane-tazobactam; FDC, cefiderocol; OMC, omadacycline; ERV, ervacycline; DLX, delafloxacin; CST, colistin; PLZ, plazomicin; FOS, fosfomycin.



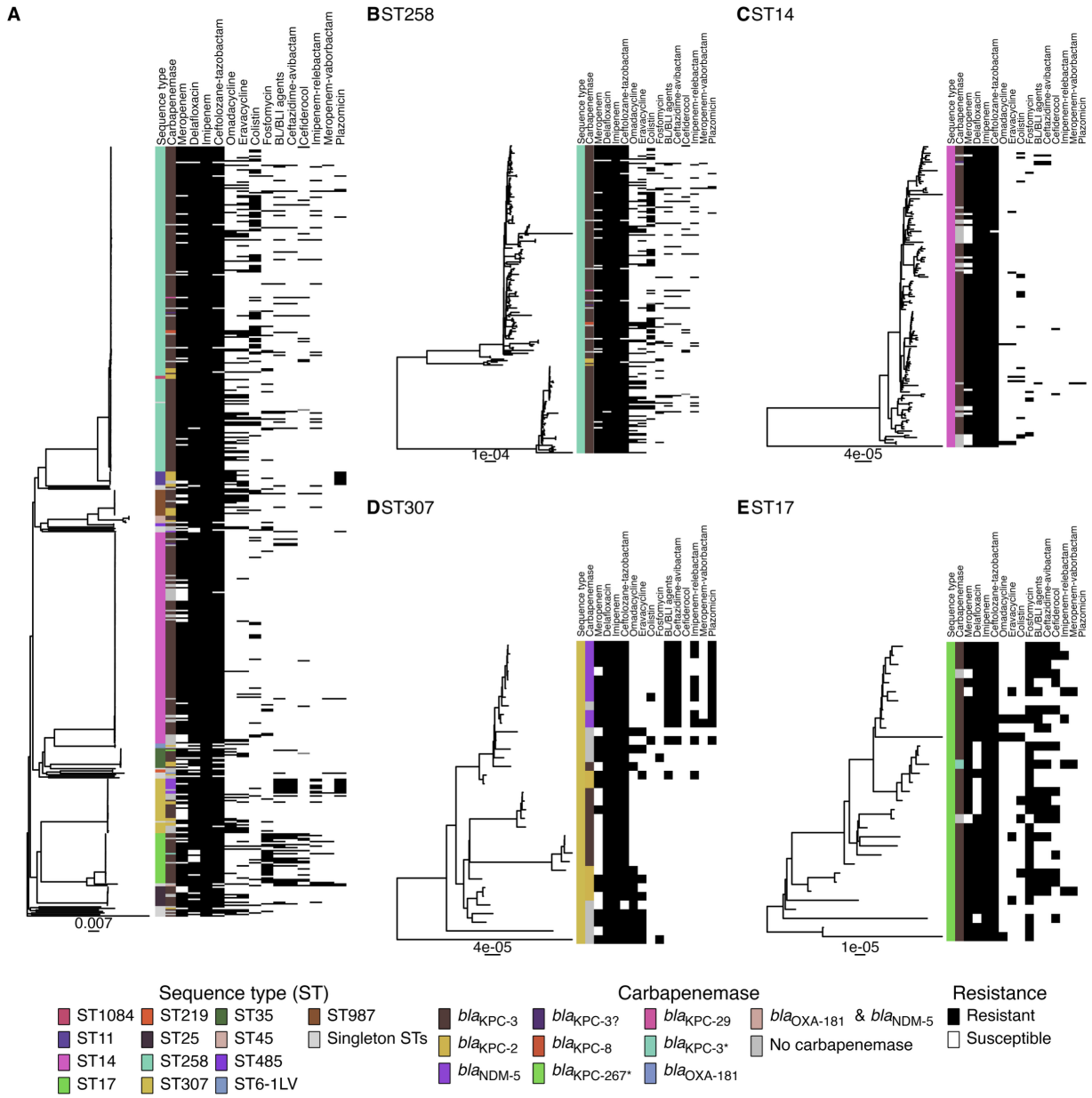
Appendix C Figure 11 Distribution of Resistance Across Sequence Types in the 2014-15 Study

Heatmap detailing the distribution of resistance to last-resort and novel antibiotics among carbapenem-resistant *Klebsiella pneumoniae* sequence types collected during the 2014-15 study. Resistance to BL/BLI agents is a composite outcome that indicates resistance to at least one of the following antibiotics: ceftazidime-avibactam, imipenem-relebactam, or meropenem-vaborbactam.



Appendix C Figure 12 Distribution of Resistance Across Sequence Types in the 2021-23 Study

Heatmap detailing the distribution of resistance to last-resort and novel antibiotics among carbapenem-resistant *Klebsiella pneumoniae* sequence types collected during the 2021-23 study. Resistance to BL/BLI agents is a composite outcome that indicates resistance to at least one of the following antibiotics: ceftazidime-avibactam, imipenem-relebactam, or meropenem-vaborbactam. Isolates with one allelic difference from a known sequence type were denoted with "-1LV."



Appendix C Figure 13 Phylogenetic Distribution of Resistance Among 2021-23 Study Isolates

(A) Resistance to last-resort antibiotics was overlaid on the 2021-23 carbapenem-resistant *Klebsiella pneumoniae* phylogeny. Next, the phylogeny was restricted to the four major STs: (B) ST258, (C) ST14, (D) ST17, and (E) ST307. Resistance to BL/BLI agents is a composite outcome that indicates resistance to at least one of the following antibiotics: ceftazidime-avibactam, imipenem-relebactam, or meropenem-vaborbactam.