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Review

Genomic Prediction of Antimicrobial Resistance: Ready or Not, Here It Comes!

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BACKGROUND: Next-generation sequencing (NGS) technologies are being used to predict antimicrobial resistance. The field is evolving rapidly and transitioning out of the research setting into clinical use. Clinical laboratories are evaluating the accuracy and utility of genomic resistance prediction, including methods for NGS, downstream bioinformatic pipeline components, and the clinical settings in which this type of testing should be offered.

CONTENT: We describe genomic sequencing as it pertains to predicting antimicrobial resistance in clinical isolates and samples. We elaborate on current methodologies and workflows to perform this testing and summarize the current state of genomic resistance prediction in clinical settings. To highlight this aspect, we include 3 medically relevant microorganism exemplars: *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Neisseria gonorrhoeae*. Last, we discuss the future of genomic-based resistance detection in clinical microbiology laboratories.

SUMMARY: Antimicrobial resistance prediction by genomic approaches is in its infancy for routine patient care. Genomic approaches have already added value to the current diagnostic testing landscape in specific circumstances and will play an increasingly important role in diagnostic microbiology. Future advancements will shorten turnaround time, reduce costs, and improve our analysis and interpretation of clinically actionable results.

Antimicrobial resistance is one of the single greatest concerns for human health globally *(1)*. In the setting of infection, clinical microbiology laboratories are tasked with generating clinically relevant results, including antimicrobial susceptibility profiles, to guide patient care decisions. Antimicrobial susceptibility testing has evolved over the years, and now laboratories have access to genomic approaches like whole-genome sequencing (WGS) and metagenomic next-generation sequencing (mNGS). We review genome-based antimicrobial resistance prediction in relation to clinical microbiology: how it is performed, when it may be useful, and what opportunities exist for the future.

Methodology

OVERVIEW

The first step of sequencing should be clearly identifying the goal of the analysis. This review is focused on genomic prediction of antimicrobial resistance. Another early decision point is choosing the appropriate next-generation sequencing (NGS) technology. The most prevalent in use is the second-generation short-read sequencing technology (i.e., Illumina) or third-generation long-read sequencing technology (i.e., PacBio, Oxford Nanopore). Generally, Illumina sequencing has higher accuracy of reads (approximately 1% error rate) than PacBio (approximately 10% error rate) or Oxford Nanopore (approximately 5% error rate) but at the expense of length, with both long-read sequencing technologies often able to produce contiguous reads >10 kb in length *(2)*. In addition, they differ in turnaround time: Illumina sequencing runs generally take at least 24 hours, whereas PacBio runs are often 0.5–10 hours, and a major advantage of Nanopore systems is the real-time results reporting. Typically, long-read sequencing technologies have a higher cost per base *(3)*. These sequencing technologies, their respective library preparation methods, and metagenomic alternatives are discussed below and summarized in Fig. 1. Following any sequencing, data analysis typically includes in silico refinement (performed computationally) of partial or completely assembled bacterial genomes, which can then be annotated for antibiotic resistance genes (ARGs). Table 1 includes a list describing examples of commonly used bioinformatic software in microbiology that have been reviewed comprehensively *(4)*.

WGS AND ASSEMBLY

For culture-dependent Illumina sequencing, genomic DNA representing the microbial chromosome and

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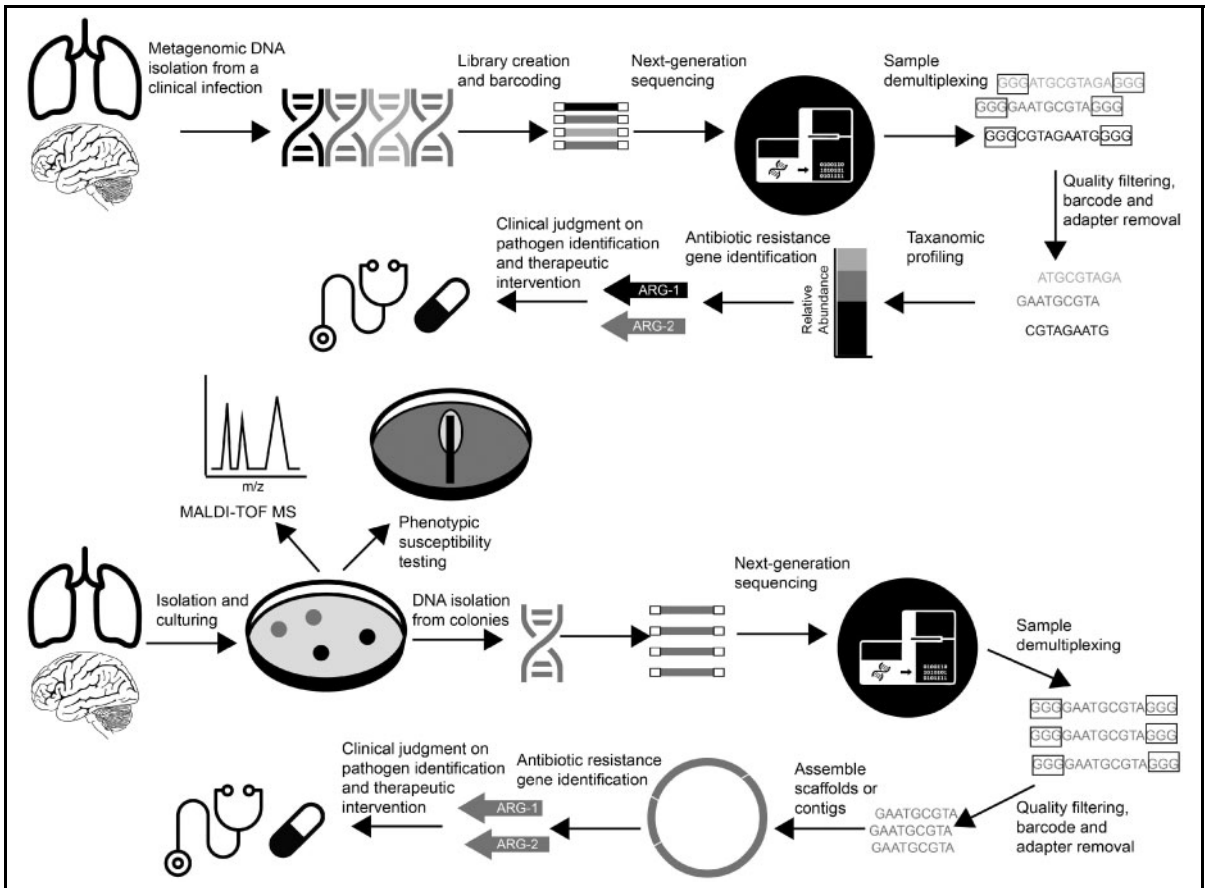

Fig. 1. Overview of culture-independent (upper) and -dependent (lower) sequencing approaches. Culture-independent techniques rely on mNGS and are capable of sequencing clinical specimens containing human and microbial DNA. After sequencing and quality processing, this information can be used to identify bacteria within genus or species taxonomic resolution and identify ARGs. Culture-dependent techniques rely on sequencing a cultivated organism but have the benefit of confirming genomic resistance prediction with true phenotypic resistance.

plasmids can be isolated using multiple different methods that may require species-specific optimization *(5)*. Next, DNA is sheared typically using enzymatic digestion or sonication into fragments of specific size depending on the desired read length *(6)*. Enzymatic shearing methods have been demonstrated to be more rapid than manual protocols; however, they may lead to sequence read bias for coverage of genomes with a high percentage of GC content, such as *Mycobacterium tuberculosis*, or AT content, such as *Plasmodium falciparum (7–9)*. Following the shearing process, the DNA is modified to include Illumina adapters, which are important for binding to the flow cell, and unique bar codes (or indexes) ligated onto the ends of the DNA *(6)*. The unique bar codes enable multiplexing of hundreds of bacterial samples together in a typical sequencing lane, drastically reducing the cost per genome assembly *(6)*. After the sequencing libraries are prepared and pooled, they can be submitted for a sequencing lane. The choice of specific Illumina technology depends primarily on the desired read length (most often 50-, 75-, 150-, or 300-bp length) and desired number of reads. Following the completion of the sequencing run, the reads can be demultiplexed by bar code, and then the bar codes and adapter sequences can be computationally removed by programs such as trimmomatic or fastp *(10, 11)*. At this stage, read quality can also be evaluated and low-quality reads discarded by programs such as FASTQC. These filtered reads can be used as input for de Bruijn graph–based assembly programs such as SPAdes, SOAPdenovo, or Velvet, which will assemble the reads into larger contiguous sequence fragments (contigs) and, depending on the program, order the contigs into scaffolds *(12–14)*. A metacomparison of multiple de novo

Table 1. Highlighted sequencing analysis pipeline programs.

Software	Utility	Input	Output
FASTQC	QC check of sequence data	Raw FASTQ files	Report on GC%, adapter contig, phred scores
Kraken	Taxonomic classification	Processed FASTQ files	Relative abundance of microbial taxa present
SPAdes	Genome assembly	Processed FASTQ files	Scaffolds, contigs, assembly graph
Velvet	Genome assembly	Processed FASTQ files	Scaffolds, contigs, assembly graph
Resfinder	Antimicrobial resistance detection	Scaffolds or contigs FASTA file	Report on presence of ARGs or alteration in target sequences
Bowtie	Sequence alignment	Processed FASTQ files	Files showing alignment information and unaligned or aligned reads
Prokka	Genome annotation	Scaffolds or contigs FASTA file	Protein coding and RNA coding information in multiple filetypes
ResFams	Antimicrobial resistance detection	Scaffolds or contigs FASTA file	Report on presence of ARGs

assemblers found that no single program was superior regarding multiple quality metrics (i.e., longest contig, number of contigs, N50 (the shortest contig length needed to cover 50% of the genome), indicating that multiple tools could be used for each specific study *(15)*. One drawback of this sequencing technology is that the microbial chromosome and large plasmids are likely to be assembled into hundreds of scaffolds, with assembly breaks at long repetitive genomic regions; however, a growing number of specialized programs are emerging that attempt to identify and annotate plasmidic regions from unassembled short-read data (e.g., PlasmidSeeker and plasmidSPAdes) *(16, 17)*.

For long-read sequencing (defined broadly in this review as >500 bases), considerable additional care is often warranted during the genomic DNA extraction process to shear the DNA properly; many extraction methods optimized for short-read sequencing result in highly fragmented DNA *(18)*. Another important difference is to ensure that plasmid DNA is nicked or decircularized for long-read sequencing; otherwise, it may not be included in the analysis. Following sequencing of the high-molecular weight DNA, initial quality assessment can be used to determine the average length of the sequence insert *(19)*. The long reads can be assembled by themselves or in conjunction with short reads (e.g., Illumina) in a hybrid assembly that combines the accuracy of Illumina reads with the contiguous scaffolding of long Nanopore or PacBio reads *(19, 20)*. Given that long-read sequencing will often produce reads that are sufficient in length to span repeat regions, a successful long-read sequencing run will produce a completely closed chromosome and closed plasmids if they are also present in the organism *(21)*. The MinION sequencer (Oxford Nanopore Technologies) was found to have high reproducibility when an identical *Bacillus anthracis* strain was sequenced by 2 different laboratories from the Public Health Agency of Canada, providing strength for the use of this technology by multiple groups during an outbreak investigation *(22)*.

Differentiation between plasmid-borne and chromosomally located ARGs is important for identifying the horizontal gene transfer potential of ARGs and infection control *(23)*. A caveat with realizing this

advantage is that considerable effort is required during the genomic DNA extraction protocol to not shear the DNA (21). A major advantage of the Oxford Nanopore MinION system is that the reads can be analyzed while the sequencing run is ongoing, providing for an extremely fast turnaround time that holds potential promise for the utility of this technology in the point-of-care diagnostic setting (24, 25). Conversely, the major advantages of Illumina sequencing are that it is cost-effective and the library preparation and genomic DNA extraction are more straightforward and less dependent on a skilled worker for extraction (26). However, these reads are too short for resolving chromosome and plasmid sequences or producing a single chromosomal contig. Illumina sequencing can have greater batch sizes, which may be compatible with current batching of molecular tests in clinical laboratories, but this would have a negative impact on turnaround time (interval from sample received by the laboratory to a result in the medical record) (26).

ARG ANNOTATION

Following production of whole-genome scaffolds or complete chromosomes and plasmids, multiple programs can be used to computationally identify ARGs. Typically, each program works by first identifying open reading frames or by accepting as input a multi-FASTA (file format containing multiple nucleotide sequences in the FASTA format) composed of the open reading frames within a bacterial genome. ResFinder is an easy-to-use program hosted on a web server that can also be downloaded for batch use on Linux systems (27). It is updated with verified ARGs multiple times per year and can be used to identify acquired ARGs (including most antibiotic-modifying enzymes) but not multicomponent efflux pumps or modifications of antibiotic resistance targets. However, the same authors have also created PointFinder, which can be used to identify resistance-conferring single-nucleotide polymorphisms (SNPs) in antibiotic-determining regions for a variety of gram-negative and gram-positive human pathogens (28). The Comprehensive Antibiotic Resistance Database has also produced a tool (Resistance Gene Identified) for annotation of genomes that includes acquired ARGs, polymorphisms in resistance-determining regions, and multicomponent efflux pumps (29, 30). Both programs rely on BLAST alignment (Basic Local Alignment Search Tool, program that identifies sequence similarity of nucleotide sequences) of input sequences against a curated database of known ARG sequences and thus are rapid and specific. However, one limitation is that genes that are divergently related to previously discovered ARGs may not be considered. To address this issue with remote-homolog detection, ResFams is an ARG-identification tool that relies on hidden Markov models for annotation of ARGs (31). This approach has been useful for identifying a novel class of tetracycline-inactivating enzymes initially from soil metagenomes that have now been identified in *Escherichia coli* and *Acinetobacter baumannii* isolates from humans, animals, and meat in China (32–34). This finding is particularly important under the One Health concept because bacteria in human-adjacent environments may transfer ARGs to those that are more closely associated with humans (35). The aforementioned identification programs rely on rules-based classification in which the presence of the determinant (either an SNP in a core gene or acquired resistance gene) indicates resistance (36). In certain studies, this analysis has proved superior to advanced machine learning methods at prediction of antimicrobial resistance (37). However, because the critical limitation of a rules-based approach is that it is unable to predict novel resistance, an ongoing avenue of research is to develop accurate model-based methods for prediction of phenotypic resistance. Two major strategies for this approach rely on variant calling of genetic diversity within a species- and population-level pan-genome analysis (38, 39). A continuing challenge in the application of NGS methods in the clinic is accurate inference of phenotypic susceptibility from these resistance predictions and conveying the information appropriately to clinicians. Current approaches largely resemble traditional susceptibility reporting as susceptible, intermediate, or resistant.

METAGENOMIC SEQUENCING

Rather than sequencing pure cultures of an isolated microorganism, an alternative approach is to perform mNGS directly from a clinical sample, capturing the entirety of the pathogen nucleic acid, other microorganism nucleic acid, and the patient's human nucleic acid (40). A major advantage of this unbiased approach to pathogen detection is forgoing culture bottlenecks; accordingly, interest in the application of clinical metagenomic sequencing is growing (41–43). This approach can be extremely advantageous for slowly growing pathogens such as *Mycobacterium* species (44). A prospective analysis of cerebrospinal fluid for metagenomic sequencing found that 22% (13/58) of infections could be identified by sequencing but not traditional laboratory methods (42). In addition, metagenomic sequencing provided auxiliary benefits to clinicians including identification of *Klebsiella aerogenes* (formerly *Enterobacter aerogenes*) ARGs and prediction of drug resistance by HIV-1 (42). However, a drawback is that this would prevent validation of ARG-based resistance prediction because there is no isolate for phenotypic susceptibility testing. Moreover, for cases in which unique reads for multiple suspected pathogens have been detected, clinical interpretation of the findings can be

challenging (45). Previous implementation of mNGS pipelines have required knowledge of bioinformatics, which may not be accessible in some laboratories. As a means to circumvent this technological hurdle, user-friendly commercial services such as IDbyDNA, CosmosID, and One Codex are emerging (46). It was demonstrated that these platforms may differ in identification of low-abundance organisms but were similar for the most prevalent organisms within a previously investigated sample of prosthetic joint fluid (46). Other commercial solutions such as Karius are being used clinically (47).

Similar to genomic analysis, the initial step is to obtain the total DNA from the sample, representing the microbial DNA of interest and human DNA from contaminating cells. Most work on the use of metagenomic diagnosis relies on short Illumina reads; however, a number of studies are emerging on the use of long-read technology. One investigation found that Nanopore-based metagenomic sequencing for lower respiratory tract infections had high sensitivity (96.6%) but low specificity (41.7%) for pathogen detection (48). One allure is the extremely rapid turnaround time and small instrument size of Oxford Nanopore sequencing. This has enabled rapid identification of suspected pathogens from contaminated orthopedic devices (49). A drawback of NGS technology, and especially Nanopore sequencing, is the requirement for data storage. One solution includes the use of compression algorithms like picopore, which can drastically reduce the total file size (50). In addition, it may be up to the laboratory director to decide on a fixed cutoff for discarding processed reads after an allotted time. Following sequencing, human DNA is computationally separated (and typically discarded) so that analysis can be performed on the remaining microbial reads (49). These reads can then be used for species classification via tools such as MetaPhlan2, Kraken, or Centrifuge (51–53). Furthermore, the reads can be mapped to databases of known ARGs so that the antibiotic resistance potential of the identified organism may be inferred. Moreover, because the data obtained from direct-sequencing technologies likely contain human reads, it is important that hospitals and laboratories spend adequate resources necessary to ensure that this health information is secure and deidentified. Last, a major limitation of mNGS is the higher limit of detection for ARGs in complex specimens (there are technical challenges to obtaining the necessary reads among a plethora of DNA).

CURRENT STATE OF GENOMIC PREDICTION IN CLINICAL LABORATORIES

To date, most clinical microbiology laboratories predict antimicrobial resistance using a combination of conventional culture-dependent phenotypic methods (e.g., broth microdilution, disk diffusion, gradient diffusion, automated systems) and culture-independent rapid molecular methods. This approach maintains the historical outcomes data and species breadth of conventional testing while offering shorter turnaround times using molecular methods for a subset of targeted pathogens. It is worth noting that this approach is imperfect. For example, rapid molecular testing is available for only a small subset of prominent resistance markers from a relatively limited number of clinical pathogens. Any culture-dependent method also has limitations: reliance on in vitro growth, extended turnaround times for slow-growing microbes, bias toward predominant microbial populations in a culture, and risk of contamination overgrowth. Even so, the field of clinical microbiology has been somewhat slow to implement genomic-based resistance prediction. Major points of contention include turnaround time and added costs compared with conventional methods, as well as lack of robust outcomes data demonstrating clinical utility. However, studies have shown that these factors may be less concerning than initially theorized (54). Another valid point is the lack of robust predictive tools for a substantial number of pathogens, particularly gram-negative ones (e.g., *Pseudomonas aeruginosa*).

There are advantages for a clinical microbiology laboratory in implementing genomic-based resistance detection. First, turnaround times could be improved for slowly growing pathogens. Second, laboratories may be able to test a wider breadth of microbes. This could include fastidious bacteria and other types of microbes like fungi or parasites. Third, clinical laboratories could determine isolate relatedness. This would be important for infection control and prevention practices, assessment of failed therapy versus new infection, and identification of contamination in the laboratory. Such isolate-relatedness data could also be sent to public health directly instead of sending an isolate, which may result in more rapid detection of outbreaks. In addition, combination efforts with WGS in conjunction with phenotypic analysis can be used to identify novel resistance determinants, as shown by recent studies of *Elizabethkingia* and *Salmonella* (55, 56). Last, genomic data can be reviewed retrospectively when new drugs come to market to predict efficacy without retesting. It is worth noting that we may not fully understand the benefits and limitations of genomic resistance prediction until it is implemented in clinical microbiology laboratories and directly compared with the status quo.

MICROBE VIGNETTES

Next we describe the role of genomic-based resistance prediction using 3 microorganisms. The first example is *M. tuberculosis*, for which genomic methods will likely displace current testing in the near future. The

subsequent exemplars *Staphylococcus aureus* and *Neisseria gonorrhoeae* depict some of the limitations preventing genomic resistance prediction from displacing current technologies.

Mycobacterium tuberculosis *M. tuberculosis* is a slowly growing, acid-fast bacillus that is notoriously challenging to treat and is of great concern for drug resistance. The 2019 CDC Antibiotic Resistant Threat report listed drug-resistant *M. tuberculosis* in the category of "Serious Threat" *(1)*. Multidrug-resistant tuberculosis made up 1.9% of the 9029 US cases in 2018 but is much more common globally. Treating multidrug-resistant tuberculosis requires timely drug-susceptibility testing; however, culture-based drug-susceptibility testing takes weeks to complete and is available only in selected, specialized laboratories. This lengthy turnaround time has led the field toward rapid molecular resistance prediction for *M. tuberculosis*.

Pyrosequencing and Sanger sequencing have been used for years to predict drug resistance in *M. tuberculosis (57)*. These first-generation sequencing technologies use a targeted approach to detect mutations in predetermined genomic resistance markers (Fig. 2C). A major advantage of targeted sequencing is that it can be performed directly on processed sputum sediments, substantially reducing turnaround times. One study found that effective patient therapies were initiated 5 weeks earlier for MDR infections due to pyrosequencing *(58)*. One limitation of sequencing directly from sputum sediments is reduced sensitivity compared with culture. Another limitation of targeted sequencing is the inability to detect off-target (often novel) resistance markers. This is less of a concern in *M. tuberculosis* because it does not readily acquire horizontally transferred resistance mechanisms like other clinically relevant microorganisms (e.g., gram-negative bacilli). Other limitations of Sanger sequencing and pyrosequencing are expense, minimal detection of heteroresistant populations, and widespread phasing out of first-generation sequencing technologies.

NGS technologies have been frequently used to predict drug resistance *(36, 59, 60)*. To date, the majority of genomic-based resistance prediction has focused on culture-dependent WGS. This approach offers comprehensive data for resistance profiling and epidemiological purposes *(36, 61, 62)*. However, the lengthy turnaround time is similar to that of phenotypic drug-susceptibility testing. The largest culture-dependent WGS study is from Comprehensive Resistance Prediction for Tuberculosis: an International Consortium (CRyPTIC) *(63)*. CRyPTIC evaluated >10,000 isolates and found genotypic predictions for first-line drugs correlated with phenotypic susceptibility (>90%). The ability to predict resistance in second-line drugs was less robust (<90% accuracy for some drugs). National public health institutions in the United Kingdom and the United States also perform culture-dependent WGS on isolates from domestic tuberculosis cases. These remarkable surveillance data sets promise to strengthen our understanding of current genomic resistance markers and may uncover novel resistance mechanisms. Today, few clinical laboratories have the resources, specimen volume, and turnaround tolerance to justify culture-dependent WGS for *M. tuberculosis* drug-susceptibility testing.

An alternative approach to detecting *M. tuberculosis* drug resistance is culture-independent, targeted sequencing from processed sputum *(44, 64)*. This approach has a rapid turnaround time and decreased cost because only relevant genomic regions are sequenced. This approach also significantly increases coverage depth, which is particularly important for detecting heteroresistant populations in *M. tuberculosis*. A limitation of targeted sequencing is low sensitivity due to the low bacterial biomass in clinical samples. Another limitation of targeted sequencing is missing an unforeseen resistance mechanism. The aforementioned epidemiological work by national surveillance programs should ensure that targeted sequencing is focused on specific genomic resistance markers. Another limitation of targeted sequencing is that most studies to date are from research settings, and clinical implementation may require further research and development, including validation, competency assessments, and quality assurance and QC measures. It is likely that, in the near future, clinical laboratories in MDR-prevalent areas will implement culture-independent, targeted sequencing to provide clinicians with rapid drug-susceptibility testing.

Oxford Nanopore is a long-read sequencing technology that has also been used for *M. tuberculosis (65, 66)*. This technology excels at homopolymer stretches, has a low cost per run, and is highly portable. The major limitation of Nanopore is the high sequencing error rate, making it less suitable for the single-nucleotide mutations and heteroresistant populations in *M. tuberculosis*. Nanopore technology must advance before it can be a practical option for clinical laboratories.

Traditional *M. tuberculosis* susceptibility testing will likely be displaced by sequencing-based resistance prediction. This approach is especially feasible for *M. tuberculosis* because of the limited number of resistance determinants, many of which are well characterized (Fig. 1C). Strong evidence suggests that for the majority of isolates, data are comparable to phenotypic testing but with a markedly reduced turnaround time. However, noteworthy phenotypic and genotypic discrepancies have been reported for the key antituberculosis drugs isoniazid and rifampin, even in multidrug-resistant tuberculosis

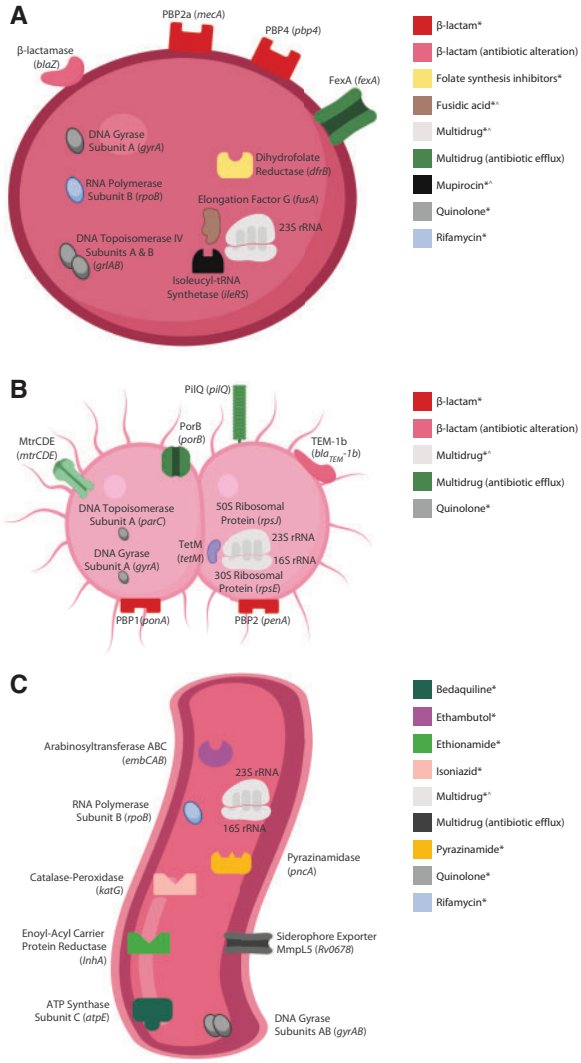

Fig. 2. Highlighted resistance determinants in *S. aureus* (A), *N. gonorrhoeae* (B), and *M. tuberculosis* (C). Because genes with target alterations are predominantly found within the core genome of bacterial pathogens, genomic-based resistance predictions are able to identify known SNPs within most isolates of a given species. Conversely, prediction of genes within the accessory genome for organisms (e.g., genes that encode efflux pumps or antibiotic altering enzymes in *S. aureus* or *N. gonorrhoeae*) may work on a presence/absence basis if the ARGs are strong determinants for phenotypic resistance. Colors link antibiotics to their respective resistance determinants. Mechanisms of target alteration and inhibition of protein synthesis are indicated by asterisks and carets, respectively.

(67–69). Because patients receive a multidrug treatment regimen, it is difficult to determine the clinical impact of discrepant results, and further work is required to understand the cause of these discrepancies.

Staphylococcus aureus *S. aureus* is a Gram-positive opportunistic microbe that is a common skin colonizer but can also cause serious health conditions including bacteremia, endocarditis, pneumonia, skin and soft tissue infection, and osteomyelitis. Treating *S. aureus* infections is often complicated by antibiotic resistance (see highlighted resistance determinants in Fig. 2A). Important resistance types include methicillin-resistant *S. aureus*, vancomycin-intermediate *S. aureus*, and vancomycin-resistant *S. aureus*. Clinical microbiology laboratories characterize *S. aureus* isolates using a combination of culture-dependent phenotypic antimicrobial susceptibility testing methods, specialized media (e.g.,

chromogenic agar and mannitol salt agar), and rapid phenotypic tests (e.g., PBP2a to detect methicillin resistance) *(70)*. Rapid molecular tests to detect methicillin-resistant *S. aureus* have also become common for nares surveillance screening and positive blood cultures. All of these current approaches have been developed and optimized for high testing volumes and timely results.

The overwhelming majority of sequencing in *S. aureus* has been for epidemiological purposes, but NGS technologies have been used for resistance prediction *(71–73)*. In general, there is a strong correlation between WGS resistance prediction and traditional phenotypic testing for *S. aureus*. Gordon et al. *(72)* used several hundred isolates and found >95% agreement between WGS and routine clinical laboratory testing (using automated systems and disk diffusion) for 12 antimicrobial agents. Resistance was predicted by a self-developed tool that searched genetic mechanisms reported in clinical isolates rather than existing resistance databases. Babiker et al. (*73*) found that genomic resistance prediction for methicillin, vancomycin, linezolid, tetracycline, gentamicin, trimethoprim–sulfamethoxazole, and rifampin all had 100% concordance with disk diffusion results. Mason et al. *(74)* used 3 programs (GeneFinder, Mykrobe, and Typewriter) to assess 84 resistance determinants and found highly similar resistance predictions (99.5%) that also correlated very well (98.3%) to the laboratory phenotype (determined by automated systems and disk diffusion) of >1300 *S. aureus* isolates. *vanA* and *vanB* were not found in any teicoplanin-resistant *S. aureus* genomes from India, indicating that these proxies for vancomycin resistance cannot predict teicoplanin resistance. Of note, *vanB*-harboring isolates are often teicoplanin susceptible *(75)*. However, the authors identified a number of mutations including some previously reported in the teicoplanin-resistance operon genes *tcaA* and *tcaB* (which encode for a zinc finger domain membrane-associated protein and Bcr/CflA family efflux transporter, respectively) *(76)*. Bradley et al. *(77)* developed a software package using Illumina reads as input that had a 100% positive predictive value for detection of methicillin and erythromycin resistance in *S. aureus*. The lowest positive predictive value obtained for this package was 91.1% for fusidic acid *(77)*.

Implementation of genomic sequencing approaches for *S. aureus* is tractable because most resistance is well characterized, and this technology could detect clones in outbreaks *(78)*. Data could then be shared with public health laboratories instead of an isolate, reducing unnecessary testing and shipping hazards. Hospital-acquired infections are a serious threat to patient health, and prevention is financially incentivized for patients and hospitals.

Displacement of current *S. aureus* testing in clinical laboratories by genome sequencing has obvious challenges. There is a lack of literature that clearly demonstrates clinically relevant WGS superiority (i.e., improvement of turnaround time, cost, or susceptibility prediction) over the aforementioned rapid susceptibility prediction methods. WGS can struggle with pertinent resistance mechanisms in *S. aureus*: plasmid-mediated resistance mechanisms (particularly short-read sequencing), altered expression of resistance genes (e.g., encoding efflux pumps or some oxacillinase genes), and uncharacterized mechanisms of resistance *(72)*. Substantial testing and personnel resources would also be needed to accommodate such a large shift to sequencing because *S. aureus* is one of the most commonly encountered isolates in a clinical microbiology laboratory. Although genomic-based resistance prediction could be used for *S. aureus*, it is unlikely to displace current approaches.

Neisseria gonorrhoeae Drug-resistant *N. gonorrhoeae* was categorized as an "Urgent Threat" in the 2019 CDC antibiotic-resistance threat report *(1)*; this category is the highest priority assigned. Given the fastidious nature of *N. gonorrhoeae*, diagnosis of active infection has relied on nucleic acid detection methods (nucleic acid amplification tests [NAATs]) *(79)*. Although NAAT use has shown high sensitivity and quick turnaround time for detection of *N. gonorrhoeae*, it does not provide information on the burden or repertoire of ARGs and may cross-react with commensal *Neisseria (79)*. Therefore, a plethora of efforts have been undertaken to use NGS methods for the detection of *N. gonorrhoeae* and characterization of ARGs and antibiotic resistance determining SNPs. Figure 2B highlights some of the acquired and intrinsic antibiotic-resistance determinants in *N. gonorrhoeae* that are potential targets for NGS-based prediction sets.

The fastidious nature of *N. gonorrhoeae* has complicated development of WGS-based diagnosis and antibiotic resistance prediction, given the inability to produce ample, pure cultures. However, recovery of *N. gonorrhoeae* has been shown to be 371% greater using the BD Kiestra Total Laboratory Automation System compared with conventional culture-based methods *(80)*. Improved routine culturing may provide new opportunities for using genomic techniques to characterize the ARG repertoire of *N. gonorrhoeae*. Population-level genome-wide association studies could lead to identification of novel resistance determinants in *N. gonorrhoeae*, as was done previously to identify novel resistance determinants in the swine pathogen *Serpulina* (formerly *Brachyspira*) *hyodysenteriae (81, 82)*. Isolates recovered from a BD Kiestra at Barnes-Jewish Hospital

in the high-incidence setting of St. Louis (Missouri) showed a strong association between intracity phylogenetic clade-specific resistance determinants, particularly the $bla_{\text{TEM-1b}}$ β-lactamase and the *tet(M)* tetracycline resistance gene *(83)*. Continued surveillance of *N. gonorrhoeae* is especially important, given the species' frequent recombination with other *Neisseria* and its natural competence *(84)*. Because certain antibiotic resistance phenotypes could be related to transcriptional change rather than genomic mutations or acquisition of dedicated ARGs, analysis of *N. gonorrhoeae* mRNA transcripts via RNA sequencing or quantitative reverse transcription PCR holds promise for prediction of azithromycin resistance *(85)*. Availability of Nanopore sequencing is increasing, and there is strong interest in application of this technology for point-of-care testing. A modeling analysis found that point-of-care testing for *N. gonorrhoeae* required information on suspected antibiotic resistance to prevent spread *(86)*. Nanopore sequencing can provide rapid identification of all ARGs within a *N. gonorrhoeae* genome or metagenome infection, which NAAT testing is not able to achieve; however, before widespread implementation, improvements in the accuracy of Nanopore-based reads will need to occur *(87, 88)*.

Given the difficulty of culturing *N. gonorrhoeae*, the use of WGS is an enticing alternative to predict antibiotic resistance. This approach may be particularly feasible for quinolone antibiotics; a logistic regression approach found that the S91 and D95 mutations in GyrA protein provided 98.6% sensitivity and 91.4% specificity for predicting quinolone resistance *(89)*. Because ciprofloxacin is no longer the current treatment for *N. gonorrhoeae* infections, the prediction of frontline azithromycin or ceftriaxone resistance is more relevant for immediate clinical application. The same logistic regression method found that although the C261T or A2059G mutation in greater than or equal to two 23S rRNA alleles had a perfect specificity (100%) for azithromycin resistance, it had a low sensitivity (65.8%) *(89)*. One problem for prediction of SNPs or genes associated with ceftriaxone resistance is an insufficient number of ceftriaxone-resistant isolates to make accurate calls. A multivariate linear regression approach using minimal inhibitory concentration data and WGS produced from isolates collected in England, the United States, and Canada found that predictions using resistance data from cefixime, penicillin, azithromycin, ciprofloxacin, and tetracycline all had ≥91% matches within 1 doubling dilution of the observed minimal inhibitory concentration *(90)*. Although not prediction based, a cohort of 435 clinical *N. gonorrhoeae* isolates from China were found to have 25.6% (112/425) isolates with reduced susceptibility to ceftriaxone *(91)*. Within this cohort, SNP leading to amino acid changes in PBP2, PorB, MtrR, and PilQ were associated with increased ceftriaxone resistance *(91)*. A novel method for identification of resistant *N. gonorrhoeae* using genomic neighbor typing when combined with Oxford Nanopore MinION technology was able, within 10 minutes, to provide 81% sensitivity and 100% specificity for resistance prediction *(92)*.

We can foresee a future in which clinical mNGS is used to identify antibiotic resistance conferring SNPs or the presence of acquired resistance genes within *N. gonorrhoeae*. This premise relies on resistance to the frontline azithromycin–ceftriaxone double therapy increasing beyond its current low level, necessitating the use of genomic or phenotypic means to identify other efficacious antibiotics (i.e., quinolones, tetracyclines, or different β-lactams). The use of mNGS, we believe, would be superior to culture plus WGS, given the historical difficulties in growing *N. gonorrhoeae* and given the current widespread use of culture-independent NAATs. An additional benefit will be the detection of the related pathogen *Neisseria meningitidis*, which can cause urethritis.

The Future of NGS in Clinical Microbiology

NGS is currently in use as a diagnostic tool in some clinical microbiology laboratories; example cases include culture-independent microbial identification or detection from sterile sources including blood, cerebrospinal fluid, and synovial fluids using mNGS *(47, 93)*. WGS for susceptibility predictions remains uncommon. For widespread implementation, a test must improve turnaround time, provide enhanced or additional clinically actionable information, or reduce costs. Reduced turnaround times are quite plausible for slow-growing acid-fast bacilli, molds, anaerobes, fastidious bacteria, or formalin fixed-tissue specimens. Reduced turnaround times and enhanced data may be possible for positive blood-culture specimens. WGS is unlikely to displace current approaches by cost alone without major technological advancement.

The small size of the MinION (particularly when used with the Flongle adapter, which enables direct, real-time sequencing) and rapid turnaround time holds promise for its use as an implementable sequencing technology in clinical microbiology laboratories. Combined with development of novel algorithms such as those used by Břinda et al. *(92)*, it could provide information on species and ARG presence within minutes. Currently, for most nonfastidious human pathogens, targeted sequencing may prove to be a reasonable stopgap to improve sequencing efficiency, as data and space on the sequencer remain concerns.

Conclusions

Microbial sequencing is well established in research and public health settings and is emerging as a component

of clinical care in clinical microbiology laboratories. The role of resistance prediction will undoubtedly continue to expand in clinical microbiology as our understanding and appreciation of the technology grows and the workflow and analysis become aligned with routine clinical use. Because clinical microbiology and hospital systems are trending toward consolidation, having a centralized laboratory performing NGS analysis for multiple hospitals may be one compromise between cost and turnaround time. As these techniques become more readily available, it will also require that clinical microbiology laboratory directors and staff access and distill the rapidly expanding information about sequencing technologies and the computational pipelines to accurately and confidently interpret results and provide clinically accessible and actionable reports. We anticipate that, for the foreseeable future, genomic methods will continue to complement culture-based methods in the clinical microbiology setting.

Nonstandard Abbreviations: WGS, whole-genome sequencing; mNGS, metagenomic next-generation sequencing; NGS, next-generation sequencing; ARG, antibiotic resistance gene; contig, contiguous sequence fragment; SNP, single-nucleotide polymorphism; NAAT, nucleic acid amplification test.

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