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Molecular Diagnostics for *Mycobacterium tuberculosis* Infection

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Abstract

Resistance to antimycobacterial drugs is a major barrier to effective treatment of *Mycobacterium tuberculosis* infection. Molecular diagnostic techniques based on the association between specific gene mutations and phenotypic resistance to certain drugs offer the opportunity to rapidly ascertain whether drug resistance is present and to alter treatment before further resistance develops. Current barriers to successful implementation of rapid diagnostics include imperfect knowledge regarding the full spectrum of mutations associated with resistance, limited utilization of molecular diagnostics where they are most needed, and the requirement for specialized laboratory facilities to perform molecular testing. Further understanding of genotypic–phenotypic correlates of resistance and streamlined implementation platforms will be necessary to optimize the public health impact of molecular resistance testing for *M. tuberculosis*.

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INTRODUCTION

Tuberculosis (TB), caused by bacteria belonging to the *Mycobacterium tuberculosis* complex, is one of the world's deadliest diseases. The numbers speak for themselves: TB is the most common infectious cause of death in adults worldwide, with an estimated 10.4 million new cases diagnosed worldwide and 1.7 million deaths in 2016 (1). While effective treatment regimens have been available since the 1950s, barriers to tuberculosis elimination persist. These barriers include the ability of persons with TB, who often are socioeconomically disadvantaged, to access healthcare; timely diagnosis once such persons do access healthcare; and the ability to obtain and administer appropriate medications to cure TB once diagnosed. The latter is made more challenging by the emergence of drug-resistant TB, which requires different and often more expensive and toxic therapies. Failure to ascertain in a timely fashion whether drug resistance is present may result in suboptimal outcomes including amplification of drug resistance, morbidity, and mortality (2–4).

TB control efforts have been severely limited by imperfect diagnostic testing. The gold standard method of diagnosing TB is acid-fast bacilli (AFB) smear microscopy and sputum cultures for *M. tuberculosis*, followed by growth-based drug-susceptibility testing (DST). The sensitivity of AFB smear microscopy ranges from 45% to 80% and can be affected by specimen concentration and laboratory experience (5, 6). The sensitivity of AFB smears is lower in patients with paucibacillary disease, such as children and patients with HIV (7–9). Culture-based diagnostic testing requires an average of 2–3 weeks of incubation time, and smear-negative specimens may take more than 4 weeks to grow in culture. After *M. tuberculosis* is isolated in culture, growth-based DST can take an additional 1–4 weeks depending on the growth medium. In all, our current growth-based diagnostic testing often takes months to complete. Furthermore, mycobacterial culture and DST require highly trained laboratory personnel that are often unavailable in primary healthcare settings in low- and middle-income countries, so frequently culture and DST are reserved for cases of treatment failure or disease recurrence. By the time DST is performed, the organism may have developed further drug resistance.

Drug resistance adds another layer of complexity in the diagnosis and treatment of TB. Multi-drug-resistant (MDR) TB is defined as TB that is resistant to at least isoniazid and rifampin, the two most important first-line antituberculosis drugs. Extensively drug-resistant TB is defined as MDR TB that is also resistant to both a fluoroquinolone and a second-line injectable agent. Patients with drug-resistant TB have longer treatment courses and more side effects, and they are less likely to achieve cure in the absence of individualized regimens (10, 11). Rapid DST is crucial to getting patients on appropriate therapy as quickly as possible and to preventing the development of further resistance. The expansion of rapid testing and detection of drug-resistant TB cases is a priority in addressing the global TB crisis.

Molecular diagnostic testing for the detection of *M. tuberculosis* complex and rapid DST offer possible solutions to our current TB diagnostic shortcomings. These tests are based on genetic information rather than growth-based assays and have a potential turnaround time of hours rather than weeks. These tests have transformed the TB diagnostic landscape, providing rapid and accurate diagnosis in TB-endemic settings where culture-based diagnosis was not previously available. This review outlines commercially available and promising investigational molecular methods of TB diagnosis, as well as limitations that will need to be addressed before these diagnostic methods can be fully implemented in the fight against TB.

PHENOTYPIC SUSCEPTIBILITY TESTING AND LIMITATIONS

The agar proportion method on solid media is the reference standard for conventional growth-based DST (12). Cell suspensions prepared from pure culture are placed on three quadrants

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containing a drug at its critical concentration and on one drug-free control quadrant. Colonies are counted after 21 days of incubation. The number of *M. tuberculosis* colonies that grow on drug-containing media is compared to the number of colonies on the drug-free control quadrant. A colony count of 1% or greater on a drug-containing quadrant compared to the control quadrant indicates resistance. Pyrazinamide cannot be tested using the agar proportion method because the acidic pH required to test this drug prevents many isolates from growing. Indirect DST refers to testing on subcultures after isolation of the organism from the primary specimen, while direct DST is performed on AFB smear-positive sediments. Direct DST can yield results in a shorter time but is more prone to bacterial contamination, yielding uninterpretable results. Expected turnaround time for solid-media growth-based DST is 3–4 weeks.

More modern phenotypic DST methods include liquid media and time-to-growth indices. The liquid DST method most frequently used in the United States is the Mycobacterial Growth Indicator Tube. This fully automated system detects mycobacterial growth through an increase in a fluorescent indicator in each tube. The system tests for susceptibility to rifampin, isoniazid, ethambutol, and streptomycin and is the preferred DST method for pyrazinamide. Results from liquid media are typically available within 1–2 weeks.

DST on solid and liquid media is limited in terms of turnaround time, availability, and accuracy. It can take weeks to months to go from making a TB diagnosis to obtaining susceptibility results, and this lag may result in inappropriate regimens and amplification of resistance. Many resource-limited countries do not have the laboratory capacity or infrastructure to support phenotypic DST. Reproducibility is suboptimal for certain drugs, such as pyrazinamide, streptomycin, and ethambutol. Additionally, phenotypic DST cannot be performed on mixed cultures. Contamination can occur at the time of phenotypic DST set-up or when nontuberculous mycobacteria are also present in culture. This confounding will lead to false-positive resistance results. Ideally, drug-resistance results should be verified to rule out contamination with other bacteria.

MUTATIONS ASSOCIATED WITH DRUG RESISTANCE

TB becomes phenotypically resistant by selection for preexisting resistance-associated mutations. Each patient with TB harbors a mixed bacterial population containing small numbers of organisms that have mutations conferring resistance to various drugs. For example, 1 in 10^6 wild-type TB organisms are thought to have isoniazid resistance mutations, and 1 in 10^8 wild-type TB organisms are thought to have rifampin resistance mutations. Resistance can also occur by spontaneous mutations during replication. Organisms carrying resistance mutations are positively selected during periods of ineffective therapy, such as improper treatment regimens or patient nonadherence. With a sufficient period of ineffective therapy, bacterial cells carrying mutations eventually replace the drug-susceptible bacterial populations.

The phenotypic manifestations of TB resistance–conferring mutations are varied and complex. Some mutations cause high-level resistance to a drug while other mutations cause low-level resistance. Some mutations do not cause phenotypic resistance at all. A mutation associated with drug resistance should be associated with worse clinical outcomes, but this is often hard to appreciate due to multidrug regimens that can compensate for this resistance. For example, isolated resistance to ethambutol has little impact on the efficacy of standard treatment regimens. Additionally, some isolates that have proven phenotypic resistance respond to treatment with drugs to which they are resistant; for example, 25% of patients with MDR TB are cured by standard treatment. This is not unique to TB; the concept that infections may respond to treatment when phenotypic resistance is present has been codified as the "90–60 rule" (13) (i.e., ~90% of infections with organisms that are susceptible to a given drug in vitro will respond positively to treatment with

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Table 1 ***Mycobacterium tuberculosis*** **genes associated with drug resistance**

Drug	Gene (mutation)	Clinical implications	Approximate sensitivity of molecular testing for phenotypic resistance
Isoniazid (INH)	*katG*	High-level INH resistance	87%
	inhA	Low-level INH resistance and ethionamide resistance	
	fabG1	Promotes upregulation of *inhA* expression	
	ahpC	High-level INH resistance	
Rifampin (RIF), rifabutin (RBT)	*rpoB*	Most mutations cause both RIF and RFB resistance, although GAC516GTC causes RIF resistance only TTC514TTT is a silent mutation and is not associated with RIF or RFB resistance; falsely interpreted as RIF resistance by probe testing	96%
Ethambutol (EMB)	*embB*	Some mutations are associated with EMB resistance while others are not	79%
Pyrazinamide (PZA)	*pncA*	Mutations widely distributed, not always associated with PZA resistance	86%
Fluoroquinolones: moxifloxacin (MFX), levofloxacin, ofloxacin	*gyrA*	Increases minimum inhibitory concentration of MFX; MFX may still be effective at higher doses	79%
Amikacin (AMK)	*rrs*	AMK resistance	91%
Capreomycin (CAP)	*rrs*	CAP resistance	55%
	tylA	Mutations widely distributed, associated with CAP resistance	
Kanamycin (KAN)	*rrs*	KAN resistance	99%
	eis promotor	KAN resistance	

that drug, while ~60% of infections with organisms that are resistant to the same drug in vitro will respond to treatment with that drug).

More is known about some resistance-conferring mutations than others (14, 15). **Table 1** summarizes the known mutations associated with drug resistance. More than 95% of resistance to rifampin is caused by mutations in an 89-base-pair region of the *rpoB* gene called the rifampin resistance determining region. Not all mutations in the region are equivalent: Some mutations cause high-level resistance to rifampin and rifabutin, while others cause resistance to rifampin but not rifabutin, and "silent mutations" do not confer phenotypic resistance at all. Interestingly, another group of mutations is associated with susceptibility using phenotypic DST but is associated with worse clinical outcomes when standard rifampin-containing regimens are used (16). The majority of resistance to isoniazid is caused by alterations in the *katG* gene (causing high-level resistance) or *inhA* gene (causing low-level resistance) (17). Isoniazid and ethionamide have similar structures, and cross-resistance can occur. Single mutations in *inhA* cause low-level isoniazid resistance and ethionamide resistance, although high-level isoniazid resistance and ethionamide resistance due to a double mutation in *inhA* have been described (18). Hundreds of mutations in the *pncA* gene promote resistance to pyrazinamide, but some mutations in *pncA* do not cause resistance (19). Pyrazinamide resistance also occurs through mutations outside of the *pncA* gene, as some pyrazinamide-resistant isolates have normal *pncA* sequences (19). The most frequent mutation conferring ethambutol resistance is due to a mutation in the *embB* gene (20), but 40%

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of mutations occur in other areas, and not all mutations in *embB* are associated with ethambutol resistance (21). The majority of fluoroquinolone resistance is due to mutations in the *gyrA* and *gyrB* genes (22). Resistance to the second-line injectable drugs—amikacin, capreomycin, and kanamycin—is usually mediated through the *rrs* gene.

OVERVIEW OF MOLECULAR DIAGNOSTICS

TB molecular diagnostics are crafted around known resistance-conferring mutations. The current molecular methods for detecting rifampin and isoniazid resistance have high sensitivity and specificity because mutations tend to occur in predictable locations. However, isolates with rifampin or isoniazid resistance are occasionally missed by current molecular-based testing methods (23), and detecting resistance to second-line drugs is far more difficult because mutations are scattered and variable. Consequently, phenotypic DST remains the gold standard for determining drug resistance and ideally should be used to complement molecular-based diagnostic testing when both are available.

The two main categories of molecular diagnostic testing for active TB are probe-based and sequence-based. Probe-based testing looks for specific gene mutations but cannot provide the sequence information for the mutations. It is thus limited by our knowledge of resistance-conferring mutations. Sequence-based testing is able to describe the genetic identity of a particular mutation, thereby increasing accuracy.

PROBE-BASED ASSAYS

GeneXpert MTB/RIF

The GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, California, USA) is a probe-based, fully automated polymerase chain reaction (PCR) test that simultaneously detects *M. tuberculosis* complex and resistance to rifampin. The assay tests with five molecular beacon probes (A through E) that cover the rifampin resistance determining region of *rpoB*. The probes contain wild-type sequences, and any variability in sequence between a probe and the test organism will prevent hybridization and indicate that a mutation is present. Sample processing, PCR amplification, and detection occur in a single cartridge to minimize hands-on technical time and opportunities for contamination. Additionally, the assay's sample reagent has tuberculocidal activity, which decreases biosafety concerns during the test procedure (24, 25). GeneXpert MTB/RIF is approved by the US Food and Drug Administration for direct testing on smear-positive or -negative sputum specimens in patients who have received fewer than 3 days of antituberculosis therapy and has a turnaround time of 2 h. Additionally, one or two GeneXpert MTB/RIF tests can be used as an alternative to AFB smears to inform the decision to discontinue airborne isolation in patients with suspected pulmonary TB, thereby decreasing the length of time that a patient remains in isolation (26–29).

GeneXpert MTB/RIF has been extensively studied and validated in various clinical settings (25, 30–38). A meta-analysis that included data from low- and middle-income countries found that the pooled sensitivity and specificity for GeneXpert MTB/RIF as an initial test replacing smear microscopy were 89% [95% credible interval (CrI), 85–92%] and 99% (95% CrI, 98–99%), respectively (39). GeneXpert MTB/RIF had a higher sensitivity for TB detection in smear-positive cases than in smear-negative cases [smear-positive: 98% (95% CrI, 97–99%), smear-negative: 67% (95% CrI, 60–74%)] and in people without HIV than in people with HIV [HIV negative: 86% (95% CrI, 76–92%), HIV positive: 79% (95% CrI, 70–86%)]. Additionally, GeneXpert MTB/RIF increased TB detection among culture-confirmed cases by 23% (95% CrI, 15–32%). The pooled

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sensitivity and specificity were also high for rifampin resistance detection [95% (95% CrI, 90–97%) and 98% (95% CrI, 97–99%), respectively].

Despite its advances, GeneXpert MTB/RIF does have limitations. GeneXpert MTB/RIF detects both living and dead bacteria and should not be used to monitor response to treatment. The assay has limited sensitivity for conditions with lower levels of bacilli, such as paucibacillary disease and extrapulmonary TB (40–42). TB isolates that have silent mutations will not bind to the wild-type sequences and will yield false-positive results (43). Implementation of GeneXpert MTB/RIF can lead to considerable operational and logistical challenges, including infrastructure renovation, device training, and regular instrument troubleshooting and maintenance (34). Cost is probably the biggest limitation and has hindered widespread utilization of GeneXpert MTB/RIF. The cartridges cost on average $10.00 (an already discounted rate) and require a device for processing samples (the commonly used GX4 costs approximately $17,000), and there is an annual servicing cost (44). Countries that implement GeneXpert MTB/RIF testing have to make a substantial investment; however, multiple studies have shown that GeneXpert MTB/RIF is a cost-effective tool for the diagnosis of TB and drug-resistant TB (29, 45, 46).

The next-generation GeneXpert MTB/RIF Ultra assay (Cepheid, Sunnyvale, California, USA) is intended to improve detection of TB in patients with low bacterial burden pulmonary disease and extrapulmonary disease through improvements in cartridge design, PCR, and mutation detection. The Ultra test is more sensitive than its predecessor. When both were used to test clinical sputum samples, Ultra's overall sensitivity was 87.5% [95% confidence interval (CI), 82.1–91.7%] and GeneXpert's was 81.0% (95% CI, 74.9–86.2%). For sputum smear-negative samples, Ultra's sensitivity was 78.9% (95% CI, 70.0–86.1%) while GeneXpert's was 66.1% (95% CI, 56.4–74.9%). Both tests had a specificity of 98.7% (95% CI, 93.0–100%), and both had similar accuracies for detection of rifampin resistance (47). A more recent report also found that Ultra was more sensitive than GeneXpert (88% sensitivity for Ultra, 83% for GeneXpert) but somewhat less specific (96% specificity for Ultra, 98% for GeneXpert) (48).

MTBDR*plus* and MTBDR*sl*

GenoType MTBDR*plus* and GenoType MTBDR*sl* (Hain Lifescience, Henren, Germany) are line probe assays that are frequently used in concert for rapid screening and DST for TB (49–51). MTBDR*plus* detects and identifies mutations associated with resistance to isoniazid (*katG* and *inhA*) and rifampin (*rpoB*), and MTBDR*sl* detects and identifies mutations associated with fluoroquinolones (*gyrA*), aminoglycosides and cyclic peptides (*rrs*), and ethambutol (*embB*). Both assays can be directly used on clinical specimens with DNA extraction, multiplex PCR, reverse hybridization, and resistance gene mutation detection achieved within 5 h. MTBDR*plus* and MTBDR*sl* are endorsed by the World Health Organization for rapid screening for MDR TB (1).

The performance of GenoType MTBDR*plus* is comparable to that of the GeneXpert MTB/RIF assay (52). A 2016 meta-analysis by Bai et al. (53) showed excellent pooled sensitivity and specificity for detection of resistance to isoniazid (91% sensitive, 99% specific), rifampin (96% sensitive, 98% specific), and MDR (91% sensitive, 99% specific). This meta-analysis noted that sensitivity was more inconsistent and seemed to be higher when only liquid-medium DST studies were pooled. Indeed, other studies have confirmed that cases of isoniazid resistance are missed, likely due to the higher proportion of resistance-conferring mutations residing outside of *inhA* and *katG* (54–56). Bai et al.'s meta-analysis did not have enough data to pool data by smear status, but other studies have found that the assay's diagnostic performance, especially sensitivity, is lower and that it has a high percentage of invalid results for early detection of MDR TB in direct smear-negative sputum samples (51, 57, 58).

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GenoType MTBDR*sl* 1.0 was the first commercial line probe assay for detection of resistance to second-line TB drugs, but its updated 2.0 version has improved mutation-detection capabilities (50, 59, 60). The diagnostic accuracy of MTBDR*sl* is similar when performed directly on sputum specimens or indirectly on cultured isolates. Similar to MTBDR*plus*, the assay works better on smear-positive samples than on smear-negative samples (49). The sensitivity and specificity for direct testing of fluoroquinolone resistance in smear-positive specimens are 97% (95% CI, 83–100%) and 98% (95% CI, 93–100%), respectively, while smear-negative specimens have a sensitivity of 80% (95% CI, 28–99%) and a specificity of 100% (95% CI, 40–100%) (61). Direct testing for resistance to second-line injectable agents (kanamycin, amikacin, capreomycin) demonstrated sensitivity and specificity of 89% (95% CI, 72–98%) and 90% (95% CI, 84–95%), respectively, for smear-positive specimens, and sensitivity and specificity of 80% (95% CI, 28–99%) and 100% (95% CI, 40–100%), respectively, for smear-negative specimens. Smear-negative samples that test as susceptible using this assay must be evaluated further to rule out second-line drug resistance. The World Health Organization has endorsed MTBDR*sl* for use as an initial test to detect resistance to fluoroquinolone and second-line injectable drugs in isolates with confirmed rifampin resistance and MDR TB (62).

Nipro NTM+MDRTB

The Nipro NTM+MDRTB (Nipro Corporation, Osaka, Japan) line probe assay identifies four *Mycobacterium* species—*M. tuberculosis*, *M. kansasii*, *M. intracellulare*, and *M. avium*—and detects resistance to rifampin (*rpoB*) and isoniazid (*katG* and *inhA*) in *M. tuberculosis* (63). The mutation probes are similar in the Nipro NTM+MDRTB and MTBDR*plus*, although there are some minor variations in the codon regions covered for the wild type (64). Nipro NTM+MDRTB's performance is similar to that of MTBDR*plus* 2.0 for indirect and direct sample testing, although Nipro NTM+MDRTB showed higher rates of indeterminate results at smear grades of 1+ and scant (64). The sensitivity and specificity of indirect versus direct testing for rifampin resistance by this assay were found to be similar: Sensitivity was 92% for indirect testing and 96.5% for direct testing, while specificity was 98.5% for indirect testing and 97.5% for direct testing. The sensitivity and specificity of indirect testing for isoniazid resistance were 89.6% and 100%, respectively, while sensitivity and specificity of direct testing were 94.9% and 97.6%, respectively. In addition to the NTM+MDRTB assay, the same company has developed the Nipro Genoscholar PZA-TB II line probe assay to detect pyrazinamide resistance over the entire *pncA* gene. This assay demonstrated 93.2% sensitivity and 91.2% specificity for pyrazinamide resistance when compared with resistance determined by Sanger sequencing (see the section titled Genetic Sequencing, below) as the reference standard (65).

Anyplex Plus MTB/NTM/MDR-TB and Anyplex II MTB/MDR/XDR Kit

The Anyplex Plus MTB/NTM/MDR-TB assay (Seegene Technologies, Concord, California, USA) is a multiplex real-time PCR system that detects *M. tuberculosis* complex as well as a number of nontuberculous mycobacterial species. If *M. tuberculosis* is present, the system also detects resistance to rifampin (*rpoB*) and isoniazid (*katG* and *inhA*). The assay requires ~3 h to perform. The sensitivity and specificity for detection of *M. tuberculosis* complex determined by direct testing on respiratory specimens were 86.4% and 99%, respectively (66). The sensitivity and specificity of detecting isoniazid resistance in *M. tuberculosis* strains from respiratory specimens were 83.3% and 100%, respectively. The Anyplex Plus assay also has been tested on extrapulmonary specimens, albeit with a lower sensitivity (83.3% for TB and 50% for isoniazid resistance) (66). As compared

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to GeneXpert, the Anyplex Plus assay had significantly lower sensitivity for paucibacillary disease (smear-negative cases and cases where the time-to-culture positivity was ≥20 days) (57).

The newer Anyplex II MTB/MDR/XDR detection kit (Seegene Technologies, Concord, California, USA) is an updated multiplex real-time PCR system that detects resistance to rifampin (*rpoB*), isoniazid (*katG* and *inhA*), fluoroquinolone (*gyrA*), and aminoglycoside (*rrs* and *eis*). Its performance is similar to those of MTBDR*plus* and MTBDR*sl* (56, 67). The Anyplex II kit also suffers from lower sensitivity for the detection of isoniazid resistance as compared to phenotypic DST due to the large number of genes involved in resistance to isoniazid (56).

GENETIC SEQUENCING

Sequence-based testing is the next wave of TB molecular diagnostics. These tests identify the exact genetic make-up of a target organism, thereby increasing the accuracy of mutation detection. Three types of sequencing methods are discussed here: pyrosequencing, Sanger sequencing, and whole-genome sequencing.

Like all molecular techniques for ascertainment of drug resistance, pyrosequencing and Sanger sequencing require prior knowledge of the relationships between specific mutations and phenotypic drug resistance. Pyrosequencing sequences a short stretch of nucleotides and is capable of detecting any mutation within the targeted length if the mutation identity is provided. Sanger sequencing uses chain-terminating dideoxynucleotides coupled to dyes to obtain the sequence of nucleotides in a region of DNA targeted by specific primers; it is limited to detecting mutations that are present in at least 15–20% of genomes sequenced (68). Both techniques have been implemented in laboratory-developed, noncommercial tests that are being utilized to identify TB resistance-associated mutations and can produce results within 1–2 days (14, 69, 70). One study utilized pyrosequencing and phenotypic DST in both culture isolates and clinical specimens for detection of extensively drug-resistant TB and demonstrated agreement between the two testing modalities in the range of 94–99% (94.3% for isoniazid, 98.7% for rifampin, 97.6% for fluoroquinolones, 99.2% for amikacin and capreomycin, and 96.4% for kanamycin) (69). A combination of pyrosequencing and Sanger sequencing is being utilized at the Centers for Disease Control and Prevention for the molecular detection of drug resistance (MDDR), which is performed on primary sputum specimens that have a positive nucleic acid amplification test for *M. tuberculosis* as well as on TB isolates. MDDR tests for mutations conferring resistance to rifampin (*rpoB*; sensitivity 97.1%, specificity 97.4%), isoniazid (*inhA* and *katG*; sensitivity 86.0%, specificity 99.1%), fluoroquinolones (*gyrA*; sensitivity 79.0%, specificity 99.6%), kanamycin (*rrs* and *eis*; sensitivity 86.7%, specificity 99.6%), amikacin (*rrs*; sensitivity 90.9%, specificity 98.4%), capreomycin (*rrs* and *tylA*; sensitivity 55.2%, specificity 91.0%), ethambutol (*embB*; sensitivity 78.8%, specificity 94.3%), and pyrazinamide (*pncA*; sensitivity 86.0%, specificity 95.9%) (71).

The main limitation of pyrosequencing and Sanger sequencing is incomplete knowledge about resistance mechanisms and resistance-conferring mutations. Occasionally, these tests detect a mutation that has an unknown correlation with resistance. Additionally, since not all resistance mechanisms are known, resistance cannot be definitively ruled out for some drugs even if no mutation is detected. Furthermore, mutations residing within a minority subpopulation of a heterogeneous population could be missed by pyrosequencing or Sanger sequencing (72–74), especially if that subpopulation is present at a relatively low frequency.

Whole-genome sequencing provides the complete DNA sequence of an organism's genome at a single time, thereby removing the need to look for prespecified DNA targets. Whole-genome sequencing produces an enormous number of data, and well-defined analysis algorithms with a user-friendly software interface will be required for this sequencing method to be useful as

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a diagnostic tool. Despite these initial obstacles, whole-genome sequencing has been used to characterize common and rare mutations predicting resistance (75–81) and is being used to drive clinical decisions (82, 83).

The main limitation of whole-genome sequencing, and of molecular TB diagnostics in general, is a lack of correlation between genetic information and clinical outcomes. We are now able to obtain a large amount of information about our *M. tuberculosis* isolates, but we have limited understanding about how this information translates to successful outcomes and treatment failures. Remedying this limitation will require collaboration through a centralized TB worldwide database platform that integrates genotypic, phenotypic, and clinical data from cases of drug-resistant TB (84, 85).

THE NEXT TEN YEARS

The last decade has produced exciting advances in molecular testing for drug resistance in *M. tuberculosis*, and the next decade will hopefully produce similarly exciting progress. The first limitation of molecular DST that must be overcome is the lack of knowledge regarding underlying genetic mechanisms of drug resistance, particularly for drugs other than rifampin. While the classical approach of examining specific genes for specific mutations has produced significant advances, further progress will likely require more sophisticated tools. Some forms of resistance may not be mediated by single gene mutations but may rely on several mutations acting in concert, for example. Computational techniques that can systematically look for such patterns without prior knowledge of resistance-conferring mutations, such as deep learning, are starting to be used in other areas of medicine, including automated examination of chest radiographs for persons suspected of having TB disease (86). Such techniques will be particularly important in distilling clinically useful results from the large amounts of information produced by whole-genome sequencing. Furthermore, streamlined, user-friendly systems that automatically process whole-genome sequencing data to provide clinically relevant drug resistance reports will be needed to make the outputs of deep learning or similar machine learning algorithms useful for actual treatment of persons with TB disease.

The second limitation that must be overcome is the need for central laboratories with expensive equipment for molecular diagnosis of TB drug resistance. The quick turnaround time of rapid molecular testing is frequently nullified by delays in transporting the specimen from the primary clinic in areas of high TB prevalence to a central facility. Closed-cartridge systems such as the GeneXpert have made molecular testing simpler to perform and less susceptible to cross-contamination than open tube-systems such as line probe assays. Further progress in development of simple, closed-cartridge systems that rely on relatively inexpensive equipment near the point of care will be essential to increasing the impact of molecular diagnosis of TB drug resistance on clinical care and amplification of drug resistance.

Finally, as new drugs are developed to treat TB, understanding of molecular determinants of resistance should be an essential and early part of the drug development pathway. Molecular tests will not be useful if they are only able to examine resistance to yesterday's treatments. As an example, the early development of bedaquiline included analysis of genetic mutations associated with drug resistance (87), and subsequent work has continued to expand our knowledge of genotypic determinants of resistance to this drug (88). The pathway for new drug development must include post-rollout surveillance for resistance with systematic molecular characterization of resistant strains.

SUMMARY

Molecular DST has the potential to significantly improve both surveillance for TB resistance and treatment of persons infected with drug-resistant organisms. The last decade has witnessed

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encouraging advances in this field, with a number of new commercial and in-house laboratory assays available for clinical use. Continued work to better understand the molecular determinants of drug resistance and to make the assays more suitable for point-of-care use will be essential to realizing the potential impact of this technology in controlling TB.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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