ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Special Issue: *Antimicrobial Therapeutics Reviews*
REVIEW

Genomic epidemiology of multidrug-resistant Gram-negative organisms

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The emergence and spread of antibiotic-resistant Gram-negative bacteria (rGNB) across global healthcare networks presents 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 it 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 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.

Keywords: genomic epidemiology; multidrug-resistant organisms; hospital-associated infection; whole-genome sequencing; antibiotic resistance

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 one in 25 hospitalized patients have a healthcare-associated infection on any given day.[5] Hospitalized patients have comorbidities 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 have directly led to increases in morbidity and mortality among affected patients.[6–8] 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., multilocus sequence typing (MLST)) of microbial genetic material in order to quantify the relationships among 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 MDROs using molecular typing approaches, all classical techniques are associated with major limitations. First, methods based on

[Correction added on April 5, 2018, after first online publication: Author affiliations were corrected.]

doi: 10.1111/nyas.13672

genome structure present difficulties in interpretation, because 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 no 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] Finally, 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 facilitate 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] Finally, 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 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 also explore challenges and opportunities in maximizing the translational impact of this transformative technology in the realms of clinical practice and public health.

Hospital epidemiology and 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 multidrug-resistant (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 multistrain 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 and PFGE).

In addition to grouping patients into transmission clusters, several studies have been able to use 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]

Finally, WGS has also yielded nontrivial insights into the structure of transmission networks. Applying WGS to an outbreak of carbapenem-resistant *K. pneumoniae* allowed us to demonstrate the role of 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 intrapatient 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 intrahost 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 whole-genome multi-locus sequence typing (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.[16,19,49,52] Owing 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, one must consider whether there is benefit to having WGS embedded in clinical microbiology laboratories 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]

Regional epidemiology at different geographic scales

While the application of WGS to understand intrafacility 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 to 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*, vancomycin-resistant enterococci, and resistant Gram-negative

bacteria (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: *E. cloacae*, *Serratia marcescens,* and *K. 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 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 polyclonality 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 identify 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 as well as 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 of 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 affect 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 metadata 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.

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.[64,73,74] 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.[66,67,72,73]

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.[75] ST131 stands out among other *E. coli* lineages because of its common association with fluoroquinolone and β-lactam resistance, mediated by target site mutations and a plasmid-associated extended-spectrum β-lactamase, respectively.[72,75] Recent comparative genomics studies have revealed a nested substructure to the ST131 lineage, wherein there was a sequential acquisition of fluoroquinolone resistance–conferring mutations, followed by a *bla-CTX-M-15*–containing plasmid.[75,76] Further work has unearthed additional complexity, in that *bla-CTX-M-15* seems to be found on multiple plasmids, which vary in their cargo, indicating that there may have been multiple plasmid-acquisition events.[75] A second lineage defined by a resistance plasmid is *bla-KPC*–carrying ST258 *K. 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.[65,77] Similar to ST131, *bla-KPC* has been observed in multiple plasmid contexts within ST258.[78] 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.[79]

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 *A. baumannii* appear to be defined more by the breadth and flexibility of their resistome.[75,80,81] While ECI and ECII strains carry resistance plasmids, the defining features of these strains are massive chromosomally encoded resistance islands that contain multiple antibiotic-resistance determinants that are associated with mobile genetic elements.[82–84] These resistance islands have proved to be extremely dynamic, with many different configurations reported.[85] In addition to horizontally acquired elements, *A. baumannii* also has several intrinsic resistance genes, including β-lactamases and efflux pumps, which can become activated under antibiotic pressure by mobilization of Insertion Sequence (IS) elements that carry strong promoters.[83,86] 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.[87]

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.[65,78] It has been hypothesized that these capsular switching events are important for 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 reported in both species.[28,88,89] 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 affecting fimbriae and pili, which may provide advantages in host colonization.[65,90,91]

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 was the critical event 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.[92] 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 compared with 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 to recognize and predict the emergence of resistant organisms, with the goal of intervening before they negatively affect public health.

Evolution of antibiotic resistance

While epidemic lineages are of special interest due to their prevalence and tendency toward multidrug 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 overall to be associated with worse patient outcomes due to increased time to optimal therapy.[93] Second, resistance determinants in low-risk clones can become mobilized and be transferred to other MDRO lineages and other species.[94,95] 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 toward 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.[96–99] 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.

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, it is likely due to a small number of high-impact mutations.[98] Indeed, studies employing this approach to study resistance evolution typically only observe a handful of mutations between intrapatient pairs, which facilitates the identification of causal variants by identifying genes or pathways mutated in multiple patient time courses.[97] A drug for which several groups have studied intrapatient resistance evolution is colistin. Colistin is a last-line drug for treating MDR Gram-negatives that are resistant to carbapenem antibiotics.[100,101] 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*.[102] 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.[103–105] 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.[103,106]

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.[107] 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 outcompeted 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 (GI) tract before migrating and causing infections at other sites, such as the lungs or blood.[108,109] 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.

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.[110,111] 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 *S. marcescens* via conjugation.[110] 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.[112] 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.[113,114] For example, the outbreak investigated by Mathers *et al.* revealed that the prevalence of CRE at this institution was due to a highly complex plasmid transfer network, where intergenus transfer of a promiscuous KPC plasmid, transposition of KPC onto different plasmid 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 intrapatient 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.[115,116] One potential reservoir of resistance markers outside of healthcare settings is hospital effluent. Several groups have used metagenomics approaches to detect resistance markers and mobile genetic elements in hospital wastewater.[117–121] Growing evidence that environmental water organisms can take up clinically important resistance markers when exposed to these wastewaters further bolsters this hypothesis.[121] Recently, Rowe *et al.* used a combined metagenomic/metatranscriptomic approach to measure resistance gene abundance and expression, as well as antibiotic concentrations in effluents from different sites that varied in antibiotic use.[119] In support of hospital practices playing a role in promoting the environmental resistome, they found that catchment water from hospitals was enriched for β-lactamases compared with other sites and that hospital effluent β-lactamase levels correlated with hospital antibiotic usage over time.[119] 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 is motivated by several reports of outbreaks where resistant organisms have been isolated from these sites.[122]

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 interspecies plasmid in 2015, *mcr-1* has been identified in the human gut microbiome, wastewater, community, and animal sources.[123–127] 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 that the natural histories of various resistant organisms (e.g., environmental heartiness and colonization niche) 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.[107] Studies that examine the evolution of organisms within their

real-world context (e.g., longitudinal sampling of colonized patients and 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 decolonization 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.[128–131] Finally, 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.

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.[132,133] 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 turnaround times between sample collection, organism identification, and susceptibility testing.[26,134,135] Newer rapid-identification methods, such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF), have drastically reduced turnaround time in clinical microbiology laboratories and led to improvements in empiric antibiotic prescribing practices.[136–138] 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 not only vital information 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.[139–143] 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 turnaround 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.[57,144] 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.[145–148] 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.[149] 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.[150–153] 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 h, whereas culture-dependent phenotypic methods for *M. tuberculosis* susceptibility profiling can take weeks to months.[153] The outcomes of critically ill patients depend on how fast they can start appropriate antibiotic therapy; however, typical turnaround times for susceptibility testing in clinical microbiology laboratories for even non-slow-growing organisms range from 2 to 3 days to weeks, which may be detrimentally long in certain cases.[57,154] Further illustrating the potential for rapid turnaround, 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.[152]

Before an organism has been identified in a clinical diagnostic laboratory, patients with severe

infections (e.g., bloodstream infections) are often treated empirically, and they are then switched to an antibiotic with known efficacy once a pathogen and its susceptibilities are determined.[155] 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.[26,57,93,156,157] There are now several platforms that can be used to predict resistance phenotypes from WGS data.[158,159] 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.[160,161] For example, the epidemic lineage KPC^+ *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.[162] Rapid identification of organisms belonging to hypervirulent or highly transmissible clones could alert healthcare practitioners to place these patients into appropriate infection control precautions and 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 multiplex 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.[163,164] This application is particularly appealing because, though it is informed by WGS data, its use does not require the operator to have the skill set required to analyze genomic data, which is an additional concern to implementing genomic diagnostics into clinical microbiology workflows.[163,165] 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.[166]

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.[167] 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 laboratories 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 whether 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 whether 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.[168]

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 β-lactams in major Gram-negative pathogens, suggesting that it may be feasible to use WGS to identify resistance phenotypes in clinical settings.[169] 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 toward 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

Table 1. Open questions in the genomic epidemiology of resistant Gram-negatives

Hospital epidemiology and 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 affect 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 laboratories have for real-time epidemiological investigations?
Regional epidemiology	What are the clinical and epidemiological factors that affect regional MDRO prevalence?
	What are the networks on which different MDROs spread between regional healthcare facilities?
	How can genomics be integrated into public health workflows to affect real-time outbreak control and guide regional interventions?
Evolution and dissemination of clonal lineages	What genomic and epidemiological factors lead to the success of epidemic lineages?
	How does sampling bias affect our understanding of the genetic and epidemiological factors underlying 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 reservoirs of antibiotic resistance are sources and which are sinks?
	What is the direction of transferrable resistance flow between different reservoirs?
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 turnaround 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?

an hour with computational steps performed on standard laptop computers.[152,170,171] Whether these new technologies are best applied in everyday 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 whether the cost of implementing these technologies improves patient outcomes.

Conclusions

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 participation from experts across fields, including clinical medicine, microbiology, bioinformatics, antimicrobial stewardship, and healthcare epidemiology. Table 1 illustrates several fundamental questions in healthcare epidemiology, from before 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.

Acknowledgments

S.E.H. was supported by NIH T32-GM113900. S.E.H. and E.S.S. both contributed to the conceptualization, drafting, and editing of this manuscript.

Competing interests

The authors declare no competing interests.

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