Integration of Genomic and Epidemiologic Data to Detect Hospital Transmission of Carbapenem-resistant *Enterobacteriaceae*

by

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Dedication

For my family

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worst days for you and your families. I hope that this work will, even in the smallest way, help

us all progress to a future where healthcare associated infections are a thing of the past.

Table of Contents

Chapter 5   Cohorting KPC+ _Klebsiella Pneumoniae_ (KPC-Kp) Positive Patients—A Genomic Exposè Of Cross-Colonization Hazards In A Long-Term Acute Care Hospital (LTACH) **140**

List of Tables

List of Figures

Abstract

Carbapenem-resistant Enterobacteriaceae (CRE) are multi-drug resistant organisms (MDROs) that are resistant to nearly all antibiotics and are estimated to be responsible for 8,500 infections and 1,100 deaths in the U.S. annually. CRE have been labeled an urgent public health threat for over a decade, but despite wide-spread attention, infections with CRE have not decreased. It is established that the burden of CRE is concentrated in healthcare settings where it causes difficult to treat healthcare associated infections (HAIs), however, the burden of CRE is disproportionately distributed in different types of healthcare facilities: acute care hospitals primarily have sporadic cases of CRE, while post-acute care facilities like long-term acute care hospitals (LTACHs) have a disproportionately high prevalence and likely contribute to transmission across regions through transfer of colonized and infected patients. To reduce CRE transmission and prevent at-risk patients we must improve our knowledge of how CRE spreads in hospitals. Detection of CRE transmission, particularly in high-prevalence settings like LTACHs, is complicated by closely related strains that are difficult to distinguish by conventional molecular typing methods, as well as multiple epidemiologically plausible exposures among patients that could have led to transmission. Whole-genome sequencing (WGS) is a method that has demonstrated promise to improve detection of transmission by providing the resolution to distinguish closely related strains, however, there remains a need for studies that realize the potential of WGS to improve infection prevention practices. This dissertation develops and applies combined genomic and epidemiological methods to identify

how transmission occurs to the level needed to discern whether additional infection interventions are needed and identify new ways to prevent transmission.

Chapter two focuses on specific areas in which genomics has already made a significant impact, including outbreak investigations, regional epidemiology, clinical diagnostics, resistance evolution and the study of epidemic lineages. While highlighting early successes, I also highlight the next steps needed to translate this technology into strategies to improve public health and clinical medicine. Chapter three is a genomic epidemiological investigation of a putative CRE outbreak at the University of Michigan hospital. Our investigation revealed few plausible instances of nosocomial transmission, highlighting instead the frequent importation of CRE into our hospital, thereby informing infection prevention practitioners that additional precautions were unnecessary. Next, I delve into infection prevention improvement in LTACHs. Chapter four tackles detecting patients linked by transmission in a high-prevalence LTACH where patients harbor closely related strains. I present a method based on robust patient sampling and WGS data to detect clusters of patients linked by transmission to patients who imported CRE into the LTACH. Analysis of patient location data in transmission clusters revealed routes of transmission in the facility and highlighted exposures to patients that may increase vulnerability to transmission. Chapter five investigates the practice of cohorting CRE+ patients to prevent spread. Here I find evidence of multiple strain acquisition among CRE+ patients linked to cohorting, highlighting potential patient harms occurring during cohorting, and the importance of staff adherence to infection prevention protocols while caring for cohorted patients.

In summary, this dissertation moves forward the field of genomic epidemiology by demonstrating the power of combining WGS data and epidemiological methods to inform

infection prevention practices, reveal new insights into routes of transmission, and illuminate

new strategies to alleviate the burden of MRDOs.

Chapter 1   Introduction

1.1   Introduction

This dissertation focuses on the use of data from bacterial whole-genome sequencing (WGS), or 'genomic data', to enhance detection of transmission in hospitals and identify strategies to prevent and control healthcare-associated infections (HAIs). In this chapter, I describe the public health threat of HAIs and discuss how genomics can supplement existing frameworks in hospital epidemiology to track the spread of pathogens. Next, I introduce the group of prominent HAI-causing pathogens — Carbapenem-resistant *Enterobacteriaceae* (CRE) — discussed in subsequent chapters and describe how their burden varies across different types of healthcare facilities. Finally, this chapter concludes with a brief description of the aims of this dissertation which are examined in the subsequent chapters.

1.2   Antibiotic Resistant Healthcare-Associated Pathogens Are A Global Public Health Threat

On any given day in the United States, the safety of approximately 1 in 31 patients is threatened by health-care associated infections (HAI).(1) The burden of HAIs, which are infections patients acquire in the context of receiving medical care, highlights the need to improve healthcare infection prevention practices. Although widely implemented infection prevention practices, such as placing patients in transmission-based precautions, increased attention of healthcare staff

to hand-hygiene, and improved terminal room cleaning have reduced the spread of HAIs in some settings for some pathogens, HAIs continue to remain a threat.(1–5) The pervasiveness of HAIs, despite implementation of enhanced infection prevention strategies, suggests that there exist pathways of cross-transmission within healthcare settings that remain uncontrolled by current practices.

In conventional healthcare epidemiology when an outbreak or cluster of cases is suspected, infection preventionists search for common exposures (e.g. devices, locations, procedures) between patients identified as being positive for an organism and then evaluate these exposures for their role in facilitating transmission between patients.(6–8) Unfortunately, this framework often provides insufficient resolution to detect transmission between patients and to identify where to focus intervention resources to reduce transmission.

 Prominent HAI-causing pathogens are often capable of both colonization and infection, so in the absence of robust active surveillance schemes that incorporate colonization, patients who harbor an organism but lack an apparent infection are likely to be missing transmission links.(9–16) Additionally, there are often many common exposures that are shared by patients that could have facilitated transmission, and this -- in combination with difficulty tracking sources of transmission -- makes it difficult to identify the particular patient or epidemiologic exposures that are most impactful in driving transmission. Furthermore, the population landscape of many HAI-causing pathogens is regionally dominated by successful epidemic clones,(17–22) and conventional molecular typing methods such as multi-locus sequence typing (MLST) or pulse field gel electrophoresis often lack the resolution to discriminate between sporadic cases of

related organisms from patients who acquired them outside of a facility versus cross-transmission in a hospital, which requires intervention.(23–25)

1.3 Genomics Can Enhance Healthcare Epidemiology

In the last decade, WGS data has increasingly supplemented shoe-leather epidemiological investigations, and replaced conventional molecular typing schemes.(26–30) Unlike molecular typing, an organism's genome inherently contains information about its evolutionary history, which in combination with epidemiological data can be used to inform the likelihood of transmission links (**Figure 1**). (21,31–33) Though previous genomic epidemiologic investigations have discerned hospital transmission where conventional molecular methods failed,(34,35) so far there have been few studies that have integrated genomics into studies designed to evaluate the efficacy of existing infection prevention practices and routes of transmission within healthcare settings.(36) Designing genomic epidemiologic investigations to uncover such insights is not trivial; to be successful requires expertise in diverse fields including medicine, microbiology, bioinformatics and healthcare epidemiology. In chapters three through six of this dissertation, we hypothesize that the next steps for moving the field of genomic hospital epidemiology forward is to integrate genomic and epidemiologic data using analytical strategies that are tailored to address specific questions.

1.4 Carbapenem Resistant Gram-Negative *Enterobacteriaceae* Are A Major Cause of Antibiotic Resistant Healthcare-Associated Infections

This dissertation focuses on a group of multi-drug resistant organisms (MDROs) called carbapenem-resistant *Enterobacteriaceae* or CRE which are resistant to nearly all antibiotics and are estimated to cause 1,100 deaths in the U.S. annually.[5] The majority of the estimated 8,500 infections caused by CRE in the U.S. each year are concentrated in healthcare settings where they cause difficult to treat infections among vulnerable patient populations.[1,5,37] Despite the CDC labeling CRE as an urgent public health threat for almost a decade and wide spread attention, CRE infections have not decreased.[5]

The majority of CRE infections in the U.S. are caused by *Klebsiella pneumoniae* that carry the KPC type of carbapenemase (KPC-Kp) which confers resistance to carbapenems—a last line treatment for antibiotic resistant Gram-negative infections.[38] Though organisms that produce a carbapenemase are thought to be of the greatest concern clinically and epidemiologically,[39,40] carbapenem resistance in *Klebsiella pneumoniae* and other *Enterobacteriaceae* can be conferred by various other mechanisms including increased outer-membrane permeability, hyperproduction of endogenous beta-lactamase enzymes or extended-spectrum beta-lactamases.[41–43]

The population landscape of CRE is regionally dominated by particular successful clonal lineages of KPC-Kp as well as other members of the *Enterobacteriaceae*, such as *Enterobacter cloacae* (CREC).[19,39,44] In healthcare settings, this clonal population structure can make it impossible to discern whether strains from patients are related by in-hospital cross-transmission

without the increased resolution of WGS data in combination with patient-level epidemiologic data.(39)

1.5 Burden Of CRE Across Different Healthcare Settings

Although the burden of CRE is concentrated in healthcare settings, different types of healthcare settings are disproportionately affected. For example, previous work has demonstrated that long-term acute care hospitals (LTACHs) have a disproportionately high prevalence of CRE and likely contribute to regional spread.(16,45–47) A recent study in Chicago, which includes the dataset discussed in chapters four and five of this dissertation, demonstrated that while the average prevalence of CRE in acute care hospitals was 3%, it was 30% in LTACHs.(16) The discrepancy between the acute care hospital setting, where CRE infections primarily occur in sporadic cases and small clusters, and LTACHs where CRE prevalence is high and can be endemic, may be due to the structure of the patient-care environment in LTACHs, and the particularly vulnerable patient populations who have comparatively longer lengths of stay, increased comorbidities and more extensive previous healthcare exposure.(46,48–50) The high-burden of CRE in LTACHs as well as the potential role of LTACHs as reservoirs for regional transmission into other parts of the connected healthcare network underscores the need for improved understanding of how transmission occurs in these facilities and additional ways to reduce transmission.

1.6 Dissertation Aims

The overall goal of this dissertation is to integrate genomic and epidemiologic data to improve our understanding of how transmission of CRE occurs in hospitals to the level needed to enhance CRE infection prevention in hospitals. In chapter two, we synthesize existing literature from previous genomic epidemiologic investigations to identify knowledge gaps in the field of genomic epidemiology of antibiotic resistant Gram-negative pathogens and present strategies for optimizing the use of genomic data to improve public health and medicine.(51)

In chapter three we apply a genomic epidemiological framework to an outbreak investigation of healthcare associated carbapenem resistant *E. cloacae* at the University of Michigan hospital.(52) Here, the increased resolution gained by integrating genomic information into a conventional hospital epidemiology outbreak investigation revealed the absence of a clonal outbreak, and demonstrated that allocation of additional infection control resources to the outbreak were not necessary. Our further integration of genomic and epidemiologic data beyond the initial outbreak investigation provided new insights into the population structure of CRE, mechanisms of carbapenem resistance, and genomic signatures of bloodstream infections caused by CRE at the University of Michigan hospital.

In chapters four and five we investigate transmission of KPC-Kp in LTACHs. Chapter four develops and applies an integrated method based on genomic and surveillance data to detect groups of patients who are related by cross-transmission in an endemic LTACH. Here we take advantage of the ability to partition patients into putative transmission clusters to investigate routes of transmission within an LTACH that remain uncontrolled by previously implemented

cross-transmission prevention strategies. Chapter five explores the infection control strategy of

cohorting patients who are positive for an organism into common locations in hospitals to

prevent transmission to negative patients. We also examine the frequency of multiple KPC-Kp

strain acquisition among previously colonized patients, and acquisition of strains with diverse

antibiotic resistance potential linked to the practice of cohorting.

Chapter six concludes this dissertation with a synthesis of the current state of the field of

genomic hospital epidemiology, a discussion of the contribution of insights provided by the work

presented in this thesis, and challenges and opportunities to progress the field.

1.7   Figures
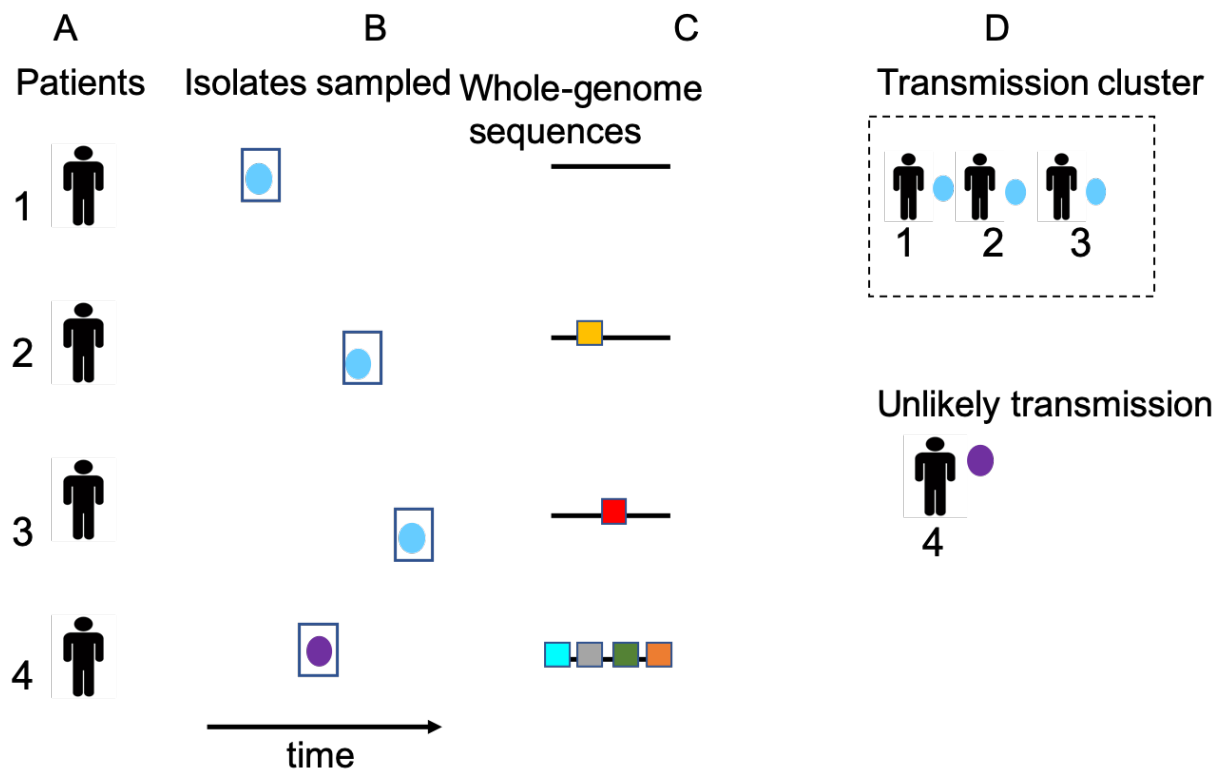
Figure 1-1 Genomics As A Tool To Supplement Infection Prevention Investigation Frameworks.

WGS Is A High-Resolution Approach That Has Been Used To Identify Transmission Events In

Hospitals. Isolates from patients (A) are collected (B) and undergo bacterial whole-genome

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Chapter 2   Genomic Epidemiology of Multidrug-Resistant Gram-Negative Organisms

2.1   Abstract

The emergence and spread of antibiotic resistant Gram-negative bacteria (rGNB) across global

healthcare networks represents a significant threat to public health. As the number of effective

antibiotics available to treat these resistant organisms dwindles, it is essential that we devise

more effective strategies for controlling their proliferation. Recently, whole-genome sequencing

has emerged as a disruptive technology that has transformed our understanding of the evolution

and epidemiology of diverse rGNB species, and has the potential to guide strategies for

controlling the evolution and spread of resistance. Here, we review specific areas in which

genomics has already made a significant impact, including in outbreak investigations, regional

epidemiology, clinical diagnostics, resistance evolution and the study of epidemic lineages.

While highlighting early successes, we also point to the next steps needed to translate this

technology into strategies to improve public health and clinical medicine.

2.2 Introduction

In recent years, multidrug resistant organisms (MDROs) that are refractory to nearly all available treatments have emerged and spread globally.(1,2) The inability of drug discovery pipelines to keep up with the pace at which resistance has neutralized existing antibiotics has created an imminent global public health crisis.(3),(4) The threat of MDROs is particularly dire within our healthcare systems, where more than 1 in 25 hospitalized patients have a healthcare associated infection (HAI) on any given day.(5) Hospitalized patients have co-morbidities that make them more susceptible to contracting infections and less equipped to combat these infections without the aid of antibiotics. Thus, increases in antibiotic resistance among healthcare-associated pathogens has directly led to increases in morbidity and mortality among affected patients.(6),(7),(8) Recently, the evolution of antibiotic resistance has reached a crucial tipping point, with the emergence of pan-resistant organisms that have caused infections untreatable with any available antibiotic.(2,9–11) In the absence of novel treatments to combat these resistant infections, there is an urgent need for the development of more effective strategies to control the spread of MDROs, and prevent patients from acquiring infections that are increasingly difficult to treat.

Over the past several decades healthcare epidemiologists have made significant strides in tracking the spread of infections within and between healthcare facilities by supplementing traditional gumshoe epidemiology with a diverse suite of molecular typing tools.(12) Molecular typing methods probe the structure (e.g. pulsed-field gel electrophoresis-- PFGE) or sequence

(e.g. multi-locus sequence typing-- MLST) of microbial genetic material in order to quantify the relationship between infectious isolates and gauge whether they are plausibly linked by transmission.(12–14) While much has been learned about the local and global epidemiology of MDRO's using molecular typing approaches, all classical techniques are associated with major limitations. First, methods based on genome structure present difficulties in interpretation due to the fact that these molecular types (e.g. pulsotype) do not evolve at a consistent rate.(14) The lack of a quantifiable relationship between variation in molecular type and historical relatedness forces investigators to apply arbitrary cutoffs in evaluating whether two isolates could be epidemiologically linked.(13) A second issue with classical methods is that there is not a single method that performs well at all time scales. For instance, MLST has been shown to be extremely powerful in characterizing regional or global pathogen populations, but lacks the resolution to discern transmission patterns within a healthcare institution.(15) Conversely, PFGE provides resolution sufficient to discern between closely related strains, but is often too dynamic to compare pathogen populations in different regions.(15) Lastly, an important limitation of all classical molecular typing approaches is that they provide no insight into how genetic changes relate to phenotypic differences among strains.

Recently, whole genome sequencing (WGS) has entered the forefront of molecular epidemiology, providing a one-size fits all tool that overcomes virtually all of the limitations of prior methods. First, WGS has been shown to provide sufficient resolution to elucidate transmission pathways within a single institution, while at the same time yielding data that facilitates the placement of global pathogen populations in the context of one another.(16–18)

Second, by probing every base pair in the genome, WGS allows investigators to translate genetic differences into historical relationships among isolates by exploiting the molecular clock at which mutations accumulate over time.(19,20) Having a molecular type that can be related to a molecular clock has allowed investigators to explicitly test whether two strains are linked on epidemiologically relevant time scales, while avoiding the need to set arbitrary cutoffs.(21) Lastly, by interrogating variation across the entire genome, investigators can leverage phenotypic information from decades of biochemical and genetic experiments to generate hypotheses regarding the phenotypic impact of observed genomic variation.(22–24)

Early work applying WGS to study MDROs has demonstrated the disruptive nature of this technology and led to fundamental insights into the evolution and epidemiology of the most significant healthcare associated pathogens. Here, we will highlight recent applications of WGS to characterize the emergence and spread of Gram-negative MDROs across global healthcare systems. While WGS has had an equally significant impact on Gram-positive MDROs, we focus on Gram-negatives to highlight some of the unique features of this increasingly burdensome class of healthcare pathogens.(25,26)   For each application of WGS we will also explore challenges and opportunities in maximizing the translational impact of this transformative technology in the realms of clinical practice and public health.

2.3   Hospital Epidemiology & Outbreak Investigation

Among the earliest applications of WGS to study the epidemiology of MDROs was to elucidate transmission networks during hospital outbreaks.(16,17,27,28) Outbreak investigations are

initiated when there is a spike in infections with an MDRO species. A typical investigation

consists of case-finding, where investigators look for additional patients who might be involved

in an outbreak, as well as contact tracing, where investigators look for common exposures or

contact between patients, with the goal of identifying contaminated infrastructure, or pathways of

patient-to-patient transmission. Traditional epidemiological investigations are often

supplemented with molecular typing, in order to narrow the focus to groups of patients that are

thought to be part of a transmission chain, based upon their harboring of a related MDRO strain.

However, this combination of contact tracing and low resolution molecular typing has been

complicated by the emergence of epidemic MDRO lineages that have become endemic in

regional healthcare institutions. In particular, the endemicity of these epidemic lineages is such

that it is not uncommon for multiple patients to enter a healthcare institution already colonized or

infected with a common strain. Thus, grouping together all patients harboring a common strain

will result in patients being grouped together who are not necessarily connected by transmission

within a healthcare institution. The inability to accurately group patients linked by transmission

can make it difficult to identify contaminated infrastructure or other potentially modifiable

factors that are mediating transmission.

Several studies have reported the successful application of WGS to partition patients into

transmission clusters when other molecular typing approaches failed. Our first application of

WGS to study a hospital outbreak was for an outbreak of MDR *Acinetobacter baumannii*.(28)

Although our hospital had not had a previous outbreak with MDR *A. baumannii*, PFGE typing of

outbreak isolates indicated that we had three different strain types simultaneously circulating in

the hospital. We therefore wondered if this outbreak was due to three contemporaneous introductions into the hospital, or if the circulating strain of *A. baumannii* had evolved in such a way that its PFGE type changed during the course of the outbreak. Application of WGS to representatives of the three outbreak strains led us to the conclusion that two importation events had seeded this outbreak. Two outbreak strains were traced back to an importation event by a single patient, with the variation in strain type believed to be due to large recombination events across the genome. The third outbreak strain was traced back to a non-MDRO strain that had been circulating in the hospital months earlier, and in the intervening time had picked up several drug resistance determinants. In a separate study, Willems and colleagues were able to partition an *A. baumannii* outbreak into two clusters, and show that transmission was largely confined within specific hospital wards, thereby focusing infection control interventions.(29) Kanamori and colleagues were similarly able to partition an *A. baumannii* outbreak into clusters due to independent importation events, and similar to our investigation, found that filtering out recombinant regions of the genome was critical to make accurate epidemiological inferences.(30)

The success of WGS in dissecting healthcare outbreaks is not limited to *A. baumannii*. Stoesser and colleagues applied WGS to isolates from an outbreak of MDR *Enterobacter cloacae* that primarily affected neonates.(31) This analysis revealed two separate clusters that were again largely confined to individual units. In addition, one of the clusters matched an isolate retrieved from a soap dispenser, implicating this contaminant as the point source seeding this cluster. Several groups have also applied WGS to study outbreaks of carbapenem-resistant *Klebsiella pneumoniae*, and other MDR gram negatives, many of which observed multiple strain

importations, followed by the preferential transmission of particular strain types.(30–33) Upon partitioning multi-strain outbreaks into clusters, these studies found that most transmission events could be accounted for by spatiotemporal overlap between patients in the facility, again emphasizing the importance of defining transmission clusters to facilitate insights into transmission pathways. Importantly, most of the aforementioned studies found that the incorporation of WGS data into the outbreak investigation facilitated insights into the origins of circulating strains and pathways of nosocomial transmission that would have been inaccessible with lower resolution typing methods (e.g. MLST, PFGE).

In addition to grouping patients into transmission clusters, several studies have been able to utilize WGS to elucidate extremely nuanced insights into the propagation of outbreaks with different MDROs. As mentioned above, multiple groups have reported that time and space overlap on hospital wards can explain the majority of transmissions for organisms such as *K. pneumoniae* and *E. cloacae*, which are thought to primarily spread patient-to-patient via healthcare worker contamination.(34,35) However, for more environmentally hearty organisms like *A. baumannii* and *Pseudomonas aeruginosa* WGS has allowed for causal links to be made between environmental contamination and ongoing transmission. In studying a prolonged outbreak of *A. baumannii*, Halachev and colleagues were able to use WGS to link contamination in an operating theater to transmission between patients who otherwise had no overlap in the hospital.(33) Several groups have linked Pseudomonas isolates from sink drains to isolates taken from patients.(36,37) While directionality was not clear in many of these cases, one report found that genetically identical isolates persisted in a sink trap months after the linked patient had been

in the room, demonstrating at the very least that infection causing isolates can persist in the hospital environment for extended periods of time.(36)

Lastly, WGS has also yielded non-trivial insights into the structure of transmission networks. Applying WGS to an outbreak of carbapenem resistant *K. pneumoniae* allowed us to demonstrate the role for asymptomatic carriers in outbreak propagation.(17) In particular, we observed that despite a 3-week gap in infections following discharge of the index patient, there had in fact been multiple transmissions from this index patient that seeded an outbreak that affected 18 patients. This observation led to the implementation of more rigorous surveillance culturing, which was critical in identifying and isolating all asymptomatically colonized patients and stopping the outbreak. In applying WGS to an outbreak of *P. aeruginosa,* Willmann and colleagues found evidence for the disproportionate role of a few super-spreaders in propagating the outbreak strain.(36) Future insights such as these into the structure of transmission networks for different MDROs will be critical in identifying and properly managing high-risk patients.

While these early studies show how powerful WGS is for outbreak investigations, there are still important challenges that need to be considered. First, several groups, including ours, have reported how intra-patient genetic heterogeneity of colonizing and contaminating populations can confound accurate descriptions of transmission networks.(17,21) The impact of this is still not fully appreciated, but the potential for many MDRO species to colonize hosts for months or years raises the possibility that certain patients may harbor extremely diverse colonizing populations.(38–42) Moreover, the potential for multiple acquisitions in high

transmission settings has been documented, and can also confound transmission inference.(43–45) While there are both analytical and sequencing based strategies to deal with these issues, they result in decreased power and increased cost, respectively.(46–48) One solution to combat decreased power of genetic inferences is to supplement transmission inference pipelines with comprehensive location or contact tracing data.(49) A second solution is to apply methods that account for potential intra-host diversity and uncertainty surrounding the potential transmission events when constructing transmission networks.(46,47)

Another challenge in standardizing WGS for clinical applications is agreement in the field regarding best practices and common analytical frameworks. Likely, the optimal framework will depend on the question at hand, where computationally friendly pipelines like WG-MLST might be preferable for real-time analyses, while more sophisticated phylogenetic approaches that take full advantage of genomic data can be applied in retrospective analyses.(46,47,50,51) A related issue is coming to a consensus as to whether the establishment of concrete variant thresholds is appropriate for evaluating whether two patients are plausibly linked by transmission .(16,52,19,49) Due to the aforementioned issue of increased variation due to prolonged asymptomatic colonization of patients, it is unlikely that a hard variant cutoff that is not overly conservative will work in all situations .(53,54) We believe that a more viable solution for distinguishing between transmission and importation is enacting more comprehensive sequencing of regional isolate collections, such that isolates from within a facility can be placed into a broader regional context.(55,56) Finally, it needs to be considered whether there is benefit to having WGS embedded in clinical microbiology labs for real-time investigation, or whether

retrospective investigations by healthcare researchers and public health laboratories are sufficient.(57) To answer this question will require well-conceived and designed studies that quantify the benefits of real-time sequencing.(58)

2.4   Regional epidemiology at different geographic scales

While the application of WGS to understand intra-facility transmission has the potential to reduce infection rates by stemming nosocomial transmission, it is increasingly appreciated that the connectivity of healthcare networks will ultimately necessitate a regional approach infection control.(59) Such a regional approach will require understanding the structure and dynamics of pathogen populations at different temporal and geographic scales. As the cost of sequencing has decreased and allowed for the application of WGS to large strain collections, it has become clear that a genomics approach can yield unparalleled insights into pathogen populations at local, regional and global scales.

In addition to targeted sequencing of suspected outbreaks, WGS has been applied more broadly at single institutions to discern local pathogen population structure and gauge the relative impacts of importation and transmission within healthcare facilities. To try and understand an observed increase in carbapenem-resistant *Enterobacteriaceae* (CRE) at their institution, Pecora and colleagues sequenced all CRE infection isolates over a 3-year period.(60) Genomic comparison revealed that there was little transmission, and that incidence of CRE at this institution was primarily driven by the sporadic importation of organisms harboring diverse mobile resistance elements. Mellmann and colleagues took this to the next level, by sequencing

all methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and resistant Gram-negative bacilli (rGNB) at their institution to discern the relative impact of transmission and importation.(58) Their genomic investigation revealed that there was little transmission of rGNB, which led to modification of infection control procedures to more effectively allocate resources. Importantly, the authors then applied WGS to validate that their procedural modifications did not have negative consequences on transmission rates. In the ultimate display of sequencing power, Roach and colleagues indiscriminately sequenced every clinical isolate taken from ICU patients over the course of a year.(43) Analysis of 1229 genomes from 391 patients revealed an unexpected level of species and strain diversity in the hospital and painted a picture of overall low transmission rates, with a handful of successful lineages being observed in multiple patients.

While sequencing isolate collections from single institutions provides insights into what is happening within the confines of a facility, understanding the ultimate origin of MDROs circulating within hospitals will require sequencing and analysis of regional isolate collections. Moradigaravand and colleagues recently took such a regional approach to understand the population structure of three MDR members of the *Enterobacteriaceae* family: *Enterobacter cloacae*, *Serratia marcescens,* and *Klebsiella pneumoniae*.(56,61,62) In contrast to MDR *K. pneumoniae*, in which epidemic clones dominate specific regions, *E. cloacae* and *S. marcescens* both exhibited a polyclonal structure across 37 hospitals in the United Kingdom (UK) and Ireland, indicating the convergent emergence and spread of multiple MDR lineages. This finding contrasts to a recent report by Hargreaves and colleagues, who observed the emergence and

spread of a lineage of *bla-KPC* carrying *E. cloacae* across multiple hospitals in North Dakota

and Minnesota.(63) Thus, polyclonal population structures are not necessarily a static feature of

a given MDRO species, with the acquisition of a key resistance determinant potentially leading

to the emergence of clonal epidemic lineages.(64–66) The emergence of epidemic clones from a

background of poly-clonality has also been reported in multiple studies applying WGS to

regional sets of *P. aeruginosa* isolates from cystic fibrosis (CF) patients. While it had been

doctrine that infections in CF patients are due to acquisition of environmental strains, Dettman

and colleagues applied WGS to demonstrate the existence of common strain infecting CF

patients in multiple hospitals in North America and the UK.(67) Work by this same group and

others went on to show that this epidemic lineage had acquired genetic variants beneficial in the

CF lung environment and spread globally.(68)

Two recent genomic epidemiological analyses of regional *Neisseria gonorrhoeae* were

among the first to move beyond the description of population structures, to identifying

epidemiological drivers of transmission.(69,70) By integrating robust epidemiological data on

timing of infections, sexual preference and past contacts into phylogenetic analyses, these studies

were able to demonstrate local geographic clustering of strains circulating among individuals

with common sexual preference. Of note, the study by De Silva et al., which focused on cases

from the city of Brighton over a 4-year period, found that local transmission networks in

Brighton were supplemented by outside importations from both geographic disparate parts of the

UK and also the United States.(69) Thus, by casting an increasingly wider net, the authors were

able to determine the relative impact of transmission and importation at different levels of geographic granularity.

These and other genomic epidemiology studies have begun to inform our understanding for the pathways by which MDRO lineages have spread at local and global scales. However, these prior studies have largely been descriptive, in the sense that the clinical and epidemiological factors that impact regional MDRO prevalence and drive the spread of MDROs across healthcare institutions remain largely hidden. For MDROs that primarily spread within healthcare settings, it is likely that patient movement between healthcare facilities drives regional spread. Through the integration of genomic and patient transfer data we were able to demonstrate that a handful of patient transfer events were sufficient to explain a regional outbreak of carbapenem-resistant *K. pneumoniae* that affected 40 patients and 26 healthcare facilities in four adjacent counties in Indiana and Illinois.(55) Moving forward, it will be important to expand on this proof of principle study, overlaying additional meta-data on clinical practices and resident patient populations, such that we can understand what drives variation in the prevalence of different MDRO species across healthcare settings, even over short geographic distances. Finally, full understanding of MDRO spread will require more comprehensive sampling. This will require consideration of not only clinical isolates, but also isolates gathered through active surveillance culturing of asymptomatic individuals across facilities comprising connected healthcare networks.

2.5 Evolution And Dissemination Of Clonal Lineages

A recurring theme from both genomic and classical molecular epidemiological studies of various MDR Gram-negative organisms is the observation of epidemic lineages that have emerged and spread globally since the dawn of the antibiotic era.(71,72) The proliferation of these lineages has prompted many investigators to search for common characteristics that might account for their success, but thus far the only characteristic that unifies these epidemic clones is their resistance to one or more common antimicrobials.(72) While antibiotic resistance is by definition necessary for a clone to become epidemic, it does not appear in itself sufficient, as there are numerous examples of less prominent clones having the same resistance determinants as their epidemic counterparts.(73,63,74) Far from being simply an academic exercise, understanding why certain lineages explode on the global scene is critical for more effective monitoring and early detection of emergent lineages of high epidemic potential. By applying evolutionary genomics approaches investigators have begun to chart the evolutionary trajectories of epidemic lineages, which is an essential first step in understanding whether the success of these clones is due to chance accumulation of beneficial mutations, or if the genetic background of these ancestral strains predisposed them to thrive in the antibiotic era. Several of these lineages have been reviewed in detail elsewhere, and we therefore provide only a brief discussion of this area.(67,72,73,75)

As alluded to above, antibiotic resistance is a common feature of epidemic clones that have emerged in the antibiotic era. However, the critical resistance determinants and the mode by which they were acquired vary among MDRO lineages. ST131 is a globally disseminated clone

of *Escherichia coli* that is associated with both community and healthcare acquired infections.(76) ST131 stands out among other *E. coli* lineages because of its common association with fluoroquinolone and beta-lactam resistance, mediated by target site mutations and a plasmid associated extended spectrum beta lactamase (ESBL), respectively.(72,76) Recent comparative genomics studies have revealed a nested sub-structure to the ST131 lineage, wherein there was a sequential acquisition of fluoroquinolone resistance conferring mutations, followed by a bla-CTX-M-15 containing plasmid. (76,77) Further work has unearthed additional complexity, in that the bla-CTX-M-15 gene seems to occur on multiple plasmids, which vary in their cargo, indicating that there may have been multiple plasmid acquisitions events.(76) A second lineage defined by a resistance plasmid is *bla-KPC* carrying ST258 *Klebsiella pneumoniae.* While ST258 can be resistant to nearly all antibiotics, its global proliferation appears to have coincided with the acquisition of a *bla-KPC* carrying plasmid.(65,78) Similarly to ST131, *bla-KPC* has been observed in multiple plasmid contexts within ST258.(79) It is noteworthy that while the plasmid backbones vary, both *bla-CTX-M-15* and *bla-KPC* are typically carried on narrow host range IncF plasmids, suggesting that these plasmids may harbor characteristics that either minimize the cost of plasmid maintenance or encode for other features that are beneficial to epidemic clones.(80)

In contrast to the ST131 and ST258 lineages that are defined by the acquisition of particular resistance elements, the European Epidemic Clones I and II (ECI and ECII) of *Acinetobacter baumannii* appear to be defined more by the breadth and flexibility of their resistome.(75,81,82) While ECI and ECII strains carry resistance plasmids, the defining feature

of these strains are massive chromosomally encoded resistance islands that contain multiple antibiotic resistance determinants that are associated with mobile genetic elements.(83–85) These resistance islands have proved to be extremely dynamic, with many different configurations being reported.(86) In addition to horizontally acquired elements, *A. baumannii* also has several intrinsic resistance genes, including beta-lactamases and efflux pumps, which can become activated under antibiotic pressure by mobilization of IS-elements that carry strong promoters.(84,87) Similar to *A. baumannii*, resistance in MDR epidemic clones ST235 and ST111 of *P. aeruginosa* is also attributable to a combination of intrinsic resistance elements and chromosomally associated mobile elements.(88)

While most attention has been given to acquisition of antibiotic resistance determinants, several MDRO epidemic lineages have also acquired foreign genetic material with the potential to confer advantages beyond survival under antibiotic pressure. Recent comparative genomic studies of *K. pneumoniae* ST258 found that among the few defining events in the emergence of this lineage were two large recombination events that resulted in altered capsular biosynthetic loci.(65,79) It has been hypothesized that these capsular switching events are important for the immune evasion and persistence in hosts. Recombination events altering antigenic molecules have also been observed in *E. coli* and *A. baumannii*, with recombinant switching of lipopolysaccharide (LPS) loci having been reported in both species.(28,89,90) In addition to altering putative antigenic determinants, horizontal transfer events have also been observed that have the potential to modify interactions with the host environment in other ways. Studies in

ST131 and ST258 have both found horizontal transfer events impacting fimbriae and pili, which may provide advantages in host colonization.(65,91,92)

Despite large comparative genomic studies charting the evolutionary trajectories of prominent Gram-negative lineages, it still remains unclear what has made these epidemic clones so successful. One fundamental question is whether the acquisition of key resistant determinants were the critical events that propelled these lineages, or if instead it was the genetic background of the ancestors of epidemic clones that primed them for success. To begin to address this question will require a better understanding of capabilities of these organisms outside of their resistance. For example, studies that assess alternate explanations for success, such as environmental heartiness, capacity for efficient colonization of the host, and the ability to outcompete resident microbiota are needed to identify factors that underlie the success of these lineages.(93) A second issue hindering our understanding of the emergence of epidemic lineages is a potential observation bias, wherein the dissemination and evolution of resistant organisms is preferentially monitored as compared to their susceptible counterparts. More comprehensive surveillance sampling of organisms regardless of resistance or virulence phenotypes would enable generation of a more complete picture of the global population structure of prevalent pathogens with epidemic lineages and facilitate the retracing of temporal events leading up to the emergence of new dominant resistant lineages. Another form of observation bias is the preferential sampling of individuals in healthcare settings, despite the existence of both resistant and susceptible strains of MDRO species circulating in the community. This makes it unclear whether these strains were previously spreading effectively outside hospitals or if the prevalence

of these lineages exploded due to acquisition of resistance and selection in the high-antibiotic environment of healthcare facilities. Once we begin to understand the basis for success of dominant resistant lineages, we may be able recognize and predict the emergence of resistant organisms, with the goal of intervening before they negatively impact public health.

2.6 Evolution Of Antibiotic Resistance

While epidemic lineages are of special interest due to their prevalence and tendency towards multi-drug resistance, the evolution and spread of antibiotic resistance in less prolific Gram-negative lineages is also of major concern for several reasons. First, increased resistance is expected to overall be associated with worse patient outcomes due to increased time to optimal therapy.(94) Second, resistance determinants in low-risk clones can become mobilized, and be transferred to other MDRO lineages and to other species.(95,96) Finally, as discussed above, it is unclear if and when the acquisition of a resistant determinant in a low-risk clone could set it on a trajectory towards becoming a significant regional or global threat. In recent years bacterial genomics has been applied to track the real-time evolution of resistance within patients, to elucidate genetic mechanisms underlying resistance in different MDROs and to characterize the relationship between antibiotic resistance determinants found in different human and environmental reservoirs.(97–100) It is hoped that these insights into the evolution and ecology of antibiotic resistance can motivate the conception and implementation of more effective strategies for impeding the proliferation of resistance.

2.6.1 Mutational Modes of Resistance

The most straightforward experimental design for studying clinical resistance evolution is the application of WGS to longitudinal isolates taken from patients in which resistance has evolved during the course of treatment. In these situations, it is presumed that if resistance has emerged during a short treatment course, then it is likely due to a small number of high-impact mutations.(97) Indeed, studies employing this approach to study resistance evolution typically only observe a handful of mutations between intra-patient pairs, which facilitates the identification of causal variants by identifying genes or pathways mutated in multiple patient time courses.(98) A drug for which several groups have studied intra-patient resistance evolution is colistin. Colistin is a last-line drug for treating MDR Gram-negatives that are resistant to carbapenem antibiotics.(101,102) The prospect of widespread colistin resistance is of great concern, as there are limited treatment options beyond colistin for the treatment of infections caused by carbapenemase producing Gram-negatives, such as *K. pneumoniae* and *A. baumannii*.(103) Interestingly, in both *K. pneumoniae* and *A. baumannii*, genomic sequencing studies have found mutations in a common regulatory pathway controlling LPS modification systems, indicating that LPS modification is key to resistance in both species.(104–106) This genome-derived hypothesis that altered LPS modification underlies resistance was ultimately confirmed for both organisms by comparing LPS modifications in susceptible and resistant isolates.(104,107)

An important caveat in studying resistance evolution in individual patients is that the larger epidemiological significance of observed resistance mutations or mechanisms cannot

necessarily be inferred. This disconnect between short-term and long-term impacts of resistance

evolution is due to fitness costs associated with resistance that might limit the ultimate viability

of resistant mutants once the selective pressure of the drug is removed.(108) In other words,

resistance alleles that emerge within patients might not be sufficiently fit to effectively spread to

other patients. To understand the fate of colistin resistant mutants in *A. baumannii*, we collected

additional patient isolates following withdrawal of colistin treatment, and found that the fitness

cost associated with resistance was so severe, that soon after colistin was withdrawn, susceptible

isolates re-emerged and out-competed resistant isolates within individual patients.(28) However,

in one patient we ultimately identified a low-cost resistance mutant that emerged and was

sufficiently fit to be detected following termination of colistin treatment. We then went on to

show that this mutant was transmitted to other patients, thereby providing additional evidence for

its relative fitness and its potential to be a resistance mutant with epidemic potential.

Another important consideration in studying resistance evolution in patients is that there

can be multiple resistance alleles present in infecting populations, which will be missed if WGS

is performed on only a few clones.(21) Moreover, it may not always be obvious from which

colonizing or infecting population within the patient resistance emerged. For example, many

MDROs initially colonize the gastrointestinal tract, before migrating and causing infections at

other sites, such as the lungs or blood.(109,110) If a patient has sequential susceptible and

resistant isolates taken from their lung, it could be that resistant isolates actually emerged in the

GI tract and migrated back to the lung. This distinction may ultimately be extremely important in

understanding the population dynamics underlying resistance evolution and gaining a better understanding of the probability of resistance emergence during treatment.

2.6.2   Horizontal Transfer and Acquisition Of Resistance

In contrast to the large number of studies documenting mutational resistance emergence in patients, there have been fewer reports documenting horizontal gene transfer (HGT) underlying resistance emergence during treatment. The difficulty in studying HGT derives from the fact that these are presumed to be rare events, and because it can be difficult to demonstrate that the transfer event occurred in a patient, even when the putative source and donor strains are isolated. Despite these challenges, anecdotal reports have begun to emerge documenting the transfer of resistance within the context of individual patients.(111,112) Through a combination of experimental and clinical evidence, Sidjabat et al demonstrated that in a single patient the KPC gene was likely transferred from an infecting *K. pneumoniae* to *E. coli* via recombination of plasmid sequences, and then subsequently transferred to *Serratia marcescens* via conjugation.(111) Hardiman et al attempted to understand drivers of resistance transfer in patients by measuring *in vitro* KPC transfer rates with different plasmid backgrounds and environmental conditions.(113) However, in this study, *in vitro* conjugation rates did not correlate with presumed *in vivo* rates of plasmid mobilization in patients during an outbreak, highlighting the need for future studies that determine factors associated with horizontal transfer during patient treatment.

The observation that the transfer of resistance elements between different MDRO strains and species may not be as uncommon as once thought, has led investigators to attempt to track

the spread of resistance proliferation in the context of hospital outbreaks.(114,115) For example, the outbreak investigation in Mathers et al revealed that the prevalence of CRE at this institution was due to a highly complex plasmid transfer network, where inter-genus transfer of a promiscuous KPC plasmid, transposition of KPC onto different plasmids backbones, and circulation of diverse KPC+ lineages all manifested during the CRE outbreak. Similarly, Conlan et al identified both shared and distinct carbapenemase carrying plasmids in several *Enterobacteriacae* species at their institution. Adding to this complexity, the authors found that while in some cases intra-patient horizontal transfer of resistance elements was likely; in other cases patients harbored multiple species with common resistance elements where HGT was clearly not the origin. Both studies highlight the complex pathways by which mobile resistance elements spread and the importance of not only monitoring prevalence of resistance, but also tracking the mobile genetic elements capable of disseminating resistance in healthcare settings.

In addition to resistance transfer between MDRO species within patients, it is thought that other reservoirs within and outside hospitals may be hubs for resistance dissemination.(116,117) One potential reservoir of resistance makers outside of healthcare settings is hospital effluent. Several groups have used metagenomics approaches to detect resistance markers and mobile genetic elements in hospital wastewater.(118–122) Growing evidence that environmental water organisms can take up clinically important resistance markers when exposed to these wastewaters further bolsters this hypothesis.(122) Recently, Rowe et al used a combined metagenomic/meta-transcriptomic approach to measure resistance gene abundance and expression, as well as antibiotic concentrations in effluents from different sites

that varied in antibiotic use.(120) In support of hospital practices playing a role in promoting the environmental resistome, they found that catchment water from hospitals was enriched for beta-lactamases compared to other sites, and that hospital effluent beta-lactamase levels were correlated with hospital antibiotic usage over time.(120) Water sources within the hospital have also been implicated as a location where resistance transfer could occur. Recent work investigating the role of sinks, drains and other hospital waterways are motivated by several reports of outbreaks where resistant organisms have been isolated from these sites.(123)

The debate over the relative contributions of different reservoirs to resistance dissemination within hospitals recently came to a head in the case of mcr-1, which confers transferrable colistin resistance. Since its initial observation on an inter-species plasmid in 2015, mcr-1 has been identified in the human gut microbiome, wastewater, community, and animal sources.(124–128) This identification of a previously unknown mobile resistance element in all previously mentioned hypothesized reservoirs demonstrates for mcr-1 what is likely true for other resistance mechanisms: that the transfer and dissemination of mobile resistance is likely due to a complex chain of events that take place across multiple ecological settings. Much of the controversy over which reservoirs are the most important for breeding resistance in healthcare settings stems from the fact that the definitive studies examining relative contributions of hypothesized reservoirs of resistance for prevalent pathogens have yet to be carried out. The reservoir is likely different for different pathogens, given the natural history of various resistant organisms (e.g. environmental heartiness, colonization niche etc.) differ significantly. Further complicating the elucidation of the role of hypothesized resistance reservoirs is that the detection

of a resistance marker in a particular location does not inform the timing or direction of resistance transfer from one putative reservoir to another.

Though it is clear that horizontal transfer of resistance is important to the epidemiology of resistant gram negatives, there are several fundamental unanswered questions regarding the mechanism and pathways of resistance transfer. For example, though there is extensive *in vitro* work examining the fitness effects of mutations contributing to antibiotic resistance, the fitness costs of carrying particular resistance elements or mutations in the context of hypothesized real-world reservoirs, and patient carriage or infection are unknown.(108) Studies that examine the evolution of organisms within their real-world context (e.g. longitudinal sampling of colonized patients, evolution on colonized hospital surfaces) are needed to address these questions. Furthermore, little is known about where resistance initially emerges and what clinical and environmental risk factors drive emergence. For example, while multiple studies have assessed the impact of targeted infection preventatives aimed at de-colonization of patients or water reservoirs independently, studies that measure the relative contribution of multiple reservoirs, as well as the cost effectiveness and efficacy of different decontamination strategies on patient outcomes are needed.(129–132) Lastly, while it is known that particular patients with specific characteristics are more prone to developing resistant infections, it has yet to be assessed how the risk of having resistance emerge through mutation or transfer during the context of particular antibiotic treatments is distributed among patients. So far, studies of resistance evolution in patients have been predominantly anecdotal, and therefore there is little insight into why resistance emerges in particular patients and not in others.

2.7   Clinical Diagnostics

A central objective of the clinical microbiology laboratory is to gather information about the causative organism of an infection in order to guide optimal therapy.(133,134) Rapid organism identification is critically important, as delays in the initiation of appropriate treatment are associated with poor patient outcomes. This urgent need has led to the deployment of technologies and workflows aimed at reducing turn-around times between sample collection, organism identification and susceptibility testing.(26,135,136) Newer rapid-identification methods, such as MALDI-ToF have drastically reduced turnaround time in clinical microbiology laboratories and led to improvements in empiric antibiotic prescribing practices.(137–139) However, while these rapid-identification methods have yielded some gains, they are often limited in their speed by the need to first culture specimens and limited in their utility by only providing species level classifications. Strain level discrimination is vital information not only for infection control teams in their efforts to determine if an outbreak is occurring, but could also be utilized in guiding treatment decisions, as certain lineages have the strong associations with resistance and virulence phenotypes.(140–144) Despite our incomplete understanding of the genetic mechanisms of virulence and resistance for prominent pathogens, genome-guided methods hold great promise as rapid clinical diagnostics with the potential to reduce turn-around times for organism identification and susceptibility testing, as well as aid infection prevention investigations, by providing the ultimate resolution for determining relatedness of strains in healthcare settings.

Reliance on microbial culture hinders rapid organism identification because culture can take days, or in some cases weeks, for some resistant organisms.(57,145) For this reason, much attention has shifted to development of culture free diagnostics, which have the ability to identify the causative agent of infections and outbreaks of unknown etiology.(146–149) For example, a recent study applied metagenomic sequencing directly to prosthetic joint infection samples, and demonstrated that this technique can be used to accurately diagnose this type of infection.(150) The application of genomics directly to patient samples is particularly attractive for prosthetic joint infections, as the organisms that cause these infections can be present at very low levels, and take 1-2 weeks to grow, whereas genomic pipelines can detect organisms and identify clinically relevant phenotypes within hours.(151–154) The management of another slow-growing organism for which resistance is a concern, *Mycobacterium tuberculosis*, is another clinical situation that might be improved with rapid genomic diagnostics. Votintseva et al recently showed highly accurate identification and susceptibility profiling of clinical *M. tuberculosis* samples in ~12 hours, whereas culture-dependent phenotypic methods for *M. tuberculosis* susceptibility profiling can take weeks to months.(154) The outcomes of critically ill patients depend on how fast they can start appropriate antibiotic therapy, however typical turn-around times for susceptibility testing in clinical microbiology laboratories for even non-slow growing organisms range from 2-3 days to weeks, which may be detrimentally long in certain cases.(57,155) Further illustrating the potential for rapid turn-around, Leggett et al recently demonstrated real-time organism and resistance profile identification from the feces of an ill infant that took less than 1 hour.(153)

Prior to an organism being identified in a clinical diagnostic laboratory, patients with severe infections (e.g. bloodstream infections) are often treated empirically, and then switched to an antibiotic with known efficacy once a pathogen and its susceptibilities are determined.(156) In addition to organism identification, genomic methods have the potential to decrease time-to-appropriate therapy initiation by identifying resistance markers and virulence factors. Culture-free susceptibility identification methods are particularly attractive, as the antibiotic susceptibility of the infecting organism is also major barrier to the rapid initiation of appropriate and successful treatment.(26,57,94,157,158) There are now several platforms that can be used to predict resistance phenotypes from WGS data.(159,160) In addition to susceptibilities, information from the genomic detection of virulence determinants could aid in the identification of high-risk strains, providing insight into the probable disease they may cause, or their transmissibility in hospital settings.(161,162) For example, epidemic lineage KPC positive *K pneumoniae* ST258 shows variation in its virulence depending on the KPC allele it carries, and is hypothesized to be more virulent because of genetic changes in its capsule locus.(163) Rapid identification of organisms belonging to hyper-virulent or highly transmissible clones could alert healthcare practitioners to place these patients into appropriate infection control precautions rapidly initiate effective therapy.

In addition to the application of genomic diagnostics to guide patient treatment, there is interest in the use of genomics in clinical laboratories for surveillance and outbreak detection. Several genomically informed rapid typing methods have been developed. For example, a genomically informed approach to design multi-plex PCR assays that was recently developed has

the potential to rapidly identify causative agents in polymicrobial infections as well as identify relatedness between strains in outbreak settings.(164,165) This application is particularly appealing because though it is informed by WGS data, it's use does not require the operator to have the skillset required to analyze genomic data, which is an additional concern to implementing genomic diagnostics into clinical microbiology workflows.(164,166) Furthermore, genomics can be used as a gold standard to validate or refute user-friendly typing schemes that are commonly used. A recent study devised a new MLST typing method for *Salmonella* and validated this method against a core genome phylogeny to demonstrate its utility in distinguishing strains.(167)

Despite advances in genomic diagnostics, there is so far only one example in the literature of a patient outcome being improved by genomics in real-time. (168) High-risk and time-sensitive infections, such as those in immune-compromised patients, or sepsis could benefit immensely from this technology, but moving real-time genomic diagnostics into clinics and public health labs will require overcoming several additional hurdles. First, analysis platforms must be adapted for use by personnel in clinical microbiology laboratories. Second, there have so far been no trials that assess if patient outcomes improve with implementation of genomic approaches. An attractive application of genomic prediction of resistance or virulence phenotypes is to target decolonization to patients who are colonized with the goal of reducing their risk for developing and spreading infections. Currently it is unknown if precision decolonization would prove to be beneficial for patients, or if the benefit would be outweighed

by the resulting increase in antibiotic use, which might increase the burden of resistance and put patients at further risk for acquisition of resistant pathogens. (169)

Recent work illustrates both the potential and challenges of the implementation of real-time genomic diagnostics in clinical laboratories. Shelburne *et al* demonstrated that WGS accurately predicted resistance to extended-spectrum beta-lactams in major Gram-negative pathogens, suggesting it may be feasible to use WGS to identify resistance phenotypes in clinical settings. (170) Still, resistance prediction methods are limited by their ability to identify known markers, and existence of unknown resistance markers is a major concern for patients, as false susceptibility identification poses a real threat to patient outcomes. If clinical workflows are to move towards phenotype-independent susceptibility prediction, more effective approaches for prediction of unknown resistance genotypes that are scalable for real-time diagnostic workflows are needed. When combined with future methods of improved susceptibility prediction, a promising technology in the realm of rapid identification and susceptibility testing is the Oxford Nanopore platform. Already, this platform has the capacity to generate sequence data sufficient for species-identification in under an hour with computational steps performed on standard laptop computers. (153,171,172) Whether or not these new technologies are best applied in every-day diagnostic workflows, or reserved for surveillance and outbreak settings remains to be evaluated. Though it is evident that genomic approaches have the potential to revolutionize clinical medicine, unlocking this potential will require key studies that determine if the cost of implementing these technologies improves patient outcomes.

2.8 Conclusion

To summarize, there is an extensive and growing body of work showing how the application of WGS can improve our understanding of the epidemiology and evolution of MDR Gram-negative pathogens. We believe that the next step for moving the field of genomic epidemiology forward is to undertake studies integrating WGS into epidemiologic frameworks from a study's first conception, such that sample collection and analysis methods can be tailored to test specific epidemiologic hypotheses and identify areas where genomics can improve health outcomes. The design and undertaking of these studies is not trivial, and will require will require participation from experts across fields including clinical medicine, microbiology, bioinformatics, antimicrobial stewardship, and healthcare epidemiology. Table 1 illustrates several fundamental questions in healthcare epidemiology, that prior to genomics were intractable, that are now within reach with the power of genomics and epidemiology combined (Table 1). With continued improvement in sequencing technologies and data analysis strategies, we are on the cusp of fulfilling the promise of genomics to elucidate the practices that drive the emergence and spread of antibiotic resistance, and guide interventions to prevent it.

Table 2-1 - Open Questions in The Genomic Epidemiology of Resistant Gram-Negatives

Category	Open Questions
Hospital Epidemiology & Outbreak Investigation	What determines the structure of transmission networks for different pathogens in different types of healthcare settings?
	What are the patient characteristics and clinical practices that impact nosocomial transmission?
	What should be the standard best practices and analytical frameworks for different types of genomic epidemiology investigations?
	What laboratory capacity for genomics should clinical microbiology, public health, or research labs have for real-time epidemiological investigations?
Regional Epidemiology	What are the clinical and epidemiological factors that impact regional MDRO prevalece?
	What are the networks on which different MDROs spread between regional healthcare facilities?
	How can genomics be integrated into public health workflows to impact realtime outbreak control and guide regional interventions?
Evolution and dissemination of cloal lineages	What genomic and epidemiological factors lead to the success of epidemic lineages?
	How does sampling bias impact our understanding of the genetic and epidemiologic factors underlieing the emergence of epidemic lineages?
	Can we predict the epidemic potential of emergent lineages and intervene to prevent their spread?
Evolution of antibiotic resistance	Can we predict the epidemiological significance of resistance that emerges within patients?
	How is resistance emergence influenced by treatment strategy and patient characteristics?
	What influences the rate of horizontal transfer of resistance to MDROs in patients?
	Which potential resevoirs of antibiotic resistance are sources and which are sinks?
	What is the direction of transferrable resistance flow between different resevoirs?
Clinical Diagnostics	Can patient outcomes be improved by implementing real-time genomic diagnostics in clinical microbiology laboratories?
	What is the capacity for genomics to reduce turn-around-time for antibiotic susceptibility testing, and initiation of appropriate therapy?
	How can genomics most effectively supplement phenotypic assays given limitations in prediction of novel resistance alleles?
	What is the value-added of real-time genomic epidemiology investigations versus designed retrospective studies of transmission?

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Chapter 3   Genomic Investigation of a Putative Endoscope-Associated Carbapenem Resistant Enterobacter Cloacae Outbreak Reveals a Wide Diversity of Circulating Strains and Resistance Mutations

3.1   Abstract

A genomic epidemiological investigation of a putative carbapenem-resistant *Enterobacter cloacae* outbreak revealed few plausible instances of nosocomial transmission, highlighting instead the frequent importation of *E. cloacae* into our hospital. Searching for genetic determinants of carbapenem resistance demonstrated that most resistance is due to convergent mutations in phylogenetically diverse *E. cloacae*.

3.2 Introduction

Carbapenem resistant *Enterobacteriaceae* (CRE) are a major cause of antibiotic resistant healthcare associated infections (HAIs). Outbreaks of CRE are most often associated with globally distributed epidemic lineages, for which resistance to carbapenems is conferred by plasmid-associated carbapenemases(1). In the US, the majority of CRE infections are due to *Klebsiella pneumoniae* harboring a *bla-KPC* carbapenemase, with 70% of these infections being associated with the globally distributed ST258 lineage(2). However, in recent years there have been increasing reports of CRE infections caused by other members of the *Enterobacteriaceae* family, with one of the most common being carbapenem-resistant *Enterobacter cloacae* (CREC)(3,4). In addition to sporadic CREC cases due to local spread of mobile resistance determinants, recent reports in the Midwestern US and elsewhere hint at the emergence of a CREC lineage, ST171, with epidemic potential(5,6).

In 2015, the infection prevention department at our institution identified CREC infections in four patients who had previous procedures with duodenoscopes. There have been several recent reports of CRE outbreaks linked to contaminated duodenoscopes, with subsequent outbreak investigations indicating that a small number of device-associated infections can be a sentinel of larger transmission chains(7). We therefore undertook a genomic epidemiological investigation to evaluate the possibility of an outbreak and the potential role of duodenoscopes as a source of CREC transmission. In addition, we sought to identify genetic determinants of

carbapenem resistance and virulence in circulating CREC, in order to better understand the evolution and epidemiology of these clinically important phenotypes.

3.3   Methods

3.3.1   Outbreak Investigation

The University of Michigan Health System is a 990 bed tertiary academic hospital. All available CREC isolates that were collected as part of the clinical diagnostic workflow from 9/2012-12/2015 were included in the investigation (Table S1). Isolates were considered carbapenem-resistant if they were resistant to ertapenem, meropenem, doripenem or imipenem, or were intermediate and displayed a positive modified-hodge test (1 isolate, UM-CRE-9). MIC interpretations were made per CLSI guidelines. Clinical meta-data were collected from the hospital's laboratory information system. The institutional review board at the University of Michigan Medical School approved this protocol.

3.3.2   Genomic Analysis

For the outbreak investigation whole-genome sequencing was performed on 42 isolates comprising putative CRECs and epidemiologically related cases, and pairwise genetic distance was used to rule out epidemiologically implausible transmission linkages. To detect variants associated with carbapenem resistance and virulence we sequenced an additional set of 38 contemporaneous carbapenem-sensitive bloodstream isolates, and searched for genes/pathways enriched in variation among carbapenem-resistant and bloodstream isolates, respectively (see

supplementary materials for detailed methods). Sequence data is available under Bioproject PRJNA401340.

3.4 Results

3.4.1 Transmission of Carbapenem-Resistant *Enterobacter Cloacae* Was Infrequent and Duodenoscopes Were Not a Prominent Mode of Transmission.

Investigation into a putative endoscope-mediated CREC outbreak led to the identification of 37 patients with 42 CREC infection isolates, with six of these patients having had a duodenoscopy prior to their infection. Overall, isolates displayed highly similar antibiotic resistance profiles (Table S1), and there was extensive spatiotemporal overlap in the hospital among case patients (Figure S1), supporting the possible clonal spread of CREC.

To evaluate whether case patients were linked by nosocomial transmission we performed whole-genome sequencing on the 42 CREC isolates. Pairwise comparison of sequenced isolates revealed few plausible transmissions, with a conservative threshold of 100 core-genome variants yielding only 8 patients with a putative transmission linkage (Figure S2). Focusing on isolates from the six endoscope exposed patients revealed only one pair differing by less than 100 core-genome variants (UM-CRE-2, UM-CRE-3, Figure 1A), with the remaining isolates differing by greater than 17,000 variants. Further investigation into this putative transmission link revealed that these patients occupied the same ward prior to their infections (Figure 1B), thus leaving no genomically plausible cases of nosocomial transmission where endoscopes were the only epidemiologic link.

3.4.2 Most *Bla-KPC* Carrying CREC Belong To The Emerging ST171 Lineage, While CREC Strains Lacking a Carbapenemase Are Genetically Diverse

With genomic analysis indicating that most patient's CREC isolates were not clonal, we next asked if the incidence of CREC in our institution was due to a circulating resistance element(1). Searching the 42 CREC genomes for carbapenemase encoding genes revealed only seven patients with isolates harboring a carbapenemase, all of which were of the KPC-type. Five of these KPC+ isolates were clustered on the phylogeny and within 100 variants of one another (Figure 1C, lower-right), with the other two isolates being distantly related and harboring distinct resistance elements. Closer inspection of this KPC+ cluster in the context of previously sequenced CREC, revealed that they belong to the emerging ST171 lineage, that appears to be stably associated with KPC-3 and has been associated with outbreaks in Midwestern healthcare systems.(6)

3.4.3 Evidence of Convergent Evolution of Carbapenem Resistance Among Highly Diverse E. Cloacae

The observation that carbapenemase acquisition could only explain resistance in seven of 42 isolates indicated that core-genome variation drove the majority of resistance. To facilitate the identification of core genomic loci associated with carbapenem resistance, we sequenced an additional set of 38 contemporaneous carbapenem-sensitive clinical *E. cloacae* isolates from our institution to serve as references, relative to which resistance-conferring variants could be identified. Next, we searched for carbapenem resistance loci by identifying genes containing an excess of indels and non-conservative amino acid changes in resistant isolates, as compared to

susceptible controls. This analysis yielded an association between carbapenem resistance and variants in *ampD* (Fisher's exact p = 0.04), which is a negative regulator of the endogenous beta-lactamase *ampC*, and has been previously implicated in carbapenem resistance in *E. cloacae*(8). Of note, all nine observed *ampD* mutations were distinct, providing strong evidence of convergent resistance evolution. Besides *ampD*, no other significant genes were identified, leaving the genetic basis for carbapenem resistance unexplained in 29 of the 42 isolates.

3.4.4 Evidence of Convergent Evolution of Bloodstream Infection Among Highly Diverse *E. Cloacae*

While there have been an increasing number of studies successfully applying genomics to identify genetic signatures associated with antibiotic resistance, there are few examples of genetic association studies yielding predictors of other clinically important phenotypes(9). In hope of prioritizing patients who are at the highest risk of invasive *E. cloacae* infections, we searched for genetic signatures associated with isolates from bloodstream infections (BSI). Examination of the distribution of BSI isolates on the phylogeny did not show evidence of invasive lineages, with BSI isolates spanning the full diversity of observed *E. cloacae* (Figure 1C). Therefore, if there are microbial factors associated with risk of BSI, these must have arisen repeatedly, either during the evolution of different lineages or within colonized patients. To test this hypothesis we searched for evidence of recurrent mutational signatures associated with BSI isolates, as compared to isolates from other types of infections. While no associations reached

statistical significance, several of the most strongly associated pathways are involved in short chain fatty acid (SCFA) metabolic pathways (Figure S3).

3.5 Discussion

In evaluating infection control measures, it is vital to know what drives the prevalence of HAI's and the extent that nosocomial transmission or importation is responsible. Here, we performed a genomic epidemiology investigation that allowed us to rule out a putative duodenoscope-mediated outbreak of CREC. Moreover, the observation that CREC at our hospital span the full diversity of previously sequenced isolates, with few cases of patient's sharing closely related strains, strongly suggests that importation, rather than transmission, has driven the incidence of CREC infection at our institution.

While a great deal is known about the epidemiology of CRE carrying carbapenemases, far less is known about the epidemiological importance of mutational carbapenem resistance (10). One hypothesis is that common mutations associated with carbapenem resistance carry a significant fitness cost, making these resistant strains less transmissible (11). In agreement with this, we found few instances of plausible transmission of CREC that lack a carbapenemase, and no cases where an identified resistance mutation was shared among multiple patient isolates. In contrast to the apparent dead-end associated with mutational carbapenem resistance, we observed multiple patients with *bla-KPC* CREC from the ST171 clonal lineage, that is stably associated with a *bla-KPC* containing plasmid and appears to be a lineage with significant epidemic potential(6).

In addition to understanding the genetic basis for antibiotic resistance, we also searched for genetic signatures associated with invasive infection. While the diversity of our isolate collection limited our power, we observed a signature of recurrent mutation in SCFA metabolic pathways among BSI isolates. This observation is consistent with a model wherein successful colonization, and potentially modification, of the gut environment precedes invasive infection(12). In particular, the SCFA butyrate is known to play a role in the reinforcement of the colonic epithelial barrier, raising the possibility that altered SCFA metabolism could compromise the intestinal barrier, and facilitate introduction of CRE into the bloodstream. It will be of interest in the future to assess the robustness of these findings and search for additional genetic signatures associated with virulence.

Our genomic epidemiological investigation provides a glimpse into the epidemiology of CREC infection, and adds to a growing body of literature demonstrating the potential for genomics to help prioritize patient risk of clinical outcomes, and guide infection control practices.

3.6   Funding

3.7 Acknowledgments

3.8 Potential Conflicts of Interests

All authors: no reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

A.

Log10 pairwise genetic distance

0 2 4 6

UM-CRE-4
UM-CRE-1
UM-CRE-8
UM-CRE-20
UM-CRE-3
UM-CRE-2

UM-CRE-2
UM-CRE-3
UM-CRE-20
UM-CRE-8
UM-CRE-1
UM-CRE-4

B.

Ward

ERCP
Positive Culture

Patient

4
1
8
18
3
2

Sept. 2012
Mar. 2013
Sept. 2013
Mar. 2014
Sept. 2014
Mar. 2015
Sept. 2015

Date

C.

Tree scale: 0.01

ampD variant

Blood isolate

Carbapenem resistant

Duodenoscope exposed
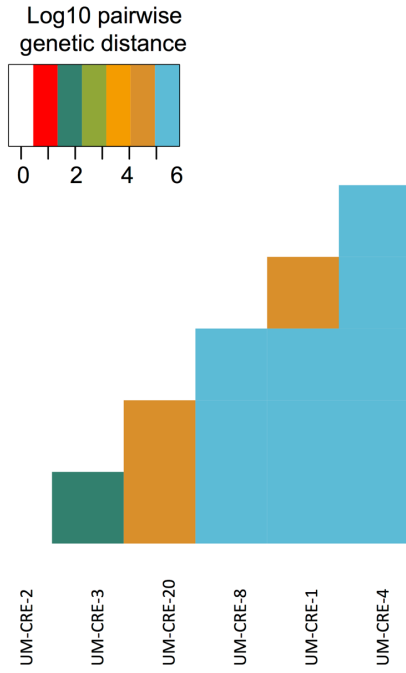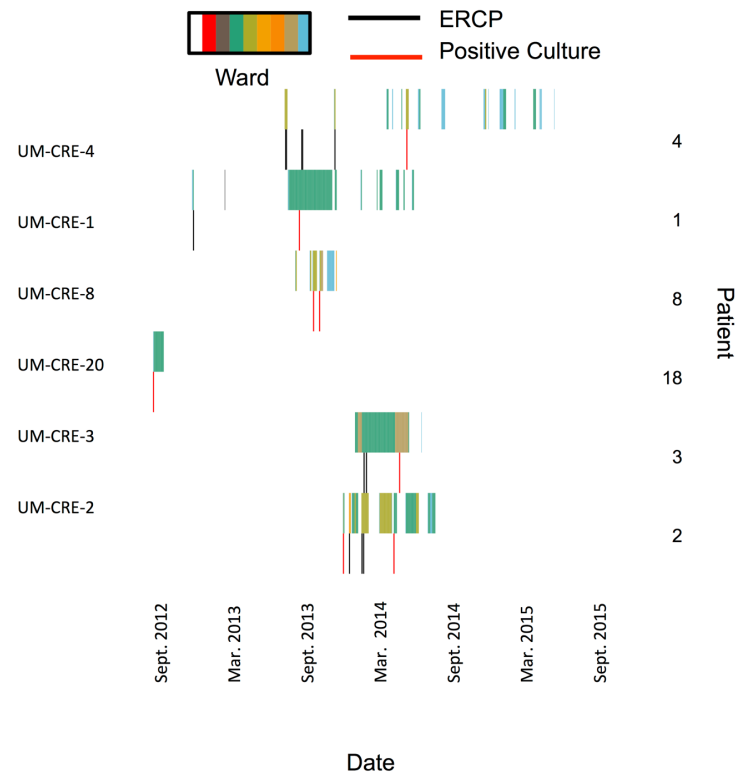
KPC
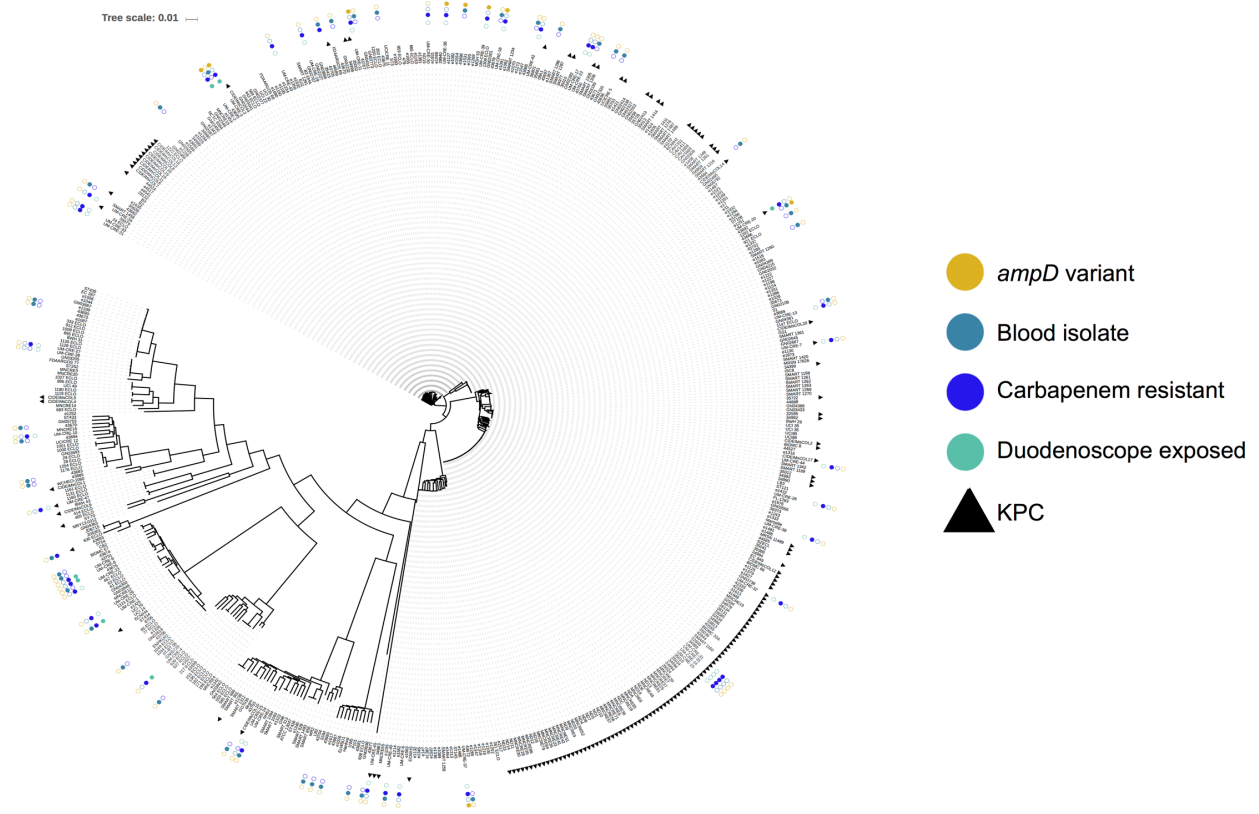
Figure 3-1: Genomic Epidemiology Investigation of CREC. **A.** The number of core genome variants between CREC isolates from patients who were exposed to the duodenoscopes was calculated, and log10 transformed for visualization purposes. Four of the six isolates from patients who were exposed to duodenoscopes prior to CREC infection are >17,000 core genome variants from the other isolates, providing strong evidence against direct transmission. In contrast, isolates UM-CRE-2 and UM-CRE-3 are only distinguished from each other by 62 core genome variants, indicating a plausible transmission relationship. **B.** Patient bed traces for the patients from panel A are shown, with the order of patients (y-axis) corresponding to the phylogenetic relationship of their CREC isolates. Each patient is represented by two rows, which correspond to the wards where patients resided while in the hospital (colors represent unique wards), and ERCP procedure/*E. cloacae* infection dates (black/red).Note that the patients 2 and 3 whose isolate's pairwise genetic distance is consistent with recent transmission, overlapped on the same ward . **C.** A maximum-likelihood phylogeny was constructed for all 80 isolates sequenced as part of this study and a representative set of *E. cloacae* available in Genbank using variants identified in the 1.4 Mb core genome (scale in mutations per site in core genome). The presence of the KPC gene is shown for all isolates (black triangle). Duodenoscope exposure (green circle) is indicated for the 42 isolates that were included in the outbreak investigation . Carbapenem susceptibility (blue circle), infection type (blue-green circle) and the presence of an AmpD variant (yellow circle) are shown only for the 80 genomes that were sequenced for the current study (42 outbreak isolates and 38 contemporaneous carbapenem-sensitive bloodstream isolates).

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Chapter 4   Genomic cluster analysis to identify modifiable unchecked routes of *Klebsiella pneumoniae* transmission during a bundled intervention in a long-term acute care hospital

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4.1   Abstract

4.1.1   Background

The implementation of infection prevention programs has yet to neutralize the threat of multi-drug resistant pathogens to hospitalized patients, suggesting that cryptic transmission pathways remain uncontrolled by current prevention strategies. Modifiable drivers of lingering transmission pathways may be detected with targeted strategies that incorporate whole-genome-sequencing (WGS), robust patient sampling and epidemiological data.

4.1.2   Methods

We leveraged KPC-Kp surveillance cultures from 94% of patients in a long-term acute care hospital (LTACH) collected during a one-year bundled intervention that reduced KPC+ *Klebsiella pneumoniae* (KPC-Kp) prevalence. We integrated WGS, and surveillance data with a single-nucleotide variant-threshold-free approach to detect clusters of patients linked by within-

LTACH cross-transmission to KPC-Kp admission-positive 'index' patients. Transmission pathways were detected using patient location data.

4.1.3 Findings

Transmission clusters (N=49) had between 2-14 patients, capturing KPC-Kp acquisitions from 100 (80%) patients who first acquired KPC-Kp in the LTACH. Within-cluster genetic diversity varied from 0-154 (median 9) SNVs. Elevated diversity was attributable to intra-patient colonization diversity and the emergence of hypermutator strains. Spatiotemporal exposures between patients in clusters could explain acquisitions in a shared room 14%, floor 66%, or elsewhere in the LTACH 81%. Sequential exposure to a common room was the only epidemiological link for a single patient. Routes of transmission that persisted during the intervention included lapses in patient cohorting, false-negative surveillance.

4.1.4 Interpretation

Integration of robust surveillance and. WGS data using a SNV threshold-free approach revealed that up to 15% of uncontrolled KPC-Kp cross-transmission occurs when patients encounter unidentified patient or environmental sources outside of their bed location. Overall, results highlight the potential for WGS to monitor and improve infection prevention and the importance of combining comprehensive sampling with SNV-threshold independent analytical strategies tailored to generate actionable hypotheses.

4.1.5 Funding

4.2 Introduction

Healthcare-associated infections (HAIs) affect one in 31 hospitalized patients on any given day in the U.S. and are a major threat to patient safety.(1) Despite increased attention to infection prevention in healthcare settings, cross-transmission between hospitalized patients still occurs, suggesting that there remain uncontrolled pathways of transmission in hospitals.(2) Encouragingly, the integration of genomics with shoe leather hospital epidemiologic investigations has proved powerful in the investigation of HAI transmission, primarily in the acute care setting where sporadic cases give rise to a healthcare associated outbreaks.(3–5)

The majority of genomic investigations have applied single-nucleotide variant (SNV) thresholds to identify cases of likely cross-transmission within a healthcare facility. SNVs accumulate over time in bacterial genomes, and the rate of SNVs that accumulate in a given timeframe can be used to determine if organisms detected from patients in a hospital are likely to be related by in-hospital transmission. Closely related organisms that are distinguished by a small number of SNVs have more likely recent transmission relationships, while more distantly related organisms are distinguished by a greater number of SNVs and are less likely to be related by recent transmission. The specific SNV thresholds applied in transmission investigations are based on a hypothesized number of SNVs to expect in a given timeframe which could be determined through calculations of when the most recent common ancestor of sampled isolates

likely existed(6) evaluation of intra-patient diversity from longitudinally sampled patients,(7) or selection of multiple colonies that are isolated from a patient at single point in time.(8)

Though the application SNV thresholds have demonstrated success in outbreak settings, where clonal relationships and short time periods limit the risk of misclassification of patients both with and without cross-transmission links, the efficacy of this method is questionable in endemic and high-transmission settings because there is the potential for this approach to yield false positive and false-negative transmission links. For example, the prevalence of prominent HAI-causing pathogens is often geographically dominated by successful epidemic clonal lineages,(9–13) which makes it difficult to distinguish closely related isolates stemming from acquisition of closely related strains at a connected healthcare facility versus cross-transmission within a facility. Small SNV distances would be observed and a false-positive transmission link would be identified if patients harbor isolates that are related by recent cross-transmission that occurred in a connected facility. False-negative transmission links could manifest as from evolution which occurs within patients during prolonged asymptomatic colonization or due to uneven selective pressures, such as different antibiotic treatments between patients, which could manifest as larger than expected SNV distances between isolates that are related by transmission that occurred within a facility.

Long-term acute care hospitals are healthcare settings that have high colonization pressure for HAI-causing pathogens and are places where multi-drug resistant organisms (MDROs) that cause HAIs are often endemic.(14,15) Carbapenem resistant Enterobacteriaceae (CRE) are MDROs that are resistant to nearly all antibiotics and cause an estimated 8,500

infections leading to 1,100 deaths in the U.S. annually.(2) Recent work points to LTACHs having a disproportionately high prevalence of CRE and suggests that these facilities contribute to regional CRE transmission when LTACH patients are transferred between facilities in connected healthcare networks.(16–18) Given the role of LTACHs as reservoirs for MRDOs like CRE, it is imperative to improve our understanding of how transmission occurs in these facilities in order to develop better methods to prevent spread.

Encouragingly, a recent study demonstrated the effectiveness of a bundled intervention that reduced a particular type of CRE, *Klebsiella pneumoniae* that carry the KPC carbapenemase (KPC-Kp), in an LTACH with high KPC-Kp prevalence.(19) Unfortunately, despite the success of the intervention, patients still acquired KPC-Kp, suggesting that routes of cross-transmission remained uncontrolled.

Here, we applied the high resolution of genomics to a comprehensive active surveillance study of KPC-Kp colonization in a Chicago LTACH where KPC-Kp was endemic in order to discern routes of transmission within the LTACH that persisted during the intervention. (19) We hypothesized that the application of a SNV threshold-independent genomic epidemiologic method that integrates robust surveillance information would enable us to identify patients related by cross-transmission of KPC-Kp in the facility (transmission clusters). Moreover, we predicted that by evaluating location data among patients in transmission clusters we would identify testable hypotheses for how to reduce uncontrolled transmission in the facility.

4.3 Methods

4.3.1 Study Design and Clinical Setting and Sample Collection

Detailed information regarding the study design, intervention bundle and data collection are available in Hayden et. al 2015.(19) Briefly and of relevance to the current manuscript, the study took place between 2011-2013 during a quality improvement project to prevent KPC-Kp colonization and infection in a Chicago LTACH where the average prevalence of KPC-Kp colonization was 30%. All location data and isolates presented here were collected from one LTACH during the intervention period, which included rectal surveillance swab culture-based screening of all LTACH patients for KPC-Kp rectal colonization at LTACH admission and every two weeks (94% adherence), and efforts to separate KPC-Kp-positive and KPC-Kp-negative patients by placing KPC-Kp-positive patients in ward cohorts (91% adherence).(19)

4.3.2 Patient Surveillance Categories

Patients were grouped into categories based on surveillance data. 'Index' patients were those who were positive at the start of the study or upon admission within three days of ever being in the LTACH during the study. 'Convert' patients converted from KPC-Kp-negative to KPC-Kp-positive during the study. If a patient was in the facility for greater than three days prior to their first surveillance sampling, they were considered a convert patient for the purposes of the transmission cluster detection algorithm (see below). When an admission-positive patient

acquired an additional KPC-Kp strain during their stay this was termed "Index with secondary acquisition."

4.3.3 Whole-genome Sequencing & Genome Processing

DNA was extracted with the MoBio PowerMag Microbial DNA kit and prepared for sequencing on an Illumina MiSeq instrument using the NEBNext Ultra kit and sample-specific barcoding. Library preparation and sequencing were performed at the Center for Microbial Systems at the University of Michigan or the University of Michigan Sequencing Core. Quality of reads was assessed with FastQC,(20) and Trimmomatic(21) was used for trimming adapter sequences and low-quality bases. Assemblies were performed using the A5 pipeline with default parameters.(22) In total, 462 samples were sequenced, with 435 yielding quality genomic that was used in downstream analyses. Sequence data are available under BioProject PRJNA603790.

4.3.4 Identification of Single Nucleotide Variants

SNV calling was performed as in Han *et al*.(16) The variant calling pipeline can be found at https://github.com/Snitkin-Lab-Umich/variant_calling_pipeline. To summarize, variant calling was performed with samtools(23) using the reference genomes listed in Supplementary table 1.

4.3.5 Whole-genome Sequence Analyses

All whole-genome sequence analyses were performed in R version 3.6.1. Whole-genome sequence alignments containing core and non-core variant positions were used to generate pairwise (genome by genome) single-nucleotide variant (SNV) matrices, shared-variant matrices, interrogate mutational biases and construct phylogenetic trees for transmission cluster detection and descriptions of genomic variants.

4.3.6 Transmission Cluster Detection

Transmission cluster detection using a SNV threshold-free approach was performed on isolates from MLSTs that were present in at least two patients including at least one convert patient, as this represents molecularly plausible cross-transmission within the LTACH (**Table 1**). Whole-genome sequence alignments including core and non-core genome variant positions were used to generate maximum parsimony phylogenetic trees, pairwise shared variant matrices and SNV distance matrices for each MLST-specific alignment. Transmission clusters were detected by probing phylogenetic trees for the maximum subtree containing admission or study-start isolates from a single index patient that was collected prior to or at the same time as acquisition isolates that was supported by at least one unique subtree-defining variant that was not found elsewhere in the phylogeny. Multiple index patients were permitted in clusters if they shared at least one unique variant as the other cluster members. Clusters with no index isolates (convert isolate only clusters) were permitted if no subtree existed that included an index isolate. Only clusters that

contained at least two patients and at least one acquisition isolate were considered valid

transmission clusters for downstream analyses.

4.3.7   Analysis of Location Data

Location data was abstracted from patient bed traces. Spatiotemporal overlap explanations for

cross-transmission between patients in clusters were defined as patients being in the same

location (e.g. facility, floor or room) at the same time during the time between when a putative

donor patient in the cluster was last negative for the isolate up until and including the day the

recipient tested positive for the isolate. Sequential exposure was evaluated for the same

timeframe, but restricted to patients being in the same location separated by time, where the

putative donor had been in a location first and the recipient later occupied the same location

while they were converting from negative to positive for the isolate, and no spatiotemporal

exposure between donors in the cluster and the recipient could explain the recipients' acquisition.

4.3.8   Statistical Analysis

Two-sample Kolomogorov-Smirnov tests were used to test for a statistical difference in pairwise

SNV distance distribution between admission and acquisition isolates. Multinomial tests were

used to determine significant biases in mutational frequencies. The Wilcoxon signed rank test

was used to detect differences in intra-patient and intra-cluster SNV distances between admission

and acquisition isolates. Permutation tests were used to evaluate enrichment in spatiotemporal and sequential exposures between patients in transmission clusters.

4.4 Results

4.4.1 KPC-Kp Endemicity in the LTACH is Due to Extensive Importation into and Acquisition Within the Facility of Diverse and Clonal Lineages

On the first day of the study KPC-Kp colonization was determined for all patients in the LTACH via rectal surveillance cultures, which yielded 51 who were positive for KPC-Kp (61 isolates). Throughout the study, admission and bi-weekly surveillance detected 77 patients (105 isolates) who were positive within three days of first admission (imported KPC-Kp). There were 128 convert patients (234 isolates) who were presumed to have first acquired colonization in the LTACH either due to their first surveillance culture being negative , or having been in the facility for more than three days before having a surveillance culture taken. Index patients who had additional secondary KPC-KP isolates collected during their stays (N=28) contributed an additional 62 isolates, bringing the total number of isolates identified after patients had been in

the LTACH for three or more days to 296 isolates from 156 unique patients. Adherence to the surveillance culturing protocol was 94% throughout the study.(19) While acquisition and importation fluctuated **(Figure 1B)** the overall colonization prevalence was relatively consistently high, averaging 32% during the study **(Figure 1A)**.

4.4.2 Strain Diversity and Surveillance Data Indicates Multiple Distinct Transmission Chains in the LTACH

Examination of strains by MLST inferred from genome sequences revealed that 62% of the isolates obtained during the study belonged to ST258, the major epidemic lineage in the U.S. (**Figure 1B, Table 1**), though other lineages were also present in smaller frequencies (**Table 1**). There were 424 (96% of all isolates collected) isolates from 7 different MLSTs that were represented by colonization of least two patients, including at least one patient who first acquired colonization during their stay in the LTACH, demonstrating molecular support for cross-transmission relationships among the majority of isolates that were collected throughout the study.

4.4.3 Application of a SNV-threshold is Inadequate to Identify KPC-Kp Cross-Transmission Links Between Patients in the Endemic LTACH

We next applied the increased resolution of genomics to discern which patients shared cross-transmission relationships within the MLSTs. First, we examined the potential to apply a

SNV threshold to identify patients with isolates linked by cross-transmission that occurred in the LTACH during the study. Robust surveillance data enabled us to identify which patients were positive on admission who brought their colonizing strains into the facility with them, and which were negative at admission, who presumably acquired KPC-Kp from cross-transmission from other patients in the LTACH. If an SNV threshold could be used to distinguish isolates from patients who acquired KPC-Kp colonization in the LTACH from patients who acquired colonization outside the LTACH, the genetic distances among admission-positive patients (e.g. pairs not related by transmission within the facility) should be greater than the genetic distances between patients who acquired KPC-Kp and their closest admission-positive patient (e.g. their putative intra-facility transmission source). Examination of the pairwise SNV distances comparing isolates imported by admission positive patients to each other and to acquired isolates from patients who converted from KPC-Kp-negative to KPC-Kp-positive in the LTACH revealed no SNV threshold that distinguished these distributions (**Figure 2**, Kolmogorov-Smirnov test p-value=0.82). These results demonstrate that a SNV threshold could not distinguish in-LTACH transmission from importation and therefore a more discriminatory approach is required to identify transmission links in this endemic setting that has both extensive importation of closely related strains and high rates of transmission.

4.4.4 Transmission Clusters Detected With a SNV-Threshold Free Approach Link the Majority of KPC-Kp Acquisitions to Importation by Admission Positive Patients

To circumvent challenges associated with applying an SNV threshold to infer transmission linkages, we took advantage of our comprehensive knowledge of which patients imported and acquired KPC-Kp and applied an algorithm whereby each acquisition isolate was grouped with the admission isolate with which it shared the most variants. In essence, this approach groups each acquisition with the admission isolate with which it shares a most recent common ancestor (**Figure 3A**). Application of this genomic cluster detection method yielded 49 putative transmission clusters grouping a median of 3 (range 2-14) patients into clusters representing at least one acquisition event and at least two patients. Overall, transmission clusters detected with this method grouped at least one isolate from 151 (60%) of the KPC-Kp-positive patients in the study including 100 (80%) of convert patients who first acquired KPC-Kp colonization in the LTACH during the study. There were 18 (14%) study start and importation index patients who imported isolates that no in-LTACH acquisitions were linked to. Transmission clusters that were traced back to importation events by a single index patient (N=23 clusters) included 41 patients who acquired colonization in the LTACH, representing 26% of acquisitions captured overall in clusters. The remaining acquisitions were grouped into 26 clusters with uncertain sources of importation including 27 (17.3%) linked to multiple index patients, 42 (27%) linked to no index patient in the study, and 26 (16.6%) linked to epidemiologically implausible index patients who were first in the facility after the convert patient had already acquired KPC-Kp colonization (**Figure 3B**).

4.4.5 Transmission Clusters Detected With SNV-Threshold Independent Approach Range in Genetic Diversity Associated with Emergence of Hypermutator Strains and Prolonged Colonization

Examination of the intra-cluster diversity revealed that the maximum SNVs separating isolates in identified clusters ranged from 0 to 154, median 7 SNVs. While the majority of clusters varied by small genetic distances, 9 clusters (18 %) had larger SNV distances (greater than 30 SNVs) (**Figure 4A**). Larger SNV distances might indicate inclusion of index patients who are not the true source of the transmission cluster (false-positive transmission links). Indeed, larger intra-cluster genetic diversity was noted among several transmission clusters containing isolates from multiple admission-positive patients (**Figure 4 A**), suggesting that one of the admission positive patients was falsely included in the cluster. Furthermore, we hypothesized that some larger distances could be due to patients with diverse intra-patient colonizing populations. In support of this, we observed a distribution of intra-patient diversity among both index and convert patients who contributed multiple isolates to a cluster (**Figure 4C**). Moreover, we observed a significantly greater intra-patient diversity among admission-positive patients, (Wilcoxon rank sum test p-value < 0.03), supporting the role of prolonged colonization driving intra-patient diversity(**Figure 4C**). In our examination of intra-patient diversity, we also observed several cases of extreme SNV distances which were inconsistent with previously reported evolutionary rates for KPC-Kp. We hypothesized that these large distances could be due to the emergence of hyper mutator phenotypes, as has been reported for other

commensal and pathogenic bacteria. Biases in the types of mutations found in bacteria have been used to infer hyper mutator strains, with mutator types showing biases towards increased AT to CG transversions and AT to GC and GC to AT transitions.(24) Examination of mutation rates among transmission cluster isolates revealed two clusters (16_16 and 258_117, **Figure 4B**) with biased mutation rates (multinomial test, p-value < 0.05), consistent with the emergence of hypermutator strains, which could explain the extremely elevated diversity in these clusters (**Figure 4A**).

4.4.6 Half of Acquisitions in Clusters Could be Explained by Spatiotemporal Exposure Between Patients in Shared Floors or Rooms

Next we evaluated shared space and time relationships between patients in clusters by examining surveillance and location data in order to identify potential routes of cross-transmission in the LTACH between patients in the same cluster. Transmission in clusters occurred for a median of 93 days (time between importation or first positive isolate and final acquisition in the cluster), but the duration of clusters varied widely from 0 (isolates being collected at the same time within a single 14-day surveillance window) to 334 days. Spatiotemporal exposures between patients were observed across transmission clusters. At different locations including facility, floor and room, 81%, 66% and 8.5% of KPC-Kp acquisitions respectively could be explained by spatiotemporal exposures between patients in the same transmission cluster. Compared to random groups of patients of the same size and patient type distribution (e.g. admission-positive and acquisition), observed transmission clusters were

109

enriched for spatiotemporal exposures between patients that could explain acquisition of KPC-Kp by convert patients (permutation tests, P-value < 0.001, all locations).

4.4.7   Sequential Exposure to Common Locations Was Not Enriched in Transmission Clusters

Sequential exposure to common locations separated by time could be a signal of prolonged contamination of the environment or other unidentified reservoir for KPC-Kp transmission that persists past a patient's stay in the LTACH. Sequential exposures explaining acquisitions among patients who did not have any spatiotemporal exposures between putative donor patients in their clusters were infrequent, with only 8.5% of acquisitions across clusters explained by sequential exposure in the facility, 4.7% floor, and 0.78% room. Sequential exposures were not statistically enriched (permutation tests, p-value >0.60, all locations). Of special note given previous reports of sinks as a vehicle for longitudinal transmission, examination of sequential exposures to common rooms, among individual cluster patients revealed only a single patient (**supplemental figure 1**, cluster 258_175, patient 174) whose sequential exposure to a room previously occupied by another patient from their cluster was the only exposure detected that could explain their acquisition.

4.4.8 Genomic Epidemiologic Transmission Cluster Detection Reveals Testable Hypotheses for How to Reduce Transmission That Persisted During the Intervention

The transmission clusters detected with our genomic epidemiologic approach fell into several categories based on the surveillance information from patients included in clusters and the exposures that could serve as transmission links between patients (**Figure 6**). Examination of location and surveillance data in these transmission clusters is suggestive of specific routes of transmission that persisted throughout the intervention study including: transmission between cohort and non-cohort locations (**Figure 6A),** lapses in cohorting e.g. transmission due to housing a known positive patient in the same location as a negative patient (**Figure 6B),** false-negative surveillance culturing (**Figure 6C**), missing intermediate link between patients (**Figure 6D**), and potential exposure between cluster patients that occurred in an outside facility (**Figure 6E**). The plausible routes of transmission illustrated in these vignettes are not mutually exclusive of one another and evidence supporting multiple routes occurred simultaneously in several clusters.

4.5 Discussion

Whole-genome sequencing has become the gold-standard for molecular epidemiologic investigations of transmission.(25) However, most previous applications have been in the context of hospital outbreaks where SNV thresholds have been used to distinguish importation of

more distantly related organisms from outside a facility from closely related organisms that are related by cross-transmission within a facility.(3,26) However, in endemic settings with high colonization pressure—such as with KPC-Kp in LTACHs—partitioning patients into transmission clusters based on a SNV threshold could misclassify patients both in and out of transmission clusters due to intra-patient colonizing diversity, prevalence of closely related strains both within and outside the facility, and the emergence of hypermutator strains, all of which can result in deviations from the expected number of SNVs.(24,26,27) Here we evaluated the potential to apply a SNV threshold to distinguish importation from acquisition resulting from cross-transmission in an LTACH with 32% prevalence of KPC-Kp during a time period where transmission persisted despite robust adherence to a bundled infection prevention intervention.(19) We demonstrated that no discriminatory SNV threshold exists in this endemic setting. We also showed that plausible cross-transmission links could be detected to explain 80% of initial KPC-Kp acquisitions by KPC-Kp-negative patients using a method that integrates robust surveillance data and a genomic algorithm that does not rely on a fixed SNV threshold. Moreover, evaluation of the genetic diversity in transmission clusters revealed evidence of closely related isolates that were likely imported from patients transferring from connected facilities, intra-patient colonizing diversity, as well as the emergence of hypermutator strains. Application of this SNV threshold-free approach enabled us to evaluate locations in the LTACH where transmission plausibly occurred between individual transmission links in clusters, allowing us to generate hypotheses for specific routes of transmission between patients that persisted despite the intervention.

Our study has several limitations related to biases in sampling. First, though the robust sampling strategy in this study captured 94% of patients who imported or acquired KPC-Kp in the facility, only a single or small number of colonies (representative unique morphologies) were collected and sequenced per patient. Therefore, we are likely to miss cases of multi-strain colonization, which could account for the 42(27%) acquisitions in clusters without an index patient and 26(16.6%) of acquisitions in clusters with index patients who are not the source of the cluster (first to test positive) in the LTACH. Additionally, our lack of knowledge of where patients were prior to admission to the LTACH prevents us from understanding how transmission at connected healthcare facilities impacts cluster detection, which could also explain inclusion of admission-positive patients who are not the first members of their cluster to test positive. Furthermore, no surveillance culturing method is perfectly sensitive, therefore there were likely KPC-Kp-positive patients in the LTACH who were detected later, or never in the study, which are alternative explanations for clusters we observed with no index patients or clusters without in-LTACH spatiotemporal exposures between patients. Additionally, because of our lack of knowledge of exposures outside of patient bed locations (e.g. physical therapy, procedure rooms), we could not gain insight into common exposures that could have mediated transmission in other locations in the LTACH.

Our study has several strengths. First, there was comprehensive admission and in-LTACH surveillance culturing to detect KPC-Kp-positive patients, which enabled our use of a SNV threshold-free approach to detect putative transmission links capturing 80% of KPC-Kp

acquisitions in an endemic setting. Application of this method allowed us to more precisely identify when common exposures between patients likely occurred to facilitate transmission in the LTACH. Furthermore, by sequencing multiple isolates from the subset of patients from whom multiple isolates were collected, we were able to gain insight into the intra-patient diversity in an endemic setting. Additionally, this study was performed in the context of a robust infection prevention intervention that reduced KPC-Kp in an endemic LTACH, affording us the unique opportunity to evaluate transmission that persisted during the intervention and highlight ways for future prevention of transmission. For example, we observed that while 81% of acquisitions in clusters could be explained by spatiotemporal exposure between patients in the facility, only 63% could be explained by patients being on the same floor at the same time, suggesting that 15% of transmission occurs outside of a patients bed location, which points towards the need for improved infection control practices when patients visit common locations in the LTACH or are cared for by staff who work on multiple floors.

Additional strengths are represented by the testable hypotheses for how transmission persisted during the study which were revealed through our evaluation of location and surveillance data in transmission clusters. We saw evidence of transmission due to lapses in the practice of cohorting where negative patients were either housed on or moved to a cohort floor prior to KPC-Kp acquisition. While transmission clusters where patients were separated by time and space are concerning because they represent unidentified intermediate sources, the detection of these clusters illuminates which patients between whom to look for additional common exposures, such as devices, shared time in a temporary unit such as physical therapy or

procedure units or shared exposures to staff that care for patients in multiple locations, such as respiratory therapists. Additionally, recent studies have highlighted the potential for environmental contamination such as hospital surfaces and plumbing infrastructure as potential reservoirs for MDROS including KPC-Kp.(28–30) However, our analysis of spatiotemporal and sequential exposures between patients revealed that only a single patient acquisition could only be explained by sequential exposure to a common room that was previously occupied by a donor in their cluster, suggesting that persistent environmental reservoirs in patient rooms contribute minimally to transmission in this setting. Recent studies have also pointed towards patient transfer as a mechanism by which MDROs spread in regions throughout connected healthcare networks.(18) We saw evidence of this manifest in transmission clusters with multiple index patients in clusters, representing importation of closely related strains from outside the facility. This suggests that improved communication between facilities when transferring patients harboring a concerning organism could serve to both alert staff at the receiving facility who could then place patients in appropriate precautions earlier to prevent onward cross-transmission, as well as pinpoint the specific facilities where transmission occurred between patients.

Overall, our results highlight the potential for WGS to monitor and improve infection prevention and the importance of combining comprehensive sampling with analytical strategies tailored to generate actionable hypotheses for how to improve infection prevention.

4.6 Acknowledgments

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4.8   Tables and Figures

Figure 4-1 Endemicity of KPC-Kp in the LTACH is due to extensive importation and

acquisition.

A. Prevalence(pink), KPC-Kp carriers (periwinkle), defined as number of patients who are or

ever had been positive for KPC-Kp during the study, and patients in the facility (tan) throughout

the 1-year study. **B.** isolates obtained through bi-weekly rectal surveillance culturing of LTACH

patients. Grey boxes indicate the study start (time 0) and 14-day surveillance periods throughout

the study. Bars indicate the MLST inferred from WGS data for isolates collected at the beginning

of the study (study start), within 3 days of the patient first entering the facility (importation), or

after negative surveillance or >3 days after ever being in the LTACH during the study (acquisition).
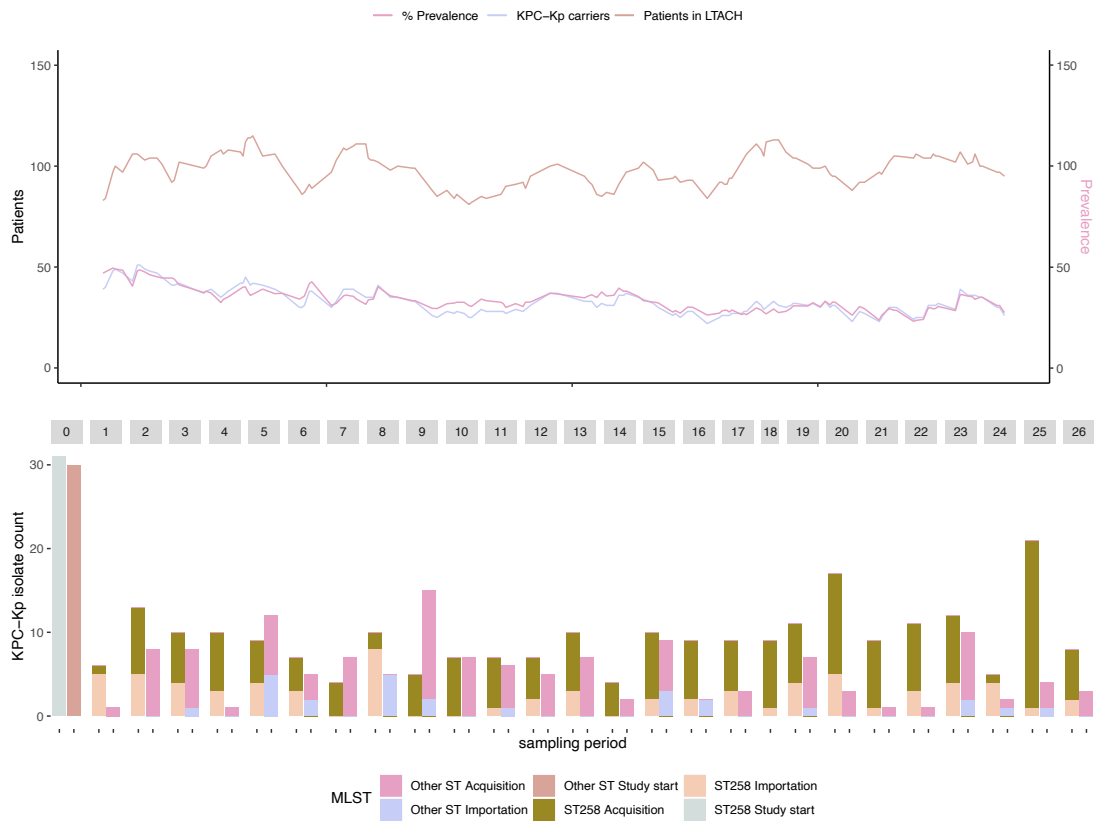
Table 4-1 Distribution of KPC-Kp Strains Isolated from Colonized LTACH Patients.

*Possible cross-transmission link in LTACH during study inferred by at least two patients with isolate of the MLST and at least one patient converting from negative to positive for colonization with an isolate of the MLST.

†Isolate total represents isolates with quality WGS data; 27 samples were excluded from the 462 total isolates obtained due to poor sequence quality.

MLST	Number of Isolates	Patients with at least 1 isolate of MLST	Patients with at least one imported isolate of MLST	Patients colonized by at least one isolate of MLST	Possible cross-transmission link in LTACH during study*
13	63	37	18	22	yes
14	7	5	1	4	yes
15	17	11	1	10	yes
16	47	32	15	20	yes
20	6	6	1	5	yes
36	1	1	1	0	no
134	1	1	1	0	no
193	2	2	2	0	no
258	271	177	83	104	yes
327	6	4	0	4	yes
834	2	1	1	0	no
874	7	5	3	2	yes
883	1	1	1	0	no
950	3	1	0	1	no
Novel	1	1	1	0	no
Total	435	285	129	172	

Figure 4-2 There Is No Single-Nucleotide Variant Threshold That Distinguishes Isolates Acquired In The LTACH From Isolates That Are Imported By Admission-Positive Patients.

Comparison of minimum pairwise SNV distances between the closest related imported isolate and acquired or imported isolates. X-axis indicates SNV distance, Y-axis indicates density of KPC-Kp isolates. Tan bars indicate the minimum distance between isolates collected from patients who acquired KPC-Kp colonization after being in the LTACH >3 days during the study. Peach bars indicate the minimum distance between isolates collected from patients who were positive upon admission to the LTACH, excluding study start positive patients who were KPC-Kp-positive on the first day of the study, as these patients represent a mixture of recent and prior colonization. Two-sample Kolmogorov-Smirnov test for differences in the distribution of pairwise SNV distances, p-value = 0.82.
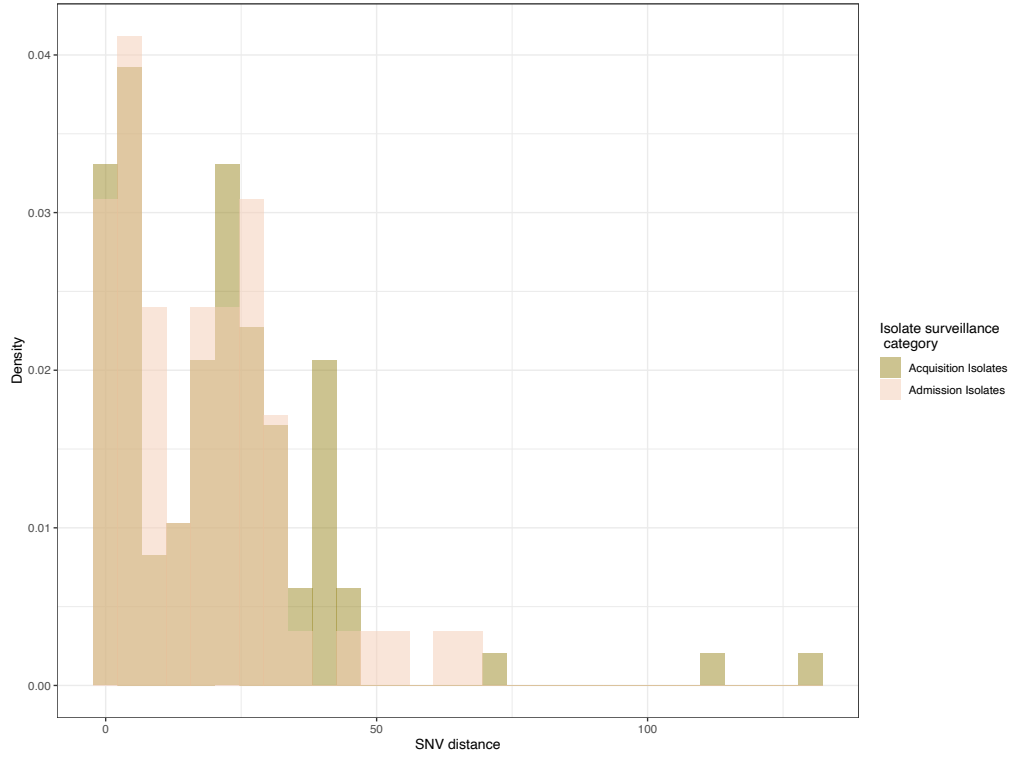
Figure 4-3. Transmission Cluster Detection Method Based on Shared Genomic Variants and Robust Surveillance Data Links The Majority Of Kpc-Kp Acquisitions During The Study.

A. Schematic of genomic transmission cluster detection strategy that integrates shared variants from whole-genome sequences with surveillance data. Shared variants in whole genome sequences(black lines, variants are colored boxes) from isolates sampled from patients are used to construct a maximum parsimony phylogeny. Transmission clusters are defined by the maximum subtree in the phylogeny that contains isolates from a single admission-positive index patient who imported the isolate from outside the facility. Valid transmission clusters must contain at least a single unique variant (yellow box) that distinguishes cluster from non-cluster isolates(A, B and C isolates vs isolate D), and at least two patients including at least one convert patient who acquired KPC-Kp colonization in the LTACH. Clusters with multiple index patients are valid if the index isolates share unique variants with other cluster members. Clusters with no index patients are returned if there existed no subtree that contained an index isolate. B. Distribution of isolates and patients in the 49 transmission clusters detected with genomic method. Admission positive patients (pink) are patients whose isolate in the clusters was obtained within 3 days of ever being in the facility. Periwinkle indicates isolates obtained from convert patients who first acquired KPC-Kp colonization in the study after 3 days of ever being in the LTACH. Tan indicates isolates from index patients that were collected >3 days after admission to the LTACH, indicating either prolonged colonization or secondary strain acquisition in the LATCH. Blue indicates patients who were first positive after being in the

125

LTACH for >3 days, but from whom no negative swab was collected prior to first KPC-Kp

detection. Grey indicates patients who were positive on the first day of the study.
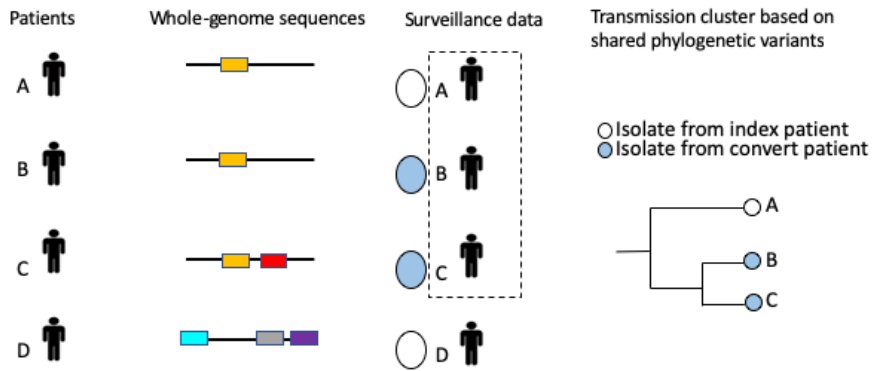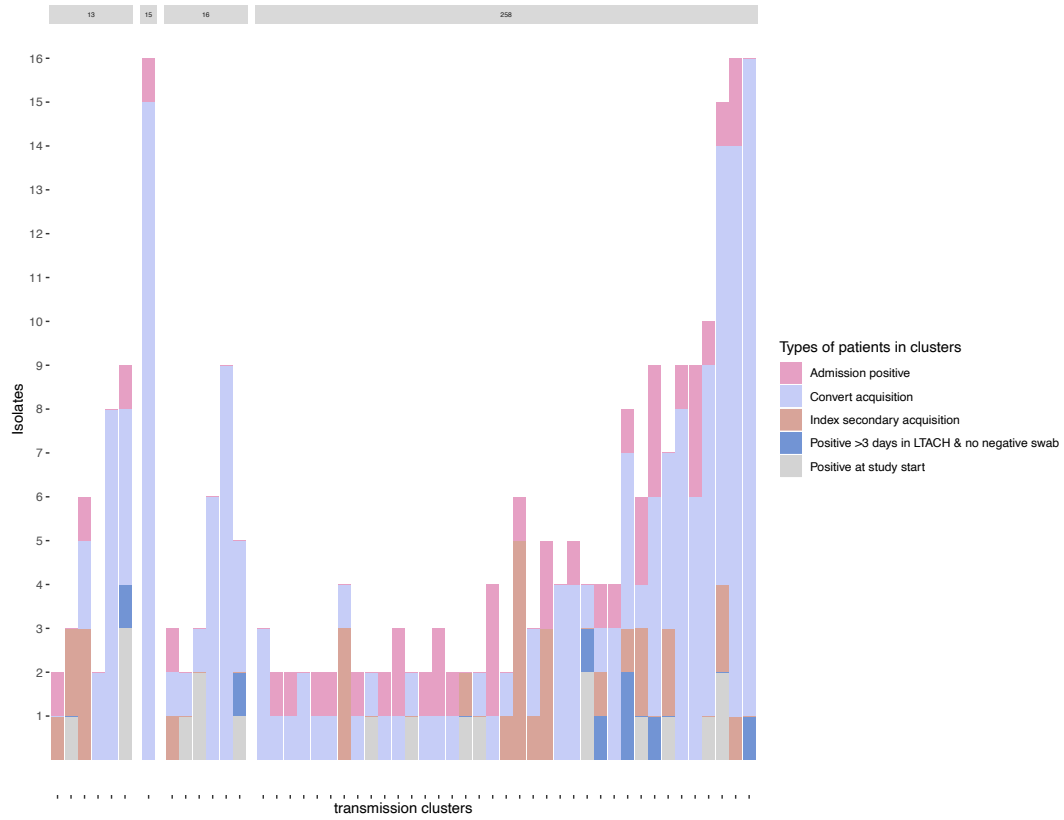
Figure 4-4: Elevated Genetic Diversity in Transmission Clusters Is Attributable To Prolonged Colonization And Emergence Of Hypermutator Strains.

Grey bars indicate the MLST of the isolates in transmission clusters A. maximum pairwise SNV distance distinguishing isolates from the same cluster. Colors indicate whether the cluster has only convert patients, multiple index patients or a single admission-positive index patient. B. Observed frequencies in mutation types across isolates included in each transmission cluster. Bars on the right of each MLST group indicate the overall population frequency of mutation types among members of that MLST in the study population. C. Maximum intra-patient intra-cluster genetic diversity among index and convert isolates. Intra-patient intra-cluster genetic diversity is greater among isolates from index patients (Wilcoxon rank Sum test, p-value < 0.03).
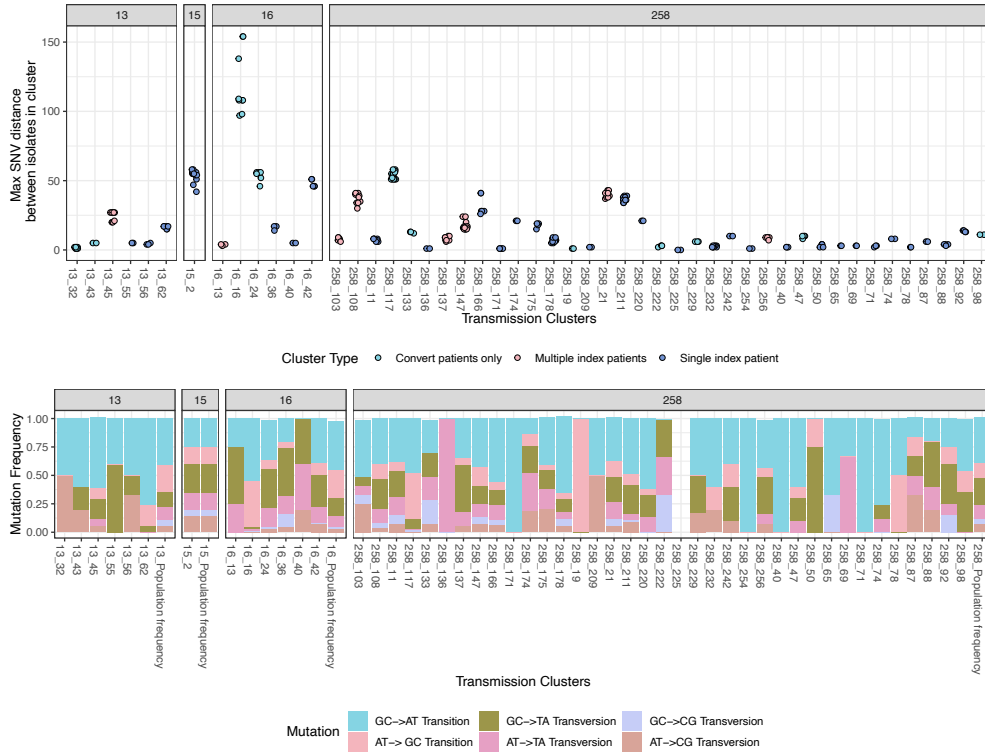
A.
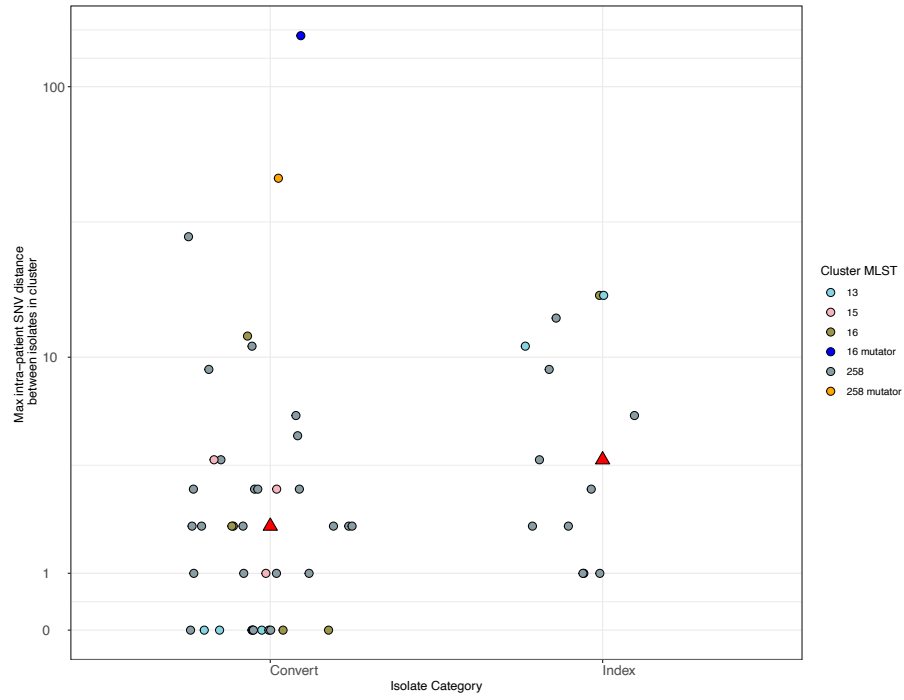
Figure 4-5 Epidemiologic Exposures Within Transmission Clusters Point to Frequent Acquisition Outside A Patient's Bed Location and Infrequent Links To Sequential Occupation Of Common Locations.

X-axis indicates locations, Y axis indicates fraction of acquisitions in transmission clusters that could be attributed to exposure between putative donor patients in the cluster and recipient (acquisition) patients being in the same place at the same time (spatiotemporal) or in the same place separated by time after a donor had left that location (sequential). Spatiotemporal exposure is enriched in transmission clusters compared to permuted groups of patients of the same size and patient (index and convert patients) distribution as the observed clusters (permutation test, $p < 0.001$).
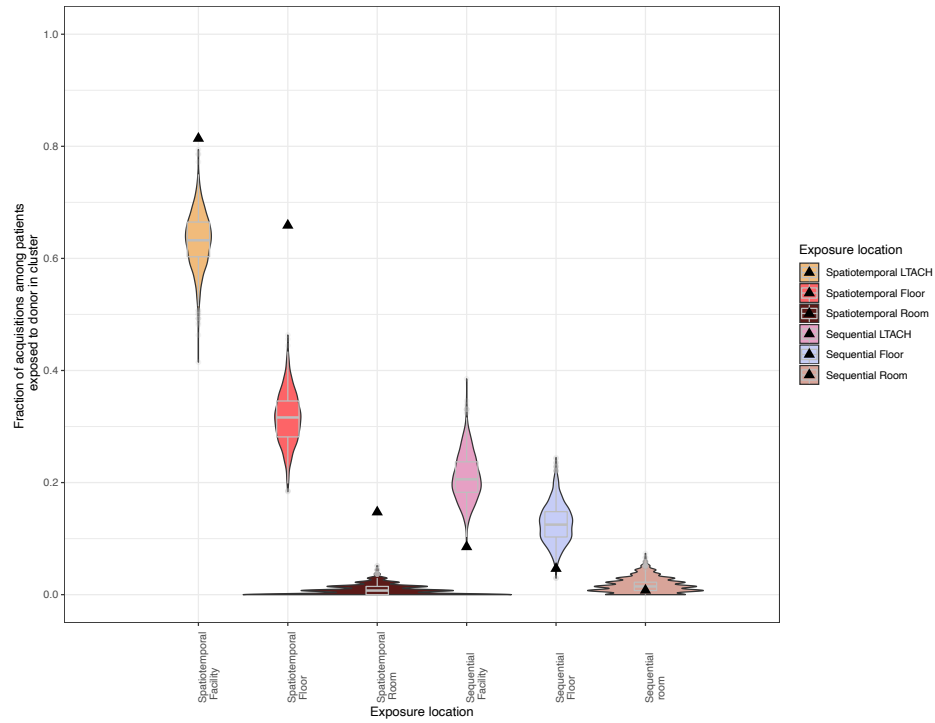
Figure 4-6: Descriptive Vignettes From Transmission Clusters Detected Through The Integration Of Genomic And Surveillance Data Illustrate Putative Routes Of Uncontrolled Transmission That Persisted Throughout The Study.

Patients are indicated on the Y axis and time is on the X -axis. A. Transmission cluster where transmission occurs from within a cohort floor to outside.

B. Patients 1 and 144 are both in the ICU (floor 6) prior to patient 144 acquiring colonization when they return to the general floor.

C. Transmission cluster with no sampled index patient source of importation into the facility in the study, suggestive of false-negative surveillance.

D. Patients 223 and 65 have a missing intermediate link (patient, environmental exposure) to explain transmission. Patient 214 is the index patient in this cluster however patients 244 and 51 (convert patients) acquired colonization prior to 214 entering the facility, suggesting a transmission link outside the study.

Surveillance culture

- Negative
- Positive: non–cluster isolate
- Positive: cluster isolate

Floor location

  0
- 1
- 2
- 3
- 4
- 6

Cluster: 16_16
Transmission Route:
Escape from cohort location
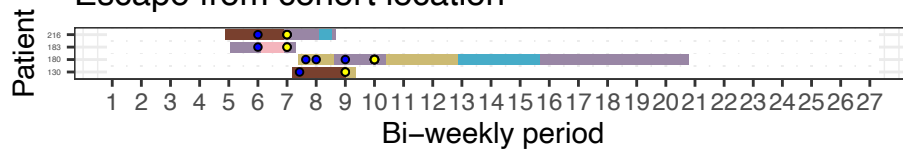
Cluster: 258_242
Transmission Route: Cohorting lapse
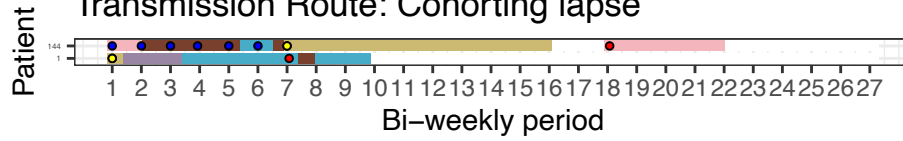
Cluster: 258_117
Transmission Route: False−negative surveillance
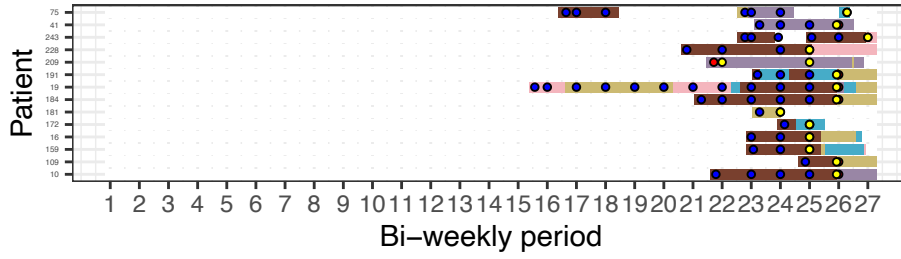
Cluster: 258_220
Transmission Route: Missing intermediate
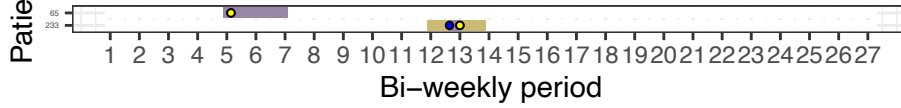
Cluster: 15_2
Transmission Route: link outside facility

136
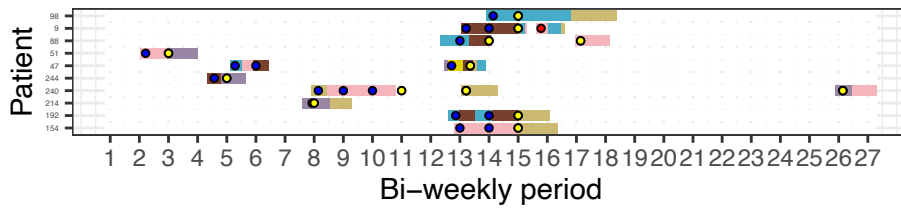
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Chapter 5 Cohorting KPC+ *Klebsiella Pneumoniae* (KPC-Kp) Positive Patients—A Genomic Exposè Of Cross-Colonization Hazards In A Long-Term Acute Care Hospital (LTACH)

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5.1 Abstract

Objective: Cohorting patients who are colonized or infected with multidrug-resistant organisms (MDROs) has been demonstrated to protect uncolonized patients from acquiring MDROs in healthcare settings. A neglected aspect of cohorting is the potential for cross-transmission within the cohort and the possibility of colonized patients acquiring secondary isolates with additional antibiotic resistance traits. We searched for evidence of cross-transmission of KPC+ *Klebsiella pneumoniae (*KPC-Kp) colonization among cohorted patients in a long-term acute care hospital (LTACH), and evaluated the impact of secondary acquisitions on resistance potential.

Design: Genomic epidemiological investigation

Setting: A high-prevalence LTACH during a bundled intervention that included cohorting KPC-Kp-positive patients.

Methods: Whole-genome sequencing (WGS) and location data were analyzed to identify potential cases of cross-transmission between cohorted patients.

Results: Secondary KPC-Kp isolates from 19 of 28 admission-positive patients were more closely related to another patient's isolate than to their own admission isolate. In 14 of these 19 cases there was strong genomic evidence for cross-transmission (<10 SNVs) and the majority of these patients occupied shared cohort floors (12 cases) or rooms (5 cases) at the same time. Of the 14 patients with strong genomic evidence of acquisition, 12 acquired antibiotic resistance genes not found in their primary isolates.

Conclusions: Acquisition of secondary KPC-Kp isolates carrying distinct antibiotic resistance genes was detected in nearly half of cohorted patients. These results highlight the importance of healthcare provider adherence to infection prevention protocols within cohort locations, and motivate future studies to assess whether multiple-strain acquisition increases risk of adverse patient outcomes.

5.2   Introduction

Cohorting of patients who are colonized or infected with high-priority healthcare pathogens has been demonstrated to prevent the spread of healthcare associated infections (HAIs).(1) Cohorting works by physically separating colonized or infected patients together in one area for care, thereby preventing contact with other patients.(1) In addition to being effective in outbreak settings,(2–5) cohorting has been demonstrated to reduce cross-transmission in endemic

healthcare settings with high colonization pressure, such as long-term acute care hospitals (LTACHs).(6,7)

Carbapenem resistant enterobacteriaceae (CRE) are multi-drug resistant organisms (MDROs) that are resistant to nearly all antibiotics and that are estimated to be responsible for 8,500 infections and 1,100 deaths in the U.S. annually.(8) CRE have been labeled an urgent public health threat for nearly a decade, but despite wide-spread attention, infections with CRE have not decreased.(8) Previous work has shown that LTACHs have a disproportionately high prevalence of CRE and that they likely contribute to transmission across regions.(9,10) Encouragingly, a recent study demonstrated the effectiveness of a bundled intervention that included cohorting CRE-positive patients to reduce a particular type of CRE--*Klebsiella pneumoniae* that carry the KPC-type of carbapenemase (KPC-Kp)-- in a LTACH with high KPC-Kp prevalence.(11) This study highlights the potential for infection prevention interventions to reduce transmission in these complex and healthcare settings with a heavy burden of MDROs.(11)

Guidelines for preventing transmission in healthcare settings recommend placing "together in the same room (cohort) patients who are infected or colonized with the same pathogen" when single-patient rooms are unavailable.(1) Yet molecular and phenotypic analyses of prominent healthcare pathogens like CRE indicate that strains of a given antibiotic resistance type are not necessarily equivalent in terms of resistance mechanisms and virulence genes.(12,13) Cross-transmission of genetically diverse strains among cohorted patients could have clinically important consequences. First, patients are often treated empirically based on

susceptibility results from prior cultures.(14–16)However, if a patient acquires new strains, this empiric antibiotic treatment strategy may fail because the secondary organism could carry different antibiotic resistance genes and therefore have a different susceptibility profile.(13,17,18) Additionally, recent reports provide evidence in support of horizontal transfer of antibiotic resistance genes within patients,(19,20) indicating that co-colonization with multiple strains can lead to entry of resistance genes into new genetic backgrounds.

Here, we examined the potential for multiple-strain colonization with KPC-Kp in a convenience sample of patients from a comprehensive surveillance study of KPC-Kp colonization in a Chicago LTACH.(11) We hypothesized that by integrating whole-genome sequencing (WGS) and patient location data we would identify KPC-Kp colonized patients with evidence of acquisition of distinct secondary KPC-Kp strains through cross-transmission from other patients co-housed in cohort locations. Moreover, we predicted that secondary acquired strains would harbor antibiotic resistance genes that were not found in the patient's admission isolate.

5.3 Methods

5.3.1 LTACH Setting, Study Design and Sample Collection

Detailed information regarding the study design, intervention bundle and data collection are available in Hayden et. al 2015.(11) Briefly and of relevance to the current manuscript, the study took place between 2011-2013 during a quality improvement project to prevent KPC-Kp colonization and infection in a Chicago LTACH where the average prevalence of KPC-Kp

colonization was 30%. All location data and isolates presented here were collected from one LTACH during the intervention period, which included surveillance swab culture-based screening of all LTACH patients for KPC-Kp rectal colonization at LTACH admission and every two weeks (94% adherence), as well as efforts to separate KPC-Kp-positive and KPC-Kp-negative patients by placing KPC-Kp-positive patients in ward cohorts (91% adherence).(7)

5.3.2 Longitudinal Convenience Sample of KPC-Kp Isolates From Previously Colonized Patients

During the course of the original study, the first KPC-Kp surveillance isolate was collected from each colonized patient.(11) Once a patient was found to be colonized with KPC-Kp, the patient was presumed to remain colonized indefinitely. Colonized patients were not rescreened systematically; however, additional 'secondary' KPC-Kp isolates were collected from a subset of patients whose prior colonization status was unclear to study staff at the time of screening.

The current analyses are restricted to this longitudinal, convenience sample of patients who were KPC-Kp positive at the study start or upon LTACH admission (within 3 days) and who also had one or more additional KPC-Kp surveillance isolates collected later. These 'index' patients were selected for study because they were housed in cohort locations during their entire LTACH stay, providing long periods of exposure to other KPC-Kp positive patients and potential opportunities for cross-transmission.

Among the index patients who had secondary isolates available, 100% were cohorted per-protocol: 21 patients with 46 secondary isolates shared a room with at least 1 patient who was KPC-Kp-positive before their secondary isolate being collected, and 8 patients with 15

144

secondary isolates did not have overlap with a positive patient before their secondary isolate was collected, but were instead housed in single patient rooms during the acquisition time frame for these isolates. Isolates from the 21 patients who shared a room with a putative KPC-Kp-positive donor prior to secondary acquisition were collected after patients shared a room with positive patients for a median of 51 days (range 1-132 days) prior to detection of a secondary isolate.

5.3.3 Whole-Genome Sequencing

DNA was extracted with the MoBio PowerMag Microbial DNA kit and prepared for sequencing on an Illumina MiSeq instrument using the NEBNext Ultra kit and sample-specific barcoding. Library preparation and sequencing were performed at the Center for Microbial Systems at the University of Michigan or the University of Michigan Sequencing Core. Quality of reads was assessed with FastQC,(21) and Trimmomatic(22) was used for trimming adapter sequences and low-quality bases. Assemblies were performed using the A5 pipeline with default parameters.(23) Sequence data are available under BioProject PRJNA603790.

5.3.4 Identification Of Single Nucleotide Variants

SNV calling was performed as in Han *et al.*(24) The variant calling pipeline can be found at https://github.com/Snitkin-Lab-Umich/variant_calling_pipeline. To summarize, variant calling was performed with samtools(25) using the reference genomes listed in Supplementary table 1.

5.3.5 Assessment of Epidemiologically Supported Secondary Acquisitions Linked To Other LTACH Patients And Roommates

Epidemiologically plausible donor patient isolates were defined as isolates collected before the recipient patient's secondary isolate collection date. To account for acquisition potentially occurring between surveillance sampling dates, the positive donor time-frame for all analyses was defined starting on the date of the donor's last negative swab before the collection date of the putative donor isolate.

The patient bed trace indicating the rooms patients were housed in during their LTACH stays was assessed to identify spatiotemporal exposures in shared patient rooms that plausibly facilitated secondary acquisition between roommates. Plausible secondary acquisitions linked to roommate exposures were defined as acquisitions between a donor and recipient patient who occupied the same room when the donor was considered positive for the putative donor isolate and prior to the collection date of the recipient's secondary isolate.

5.3.6 Genetic Relationships Between KPC-Kp Isolates Based on SNV Distance

Pairwise distances were calculated from core and accessory genome single-nucleotide variants (SNVs) in whole-genome sequence alignments for each MLST represented by study isolates (Supplementary table 1). SNV distances were compared (1) between the first (primary) and later collected (secondary) isolates from the same index patient and (2) between secondary isolates from index patients and isolates from other plausible donor patients in the LTACH.

5.3.7 Detection of Resistance Genes in Whole Genome Sequences

Kleborate (https://github.com/katholt/Kleborate) was used to screen whole-genome sequence assemblies for presence of genes and mutations known to confer antibiotic resistance in *K pneumoniae*. We used a custom R script to expand antibiotic resistance gene alleles reported from Kleborate into gene presence absence profiles (Supplementary table 1), counting only the Kleborate-reported precise matching gene hits as being present or absent.

5.4 Results

5.4.1 Almost Half of Cohorted Patients Acquired Secondary Isolates of A New Sequence Type

We considered 127 'index' patients, who were either positive at the start of the study or on first admission to the LTACH, for potential acquisition of secondary KPC-Kp strains during their stay. Although the original sampling strategy was not designed to track longitudinal colonization of KPC-Kp,(11) there were 28 index patients who in addition to their 38 'primary' isolates (earliest isolate) collected on admission or study start, also had 63 'secondary' isolates collected later during their LTACH stays (**Figure 1**). Of the 101 isolates available from these index patients, we extracted quality WGS data from 99 isolates including 38 primary and 61 secondary isolates. While the majority of primary and secondary isolates were from the epidemic ST258 strain (55% of primary isolates, 57% of secondary isolates), a diversity of other multi-locus sequence types (MLSTs) was observed among both primary and secondary isolates (Supplemental Table 1). Secondary isolates were collected from patients a median of 89 days

(range 1-310 days) after primary isolates. Evaluation of MLSTs of the primary and secondary

KPC-Kp isolates provided support for secondary acquisition among cohorted patients, with 13

(46%) patients having a distinct secondary MLST that was not detected at admission.

5.4.2 Genomic Evidence of Potential Secondary Acquisitions From Other LTACH Patients Among Admission-Positive Index Patients

To assess genomic evidence of cross-transmission in the cohort we evaluated the fraction

of patients whose secondary isolates were more closely related to another patient's isolate than to

their own primary isolate (**Figure 2**). Of the 28 index patients with one or more secondary

isolates, 19 had a secondary isolate that was more closely related to another patient's isolate than

to their own primary isolate. Of those 19 patients, 17 had secondary isolates that were more

closely related to an isolate from a patient with whom they overlapped on the cohort floor and 8

had secondary isolates that were more closely related to an isolate from a roommate. Plausible

transmission in the cohort was further supported by extremely small SNV distances in most of

these cases, with 12 patients' isolates being within 10 SNVs of another patient's isolate on the

cohort floor and 4 patients' isolates being within 10 SNVs of an isolate from a roommate (**Table
1**).

5.4.3   Patients Accumulate Diverse Antibiotic Resistance Genes in Association with Acquisition Of A Secondary KPC-Kp Isolate

There is an abundance of molecular and genomic evidence that members of the same bacterial species, including KPC-Kp, can vary extensively in the arsenal of antibiotic resistance genes encoded in their chromosomes and plasmids.(12,26,27) To determine whether secondary acquisitions resulted in increased antibiotic resistance potential we examined whether patients with high-confidence putative transmission links (<10 SNVs to another patient's isolate and >10 SNVs from their own primary isolate) acquired additional unique resistance genes in their secondary isolate. As compared to a patient's primary isolate, secondary isolates contributed a median of 2.5 additional antibiotic resistance genes beyond the primary isolate (minimum 0, maximum 10 additional resistance genes) (**Table 2**). In total, additional resistance genes were gained in 12 of the 14 patients whose secondary isolates had strong genomic links to isolates from other patients, including 3 patients whose secondary isolates were linked to patients with whom they had shared a cohort room prior to secondary isolate acquisition (**Figure 3**, supplementary table 1, Patients with unlinked secondary isolates accumulated fewer additional resistance genes (median 0, minimum 0, maximum 2 additional resistance genes) (supplementary figure 1).). This finding supports the hypothesis that these closely related isolates (<10 SNVs) represented primary isolates that accrued mutations over the course of prolonged colonization rather than that patients acquired a secondary KPC-Kp strain via transmission from another patient.

5.5 Discussion

Cohorting patients who are colonized or infected with MDROs is an effective strategy to reduce the risk of MDRO transmission to uncolonized patients. However, little attention has been paid to the potential for cohorted patients themselves to acquire secondary resistant strains through exposure to the high colonization pressure of MDROs within cohorts. Secondary strain acquisition may be particularly important in endemic settings where the MDRO for which patients are cohorted, e.g. CRE, may comprise a heterogeneous group of bacteria with varying genetic potential. In order to investigate this risk, we performed a genomic epidemiologic investigation of a longitudinal, convenience sample of KPC-Kp isolates from patients on cohort floors in a LTACH. We found strong evidence of cross-transmission within cohorts, with secondary acquired isolates often harboring antibiotic resistance genes not found within a patient's primary isolate.

Our finding that secondary isolates carry antibiotic resistance potential that is distinct from that found in patients' primary isolates is noteworthy because it suggests that multiple strain acquisition could increase risk of treatment failure. Acquisition of a secondary strain that is resistant to antibiotics to which the primary strain was susceptible could be particularly problematic for highly resistant organisms like KPC-Kp, which already have limited treatment options. For example, colistin/polymyxin E is a last-resort drug that is used to treat severe multidrug-resistant gram negative infections, such as those due to KPC-Kp.(28–31) In our study, one patient plausibly acquired a secondary isolate with predicted colistin resistance that was linked within 25 SNVs of another LTACH patient's isolate (supplementary table 1). As

150

colonization is a major risk factor for KPC-Kp infection, (32–34) and infections are thought to

arise primarily from the patient's colonizing strain, (35) the acquisition of a colistin resistant

isolate could limit efficacious treatment options and in turn increase mortality risk. (31,36) In

addition to the potential risks to multiply colonized patients, the acquisition of strains with

different resistance arsenals provides an opportunity for horizontal gene exchange and the

accumulation of resistance within a single transmissible strain.(19,20,37) Moreover, harboring

genetically diverse strains creates an opportunity for resistance alleles to find their way to strains

with other clinically relevant characteristics, such as hyper-virulence(13,38–40) or epidemic

potential.(39) Additional risk to patients could stem from the fact that different strains of the

same pathogen often carry different virulence genes.(37) Virulence factor differences in acquired

strains may predispose patients to developing infections of different types and severity.(37,38)

In addition to potentially making infections more difficult to treat, acquisition of

secondary strains could also increase a patient's time at risk of infection by prolonging the total

period of colonization. All of these potential adverse consequences of multiple strain

colonization emphasize the importance of protecting previously colonized patients from

secondary acquisition and for healthcare providers to adhere to infection prevention protocols,

even when caring for patients in cohort locations.

Our study has several limitations. First, we studied a convenience sample which

inherently precludes systematic calculation of risk. Second, we conducted limited sequencing of

multiple clones from the same sample—a single representative of unique morphologies observed

in each sample, primarily a single clone per sample--thus hindering our ability to know if a

patient was simultaneously colonized with multiple strains (e.g. colonized by both their primary and secondary strains at the same time). These sampling limitations also prevent us from determining if patients remain colonized with their primary strain when they become colonized with their secondary strain, or if colonization with both strains persists. Thus, it is possible that cohort patients entered the facility already colonized with multiple strains, and that patients did not acquire their secondary strains in the cohort. While we cannot definitively rule out this possibility, the acquisition of secondary strains in the LTACH is supported by the finding that 14 of the 28 patients with secondary isolates had strong genomic links (< 10 SNVs) to other LTACH patients. In total, these 14 strong genomic linkages account for 50% of the 28 index patients with multiple isolates available and 11% of the 127 index patients in the full study.

In summary, our study provides strong evidence for cross-transmission of KPC-Kp strains within a KPC-Kp-positive cohort, with accumulation of new antibiotic resistance genes by patients who acquire secondary KPC-Kp strains. Whether acquisition of multiple KPC-Kp strains increases risk of adverse patient outcomes needs to be studied further. In the meantime, we recommend robust adherence to infection prevention precautions within KPC-Kp cohorts to reduce the risk of within-cohort cross-transmission of KPC-Kp strains.

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5.7 Financial Support

Table 5-1 Frequency of Strong Genetic Relationships Between Secondary Isolates And Isolates From Other Patients Among Patients Whose Primary Isolate Is Most Closely Related To Another Patient's Isolate

# Index patients, N=28 (# Secondary isolates, N=63)	<25 SNV	<10 SNV	<5 SNV
Distance to closest isolate from another LTACH patient	17 (26)	14 (21)	11 (12)
Distance to closest isolate from patient on cohort floor	15(19)	12(15)	10(11)
Distance to closest isolate from roommate in cohort	5(6)	4 (5)	3 (3)

Table 5-2 Summary of Antibiotic Resistance Genes Among Primary, Secondary and All Isolates

from Index Patients Whose Secondary Isolate Is Most Closely Related To Another Patient's

Isolate

	Min.	Median	Max.
Antibiotic resistance genes detected in primary isolates	4	9.5	13
Antibiotic resistance genes detected in secondary isolates	0	2.5	10
Total unique antibiotic resistance genes in primary and secondary isolates	4	13	18

Figure 5-1 KPC-Kp Isolates From Convenience Sample Of Patients Who Were Positive At The Study Start Or Admission To The LTACH.

Patients (N=28) have primary and secondary isolates that are from the same MLST, different MLST or both same and different MLST. Y-axis indicates patients, X-axis indicates bi-weekly time-periods during the study, circles indicate positive culture dates and are colored by the MLST of the isolate collected. Grey bars indicate when patients were in the LTACH.
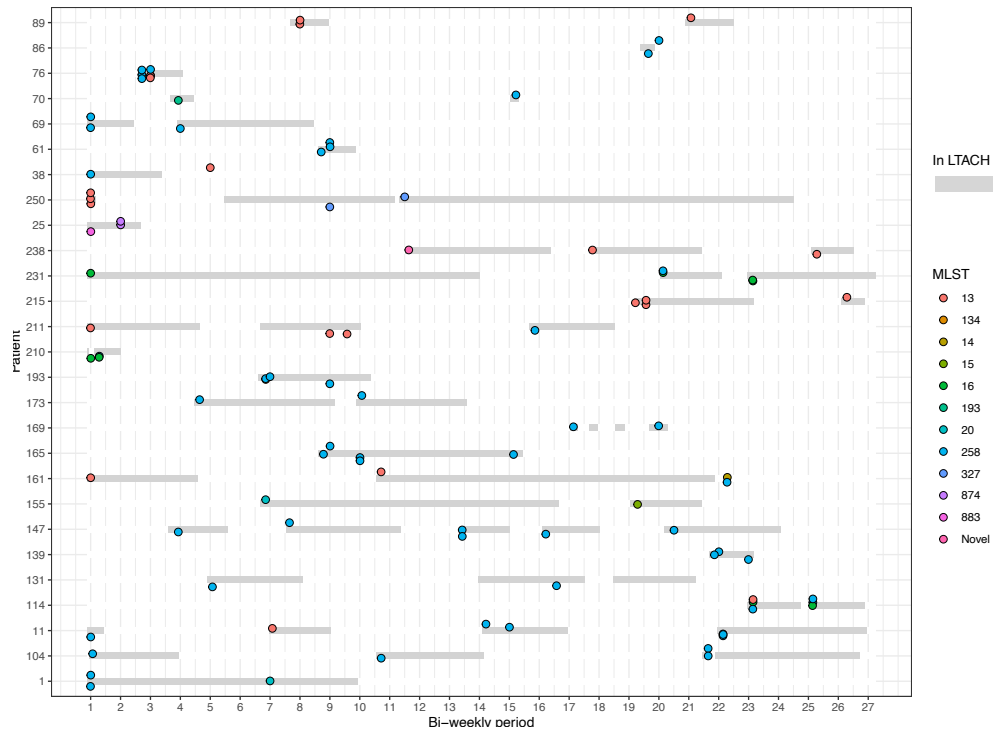
Figure 5-2 Genetic Relationship Between A Patient's Primary and Secondary Isolates Compared To Isolates From Other Patients In The LTACH And Room Cohorts.

Pairwise SNV distance between secondary isolates and closest primary isolate from the same patient compared to closest related isolate from **A.** another patient in the facility or **B.** a cohorted roommate. Diagonal line separates secondary isolates that are more closely related to primary isolates from the same patient (above the diagonal) or to another patient's isolate (below the diagonal). Colors indicate the MLST of the secondary isolate. Circles indicate the closest genetic relative to the isolate by SNV distance is from the same patient (e.g. the patient's own primary isolate) while triangles indicate that the closest relative was isolated from another patient. Comparison of isolates from different MLSTs or >100 SNVs are collapsed into the >100 SNV category for plot visualization purposes.
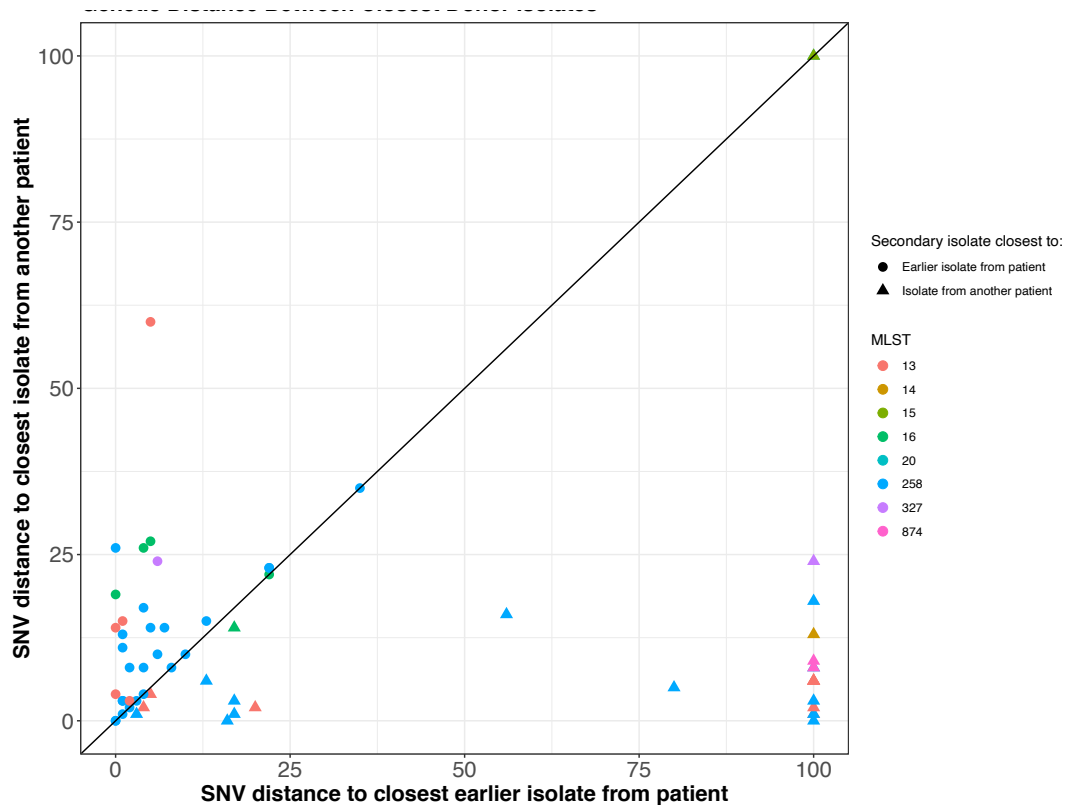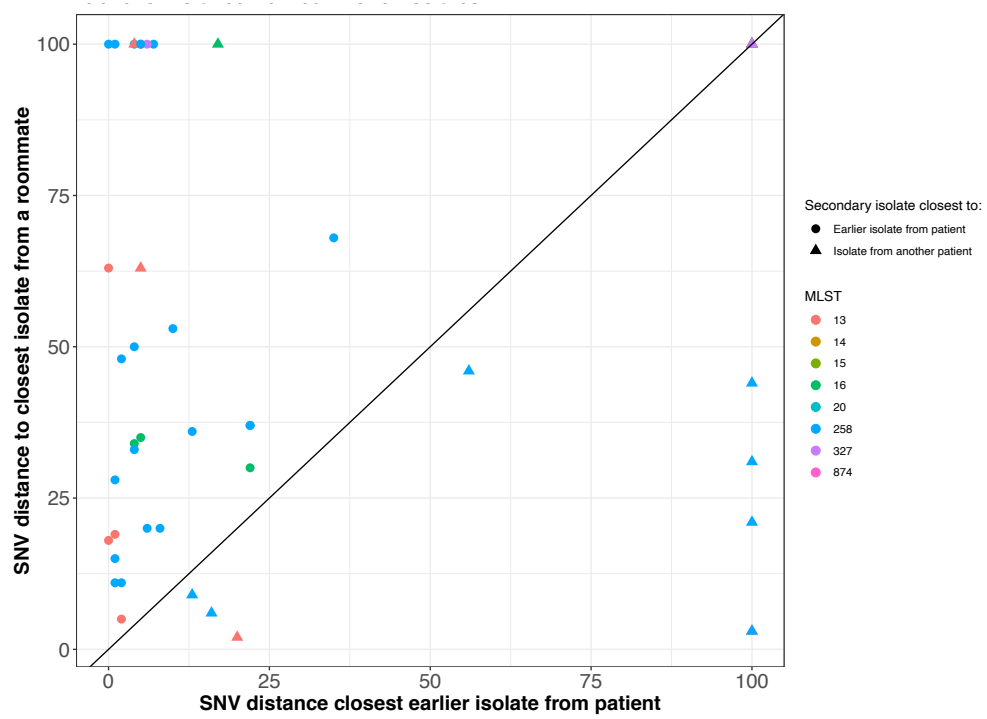
Figure 5-3 Number of Antibiotic Resistance Genes Detected in Genomes From Primary Isolates
Compared To Primary And Secondary Isolates From Index Patients Whose Secondary Isolates
Are Linked With High Confidence (<10 Snvs) To Isolates From Other Patients In The LTACH.
Y-axis indicates number of unique resistance genes detected with Kleborate (See methods,
Supplementary table 1), X-axis indicates number of unique antibiotic resistance genes detected
among primary (left) and primary and secondary isolates (right). Colors distinguish patients.
Dashed lines indicate patients whose secondary isolate is within 10 SNVs of an isolate from a
cohorted roommate.

Antibiotic resistance genes among primary and secondary isolates
from patients genomically closest and linked (<10 SNVs) to isolates from other patients
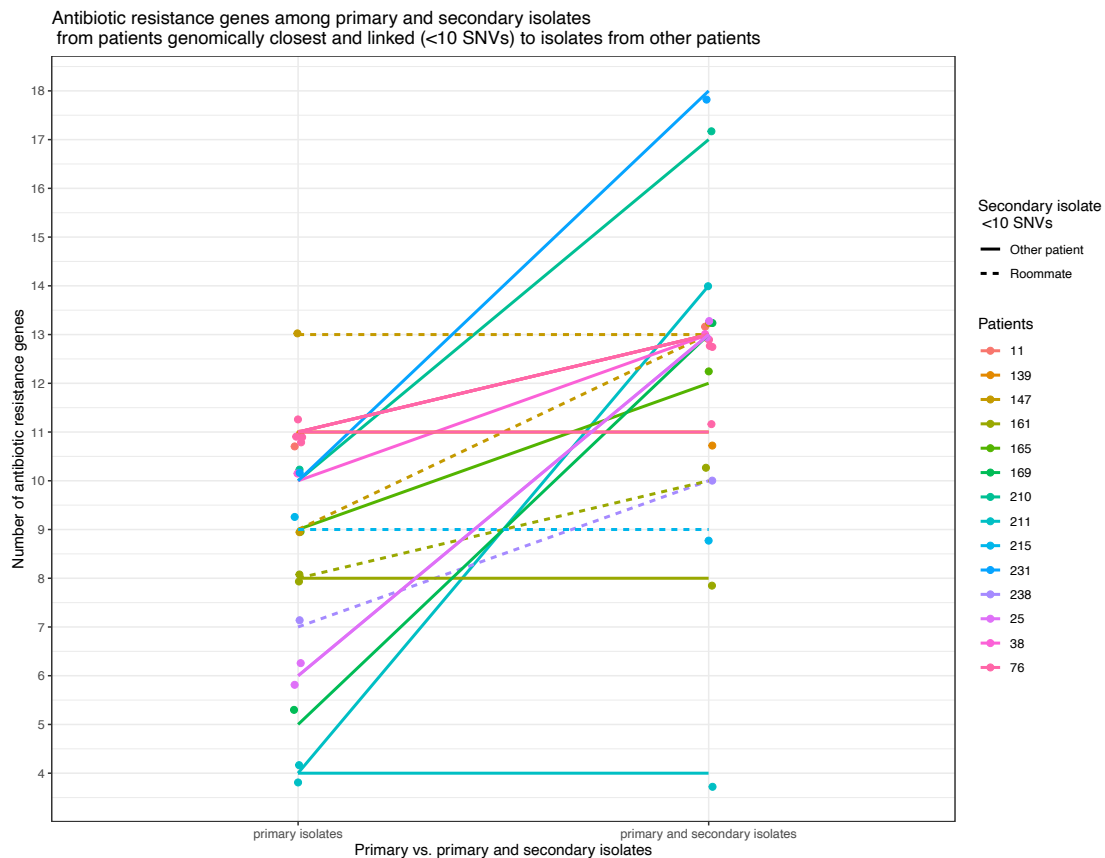
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Chapter 6   Conclusions and Discussion

6.1   Hospital Genomic Epidemiology Is Still An Emerging Field

As genomic technologies have become more accessible in the last decade, the field of genomic

hospital epidemiology has grown extensively, from a few case studies of utilization,(1–3) to

becoming established as a commonly implemented technology public health workflows.(4–6)

Despite advances, there are still fundamental hospital genomic epidemiologic questions that

remain unaddressed. For example, there still remains a lack of consensus for best practices for

integrating and interpreting WGS and epidemiologic data to evaluate existing interventions and

to infer transmission links.(7,8) In this chapter, I discuss how the main findings presented in this

dissertation contribute to progress in this emerging field. I also highlight opportunities for

advancing infection prevention with genomic data, that build upon the studies presented here.

6.2   Contribution of Findings in This Dissertation To The Field Of Genomic
       Hospital Epidemiology

6.2.1   Generation of Actionable Hypotheses for More Comprehensive Future
       Epidemiologic Studies

     The studies presented in this dissertation contribute to the field of genomic hospital

epidemiology by providing practical knowledge for improving patient safety, as well as for

establishing hypotheses for more rigorous future studies that can be used to improve our

understanding of the evolution of antibiotic resistance and virulence among healthcare pathogens.

Although at the time of our CREC outbreak investigation (chapter 3), there had already been multiple investigations which implicated endoscopes as a vehicle for transmission,(9,10) we recognized two opportunities in the dataset we had collected for the outbreak investigation which allowed us to expand our analysis to address fundamental questions regarding the population structure of CREC and virulence evolution in the university of Michigan hospital.

First, our investigation of the mechanisms of carbapenem resistance at the University of Michigan hospital was important from a practical patient safety perspective. Although CRE can be carbapenem resistant due to various mechanisms, particular clones that have become associated with carbapenemase genes have been the most prominent causes of outbreaks and HAIs.(11–13) Additionally, since CRE are capable of colonization as well as infection, a small number of device associated infections can be a sentinel of more extensive transmission, and could have indicated that CREC were transitioning from sporadic cases to becoming more established in our hospital.(10) Our identification of carbapenemase genes among isolates from CREC infections at our institution revealed that the few isolates that did harbor carbapenemases were indeed part of an emerging epidemic lineage (ST171) of CREC in the Midwest.(14) This finding highlighted a specific strain for the infection prevention team at our hospital to be on alert for, and additionally it serves as an example of how a relatively small genomic investigation can provide actionable information on the population structure of resistant organisms in an institution.

The results of our genomic investigation of carbapenem resistance mechanisms in CREC at the university of Michigan hospital also point to specific study designs that could be used to

identify drivers of CREC evolution in healthcare settings. Our genome-wide association analysis of mutations and genes associated with carbapenem resistance suggested the majority of carbapenem resistance was due to repeated convergent evolution of carbapenem resistance by diverse genetic mechanisms across phylogenetically diverse CREC isolates.(15) This observation was derived by comparing carbapenem susceptible *E. cloacae* to their closest related CREC isolate from our hospital. In combination, the observations that there were few plausible transmission links identified between CREC in our outbreak investigation, and that carbapenem susceptible *E. cloacae* were on average more closely related to individual CREC isolates than CREC isolates with mutational carbapenem resistance were to each other, further bolsters the hypothesis that non-carbapenemase mechanisms of carbapenem resistance may be selected for in the context of clinical exposures during colonization or infection and supports the hypothesis that these non-carbapenemase producing CREC present less of a risk for onward transmission, which is important knowledge for infection prevention teams as they decide where to target infection control resources.

These results suggest that future longitudinal studies have the potential to detect clinical risk factors for the evolution of non-carbapenemase carbapenem resistance. For example, collection of paired earlier carbapenem susceptible and later carbapenem resistant isolates from the same patient would improve genomic detection of mechanisms of mutational carbapenem resistance by reducing the number of mutations putatively linked to resistance. Furthermore, clinical exposures such as medications, procedures, and devices that occur between collection of a carbapenem susceptible and carbapenem resistant isolate detection could be evaluated as potential risk factors for driving the evolution of carbapenem resistance during patient treatment. Such risk factors if identified could provide opportunities for intervening in the evolution of

carbapenem resistance: for example, adjusting treatment strategies for high-risk patient populations where resistance is likely to emerge.

A fundamental question for healthcare pathogens that are capable of both colonization and infection is what determines which colonized patients will go on to acquire an infection and what factors determine infection severity.[16–18] Colonization often precedes CRE infections that can occur at multiple body sites with a range of severity, and infections are thought to arise primarily from a patient's own colonizing strain.[18,19] Among the most severe types of CRE infections are bloodstream infections (BSI) which have mortality rates reported as high as 40%.[20–23] There is an open question of what features of both the organism and patient drive progression from colonization to BSI.[16,18,19,22] Our analysis of genetic signatures of BSI isolates compared to isolates from infections at other body sites revealed a signature of recurrent mutations in short-chain fatty acid (SCFA) metabolic pathways among BSI isolates. This observation is consistent with the hypothesis that colonization and potentially modification of the gut environment precedes invasive infection. Butyrate is a well-studied SCFA that is known to reinforce the colonic epithelium, suggesting the possibility that alteration of SCFA metabolism by CRE could compromise the intestinal barrier, facilitating introduction into the bloodstream from the GI tract.[24] Though our small dataset had limited power, this finding highlights the utility of pairing even small sets of convenience samples with the high-resolution of genomic data to generate hypotheses for explaining virulence differences in pathogens. These results also provide a base upon which future studies can evaluate additional putative genomic signatures of virulence.

6.2.2 Evaluation of existing infection prevention interventions and clinical practices with genomic frameworks

In chapter five, we integrated genomics with an analysis of patient location data to evaluate potential unintentional harm to patients stemming from the widely accepted intervention of patient cohorting.(25) Here we took advantage of a convenience sample of longitudinal isolates collected from patients who imported KPC-Kp into an LTACH who also had secondary isolates collected later during their stay. Though this longitudinal portion of our dataset was small, and was not necessarily representative of the KPC-Kp colonized patient population as a whole in the LTACH, our analysis revealed instances of plausible cross-transmission linked to cohorting, as well as patients who acquired additional isolates with different antibiotic resistance potential.

Chapter five provides a case study for integrating WGS with epidemiologic data to evaluate how an existing intervention intended to reduce transmission could actually contribute to transmission and potentially adverse patient outcomes. Although the identification of additional antibiotic resistance potential among already highly resistant pathogens like KPC-Kp for which there are few treatment options may not necessarily translate to changed treatment decisions on an individual patient level, the observation that patients could acquire additional strains of an organism within a cohort is nevertheless concerning. First, it may impact antibiotic treatment decisions for other organisms that have more options available or where treatment is more dependent on individual resistance mechanisms.(21,26,27) Additionally, even for highly resistant pathogens like KPC-Kp, the limited efficacious antibiotics that we do have left could become compromised—for example despite our small sample size, we detected an instance of

secondary acquisition of a strain harboring mutations that confer colistin resistance linked to another patient in the cohort.

Despite recent large genomic studies that probe the population structure of prominent healthcare associated pathogens, there is still no consensus for where the emergence of resistant clones arises, although several studies have suggested multiple colonization could play a role.(28–32) Recent studies have also pointed to horizontal gene transfer between lineages as an important event proceeding widespread distribution of successful antibiotic resistant lineages .(12) In this context, our observation of multiple strain acquisition is especially relevant as such horizontal transfer events may be enriched when patients are multiply colonized when organisms carrying different resistance potential come into contact with each other within a patient during colonization or infection. Furthermore, patients can harbor multiple pathogens simultaneously, and therefore infection prevention breakdowns within cohort locations could in addition to facilitating spread of the organisms that patients are cohorted for, also promote transmission of additional pathogens carried by cohorted patients.

The most generalizable aspect of this study is that it provides an example for how to integrate WGS data into a standard descriptive hospital epidemiologic approach for evaluating shared space and time exposures among patients in order to examine a specific hypothesis about how an existing clinical practice works. Similar integration of genomic data into standard person, place and time investigations could be applied in the future to examine the relative contribution to transmission reduction or the burden of a particular organism in a facility that is attributable to interventions such as hand hygiene initiatives, targeted decolonization protocols, or strategies for environmental and device disinfection.(33–35) Because the added resolution provided by genomic data can assist in more accurate description of how cases of an organism in a hospital

are related to each other, studies that evaluate interventions that incorporate genomics may be more efficient in determining efficacy because they can more accurately pinpoint which organisms in a facility are related by the relationship of interest, therefore providing a more accurate measure of how well an intervention works. This improved resolution could also make the process of determining best practice guidelines for infection prevention more efficient because they can be more accurate at determining what costs are associated with case reductions that are attributable to a transmission prevention intervention.

6.2.3 Development of Analytical Frameworks That Can Be Applied to Future Genomic Epidemiology Studies

In chapter four, we developed a method based on WGS and patient surveillance data to identify patients linked by in-LTACH cross-transmission traced back to individual patients who imported KPC-Kp from outside the facility. In high-prevalence healthcare settings such as the case of KPC-Kp in the LTACH, epidemiological data alone provide limited insight into cross-transmission relationships since there are too many common exposures linking multiple positive patients. Gaining increased resolution with the addition of genomic data from isolates from positive patients would be the next step towards determining how transmission is happening in the facility. The primary method of inferring transmission that has been used in previous genomic transmission studies has been by applying single-nucleotide variant (SNV) thresholds to rule out implausible transmission links that are thought to be too distant to be reasonably related by recent transmission.(8) The application of SNV cutoffs to identify intra-facility transmission pairs can be problematic for several reasons. First, intra-patient strain diversity could increase the amount of genetic variation in transmission pairs leading to false-negative predictions of cross-

171

transmission relationships. Second, recent transmission at a connected healthcare facility, rather than cross-transmission within the facility, could lead to the importation of closely related pairs of isolates that are brought in by admission-positive patients leading to false-positive predictions of cross-transmission. Furthermore, the rate of mutations that accumulate within a patient during colonization may not be constant over time, and for example could be impacted by selective pressures such as antibiotic treatment, which could facilitate mutations accumulating more quickly or slowly than expected which in turn, could result in both false-positive and false-negative misclassification of patients with cross-transmission relationships.

Our results highlight the misclassification of patients in and out of transmission clusters that could occur if SNV thresholds are applied to determine cross-transmission, and demonstrate that there is no appropriate SNV threshold that could be broadly applied to distinguish KPC-Kp transmission in this setting. We evaluated the potential for misclassification of patients both into and out of transmission clusters by leveraging robust genomic surveillance data from a densely sampled facility. First, we observed extensive overlap in the distribution of genetic diversity among isolates imported by admission-positive patients compared to the diversity among isolates acquired in the facility, strongly supporting the hypothesis that in the KPC-Kp endemic LTACH setting, a SNV threshold could not be used to accurately distinguish importation from cross-transmission within the facility. We then observed evidence that intra-patient colonizing diversity was variable among and between patients who acquired colonization within the LTACH and patients who imported KPC-Kp from outside the facility. We also saw evidence of hypermutators which was associated with drastically increased SNV distances among colonized patients.

Since our analysis demonstrated that a SNV threshold was inappropriate to detect cross-transmission in the LTACH, we developed a method based on shared SNVs and surveillance data that makes minimal assumptions about the diversity of putative transmission links, which enabled us to detect putative cross-transmission clusters that had variable genetic diversity. We observed that variable genetic diversity in transmission clusters was associated with the emergence of hypermutator strains as well as intra-patient colonizing diversity. By grouping patients into these transmission clusters that are based on fewer assumptions about how transmission occurs in the facility, we were able to overlay patient location data to gain insight into uncontrolled routes of transmission that persisted throughout the bundled intervention study. For example, we saw that transmission occurred in clusters due to lapses in cohorting, links to unsampled patient or environmental sources outside of patient bed locations, and unidentified sources of importation into the facility that were not captured despite robust surveillance. These observed routes of cross-transmission serve as hypotheses that can be systematically tested in future studies that aim to improve infection prevention. One example of how an identified pathway of cross transmission could be tested is through an intervention that involves improved communication of prior KPC-Kp infection/colonization between facilities during patient transfer; if better identification of positive patients at admission enables healthcare workers to cohort these patients earlier in their stay prior to them being able to silently transmit to other patients, this should manifest as decreased acquisition with unidentified sources of importation in the facility.

Overall, these results exemplify how application of a SNV threshold could misclassify patients in and out of transmission clusters, and suggest that future studies should focus on collection of comprehensive samples (as we performed here), multiple isolates per patient, or

173

newer metagenomic technologies to further probe the intra-patient diversity that could be transmitted to other patients. Additionally, armed with our method that relies on fewer assumptions about intra-patient evolution to group patients who are likely to be linked by cross-transmission in the LTACH, we are now poised to test these hypotheses regarding routes of transmission in the facility to evaluate drivers of transmission in this endemic setting. For example, since we have a higher confidence about which patients are linked by cross-transmission in the facility and a better understanding of when transmission likely occurred, we can now perform studies that assess risk factors for both transmission and acquisition in the LTACH. Candidate risk factors for transmission and acquisition could include medical devices, medications or procedures or specific ward locations. Application of similar methods to datasets from other hospitals, will enable us to identify more generalizable characteristics that could be used to predict the patients and practices that are most impactful for driving transmission or acquisition in the facility, and therefore identify potential intervention targets.

6.3 Conclusions

In conclusion, the studies presented in this thesis integrate genomic and epidemiologic data to improve detection of transmission in hospitals and identify additional candidate methods to better control the spread of HAIs. We used a genomic framework to rule out a device-associated outbreak of CREC at the University of Michigan hospital, as well as performed genome-wide-association analyses that revealed insights into the population structure of CREC at our institution as well as potential pathogen genomic signatures of invasive infection. Our genomic investigations of KPC-Kp transmission in LTACHs serve as proof of principle studies for integrating epidemiologic and genomic data to evaluate clinical practices. Lastly, our rigorous integration of KPC-Kp genomic, surveillance and patient location data enabled us to develop and

174

apply a method to detect transmission in an endemic hospital, revealing routes of uncontrolled transmission in the facility while highlighting the utility of analytical frameworks to detect transmission that incorporate genomic data without imposing strict variant thresholds. Overall these studies highlight the potential for future genomic epidemiologic studies that combine robust sampling strategies and tailored analysis plans to reveal fundamental insights into the best practices for reducing HAIs.

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